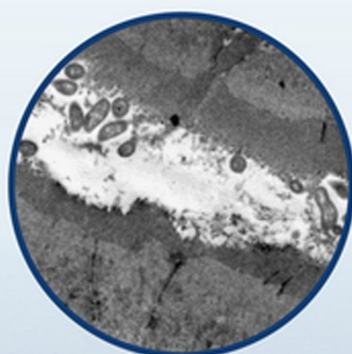
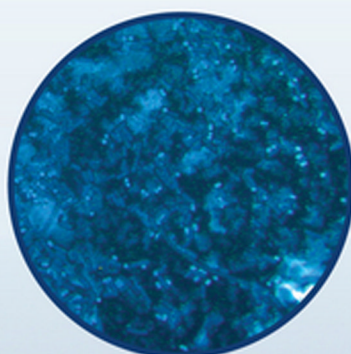


AQUACULTURE NUTRITION

Gut Health, Probiotics and Prebiotics



Edited by

Daniel Merrifield and Einar Ringø



WILEY Blackwell

Aquaculture Nutrition

Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics

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Preface

Since the initial investigations on the gut microbiota of fish some five decades ago, considerable information has been presented on their composition, abundance, diversity and activity. Numerous studies have demonstrated that these communities are complex and generally of low cultivability, containing Bacteria, Archaea, viruses, yeasts and protists. However, little attention has been paid to the Archaea, protists or viruses but several studies have revealed diverse communities of bacteria and yeast. These microbes have major implications on host health, development, welfare and nutrition and therefore great efforts have been made in the past two decades to fortify these communities and maintain microbial balance. Among such efforts the applications of probiotics and prebiotics have been at the forefront. The scientific evidence which underpins the efficacy, and to some extent elucidates their modes of action, has been comprehensive, although not always reproducible. This body of evidence has helped to create a market and drive demand for commercial probiotics and prebiotics for use in aquaculture operations globally. As such, many feed manufacturers, multi-nationals and small domestic operations, routinely add pro- and prebiotic products to their feed formulations. The extent of their economic benefits is not yet clear, as such information is not often openly discussed by fish farmers, but the increasing demand and increasing volumes of probiotic/prebiotic aquafeeds produced are positive indicators for industrial level applications. Future research efforts should focus on better understanding of the modes of action, which must include a better understanding of the composition and activity of indigenous microbiomes, as well as the effects on the host itself, so that optimisation of probiotic/prebiotic selection, dosage and application strategies can occur.

The chapters within this book address these issues and are advised reading for an understanding of the historical development of these products, their known mechanisms of action and their degree of efficacy as presently demonstrated in the literature. We also hope that the fundamental material provided on the gut microbiota itself, and more broad aspects of microbe-live feed interactions, are useful reading for researchers, academics and students. We wish to thank the authors that have contributed to this book, as well as our PhD students and post-doctoral staff whom have also assisted in the collection of data and literature. We are also grateful to the assistance of the production staff at Wiley-Blackwell for their support.

Daniel Merrifield and Einar Ringø

1 The Gastrointestinal Tract of Fish

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ABSTRACT

The organization of the gastrointestinal (GI) tract of fish follows the basic features as in other vertebrate groups with a degree of variation in phylogeny and ontogeny, feeding habits, diet, nutrition, physiological conditions and the special functions the gut may perform. There are enormous variations in the morphology of the GI tract among various fish species. The variations in the organization of the GI tract ensure optimum utilization of dietary nutrients, which in many cases means efficient primary digestion and a large intestinal absorptive surface area. Different fish species have adapted different approaches to accommodate this objective. Of particular interest to fish nutritionists is the comparison of morphological features in relation to natural diets. In order to compare data obtained from one fish species with other species, it is essential to make divisions into a broad line of common morphological features.

1.1 INTRODUCTION

Detailed descriptions of the anatomy and physiology of GI tracts of numerous fish species have been covered in several reviews (Suyehiro 1942; Barrington 1957; Kapoor *et al.* 1975; Harder 1975; Fänge and Grove 1979; Smith 1989; Stevens 1988; Olsen and Ringø 1997; Wilson and Castro 2011). Fish have the ability to rapidly and reversibly adapt GI tract characteristics to match the changes in functional demands that occur during their life history (e.g. metamorphosis, anadrome or catadrome migrations) or more frequently (day-to-day or seasonal shifts in diet or environmental conditions); this ability is dependent on endocrine signalling pathways which are augmented by the enteric nervous system (Karila *et al.* 1998). The wide diversity and levels of hormones and signalling molecules secreted by the numerous types of GI tract and endocrine pancreas cells allow fish to rapidly and reversibly alter characteristics of the GI tract

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and other organ systems to adapt to changes in the contents of the GI tract (amounts and types of nutrients, pH, ionic composition etc.) and environmental conditions (Holst *et al.* 1996).

The key feature of the alimentary tract is its ability to digest foodstuffs to make them suitable for absorption by various transport mechanisms in the wall compartments of different GI sections (Bakke *et al.* 2011). Besides the hydrolytic reactions catalysed by endogenous enzymes secreted by the pancreas and cells in the gut wall, which are considered to play the major roles in digestion, fermentation plays key roles in digestive processes in many monogastrics. The role of fermentation in fish is less clear due to a lack of knowledge, but it is considered to be of minor quantitative importance for nutrient supply in cold water species. However, qualitative importance may be significant regarding specific nutrients and immune stimulating processes.

The anatomy and physiology of the GI tract are important determinants for the establishment and for the quantitative as well as the qualitative aspects of its microbiota. The microbial communities may seem to be assembled in predictable ways (Rawls *et al.* 2006). In this study the authors showed that microbial communities transplanted from mice to gnotobiotic zebrafish (*Danio rerio*) alter quantitatively in the direction of the *normal* biota of the zebrafish species and vice versa. This indicates that environmental conditions of the intestine, determined by species-specific parameters along the GI tract such as anatomy, endogenous inputs of digestive secretions, pH, osmolality, redox potential, compartment size and structure, passage rate and residence time, help to define and shape the GI tract microbiota. However, diet composition is also an important environmental condition for fish development. Diet composition is ideally species specific regarding available essential nutrients, but supplies variable amounts of unavailable material depending on the feedstuffs used in the diet formulations. The gut microbiota is also probably inevitably linked to digestion by the production of exogenous enzymes and vitamins produced which might aid host digestive function (Ray *et al.* 2012). This chapter summarizes the current state of knowledge highlighting the morphological and histological variations in the lower GI tract of fish associated with digestion and absorption; comprehensive reviews on the gut microbiota are presented in *Chapters 4–6*.

1.2 ANATOMY OF GI TRACT

The structure and functional characteristics of the GI tract vary widely among species (Suyehiro 1942) and seem, to a great extent, to match the wide diversity of feeding habits and environmental conditions exploited by fish. The structure of the alimentary canal varies in different species of fish, and is generally adapted in relation to the food and feeding habits. Depending on feeding habits and diet, fish are generally classified as carnivorous (eating fish and larger invertebrates), herbivorous (consuming mainly plant material), omnivorous (consuming a mixed diet) and detritivorous (feeding largely on detritus) (De Silva and Anderson 1995; Olsen and Ringø 1997; Ringø *et al.* 2003), together with the genera *Panaque* and *Chochliodon* which are capable of digesting wood. However, such division may not always be correct since most species consume mixed diets or their feeding habits may change through the life cycle (Olsen and Ringø 1997). The variation becomes obvious by comparing the GI tract characteristics of carnivorous and herbivorous fish and those from freshwater and seawater. The mucosal lining of the GI tract represents an interface between the external and internal environments, and in conjunction with the associated organs (e.g. pancreas, liver

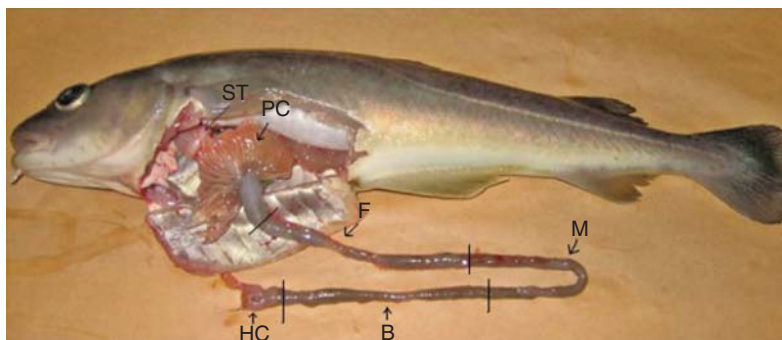


Fig. 1.1 The alimentary tract of Atlantic cod (*Gadus morhua* L.). ST, stomach; PC, pyloric caeca; F, proximal intestine; M, mid intestine; B, distal intestine; HC, fermentation chamber. (Source: Lisbeth Løvmo Martinsen.) For colour detail see Plate 1.

and gall bladder) provides the functions of digestion, osmoregulation, immunity, endocrine regulation of GI tract and systemic functions, and elimination of environmental contaminants and toxic metabolites. The GI tract is basically a tube that courses through the body. The GI tract in Atlantic cod (*Gadus morhua* L.) is shown in Figure 1.1. This tract is divided into the following characteristic regions: mouth, gill arch, oesophagus, stomach, mid intestine, distal intestine and fermentation chamber.

1.3 STOMACH AND INTESTINAL BULB

Two main groups of fish are commonly distinguished on the basis of presence or absence of stomach. The most remarkable feature of the digestive system of lampreys, hagfish, chimaeras, and many herbivorous fishes belonging to Cyprinidae, Cyprinodontidae, Balistidae, Labridae, Scomberesocidae and Scaridae, is the lack of a true stomach. In cyprinids, for example mrigal (*Cirrhinus mrigala*), the anterior part of the intestine becomes swollen to form a sac-like structure called the intestinal bulb or pseudogaster (Figure 1.2). In the absence of a stomach, the anterior intestine performs the function of temporary storage of ingested food (Sinha 1983). In stomachless fish the intestinal bulb apparently secretes mucus, and histologically the mucosa resembles closely that of the intestine and is devoid of any digestive components (Horn *et al.* 2006; Manjakasy *et al.* 2009). The mucosa of the intestinal bulb is thrown into prominent folds or villi (for lack of a better term; strictly speaking they are not true villi due to the absence of lacteals) that are lined with absorptive and mucus-secreting cells. The absence of stomach in many stomachless fish is compensated by the presence of pharyngeal teeth or gizzards for grinding food (Suyehiro 1942; Fänge and Grove 1979). Wood-eating fishes have specifically adapted spoon-shaped teeth for efficiently rasping wood (Nelson *et al.* 1999). The lack of a stomach in some species of fish raises questions regarding its significance. Several hypotheses have been put forward to explain the absence of a stomach which are often contradictory and speculative (for review, see Wilson and Castro 2011). The shape, size and structure of the stomach, when present, are related to the duration between meals and the nature of the diet (Suyehiro 1942; Smith 1989; De Silva and Anderson 1995). A stomach is defined as a portion

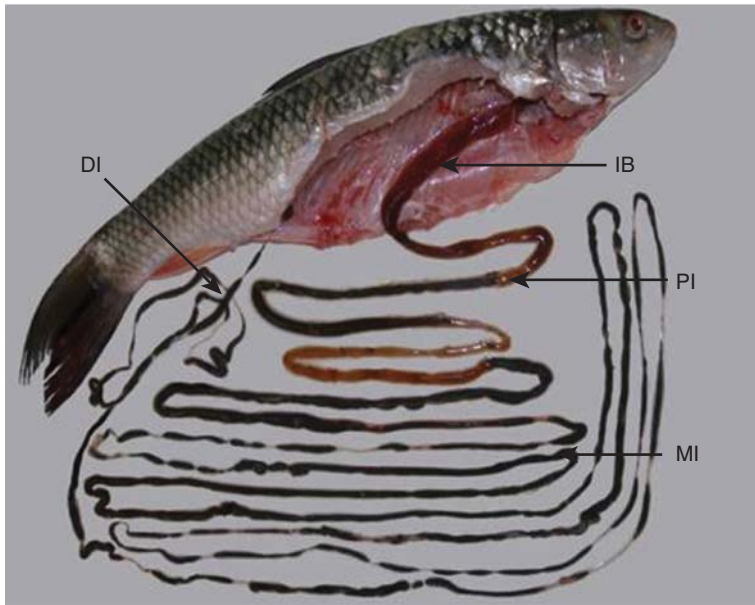


Fig. 1.2 Alimentary tract of the mrigal (*Cirrhinus mrigala*). IB, intestinal bulb; PI, proximal intestine; MI, mid intestine; DI, distal intestine. Relative intestinal length (RIL) is 14–15. (Source: Arun K. Ray.) For colour detail see Plate 2.

of the digestive tract with distinctive cell lining, where acid is secreted, usually along with some digestive enzymes like pepsin (Olsen and Ringø 1997). In his early study, Suyehiro (1942) classified stomachs of fish into five categories according to their morphological appearance: (a) straight tube (Pleuronectidae, *Esox*), (b) U-shape (Salmonids), (c) V-shape (Plecoglossidae, Mugilidae, Salmonidae, Sparidae), (d) Y-shape (Mugilidae, Clupeidae), and (e) I-shape (Carangidae, Gadidae, Scombridae, Serranidae). The highest degree of modifications of the pyloric stomach have been reported in several members of Clupeoidei, Channidae, Mugilidae, Acipenseridae, Coregoninae and Chanidae (milkfish, *Chanos chanos*) where it acts as a ‘gizzard’ for trituration and mixing (Fänge and Grove 1979; Kapoor *et al.* 1975; Buddington 1985; De Silva and Anderson 1995). This development of a ‘gizzard’ has been attributed to microphagy, and is thought to partly compensate for poor dentition (Pillay 1953). The anterior part of the stomach (cardiac or fundic region) is characterized by the presence of gastric glands (Figure 1.3A) and the musculature is also usually more prominent (De Silva and Anderson 1995). The stomach mucosa is lined with columnar epithelium and studded with minute depressions, the gastric crypts or pits that lead into the tubular or alveolar gastric glands. Gastric glands are present in abundance throughout the cardiac stomach, so much so that they occupy the entire mucosal layer beneath the superficial epithelium (Figure 1.3A). This part of the stomach is secretory in nature and is responsible for storage and initial physical and enzymatic breakdown of the diet; readers with special interest in this topic are referred to the comprehensive review of Bakke *et al.* (2011). The mucosa of the posterior part of the stomach (pyloric stomach) contains many mucus-producing tubular mucus glands or pyloric glands (Figure 1.3B). The number of these glands decreases considerably in the middle region and they are completely absent in the posterior region. The pyloric stomach is completely devoid

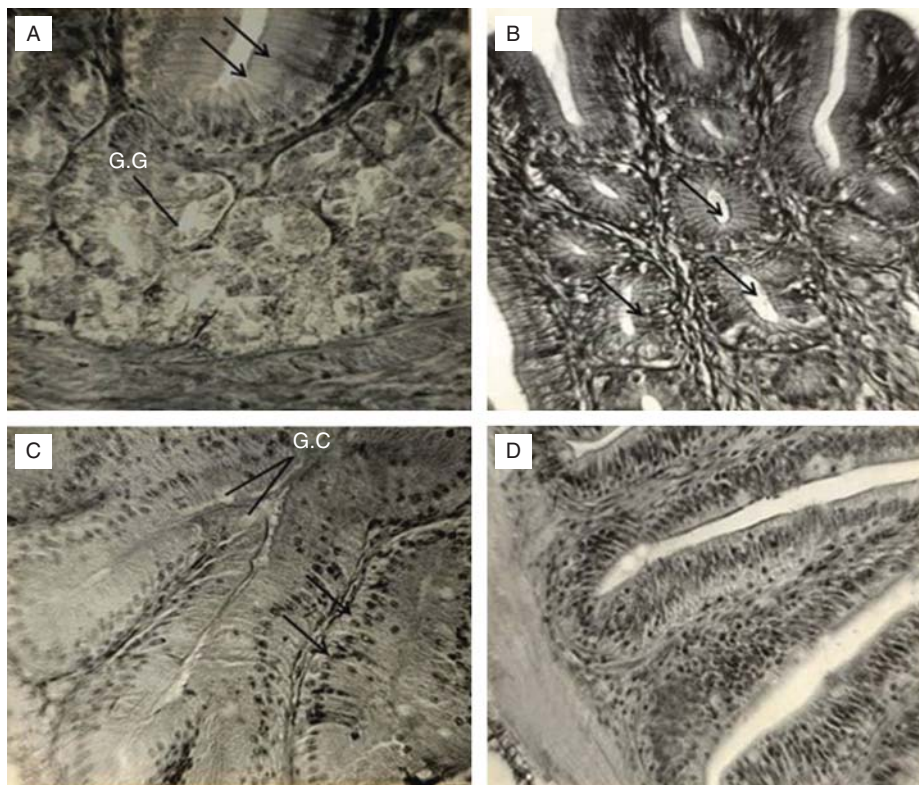


Fig. 1.3 Transverse section through different regions of the GI tract of climbing perch, *Anabas testudineus*, a carnivorous perch. (A) Cardiac stomach. GG, gastric glands. Arrows indicate mucus-secreting cells at the free borders of columnar epithelial cells. $\times 400$ magnification. (B) Pyloric stomach. Arrows indicate tubular mucus glands or pyloric glands. $\times 400$ magnification. (C) The intestine. GC, goblet cell. Arrows indicate absorptive cells. $\times 400$ magnification. (D) The pyloric caeca. $\times 400$ magnification. (Source: Ray and Moitra 1982.)

of gastric glands. The pH of the stomach therefore varies and in salmonids it is between 3.0 and 4.5 (Ransom *et al.* 1984; Gislason *et al.* 1996).

To our knowledge, the stomach microbiota is less investigated. Austin and Al-Zahrani (1988) evaluated bacteria in the stomach of rainbow trout (*Oncorhynchus mykiss* Walbaum) by using electron microscopy, while Navarrete *et al.* (2009) and Zhou *et al.* (2009a) evaluated the stomach microbiota of Atlantic salmon (*Salmo salar* L.) and emperor red snapper (*Lutjanus sebae* Cuvier), respectively, by molecular methods.

1.4 PYLORIC CAECA

In a number of fish species, several finger-like outgrowths develop from the anterior part of the intestine in the region of pylorus. These are called pyloric caeca or intestinal caeca, and open into the lumen of the intestine. They are located proximal in the midgut region, and, when

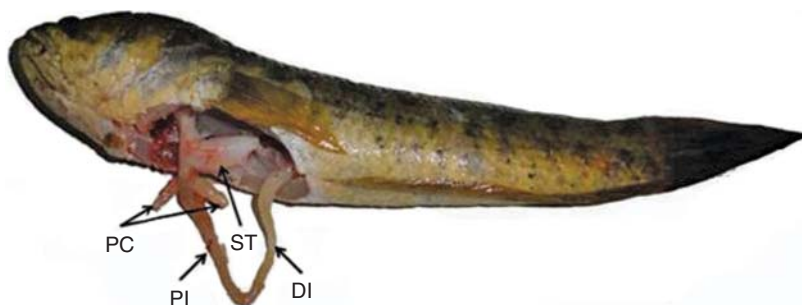


Fig. 1.4 Alimentary tract of murrelet (*Channa punctatus*). ST, stomach; PC, pyloric caeca; PI, proximal intestine; DI, distal intestine. Relative intestinal length (RIL) is 0.5. (Source: Arun K. Ray.) For colour detail see Plate 3.

present, number from a few as in murrelet *Channa punctatus* (Figure 1.4) to several hundred as in Atlantic cod (Figure 1.1). The caeca of different species vary considerably in size, state of branching and connection to the gut (Suyehiro 1942; Olsen and Ringø 1997; Ringø *et al.* 2003). Histologically, they closely resemble the intestine (Figure 1.3D), and possibly serve to increase the absorptive surface of the gut (Bergot *et al.* 1975). The pyloric caeca are always absent in stomachless fish (Barrington 1957; Kapoor *et al.* 1975). Although the presence or absence of the pyloric caeca has no apparent correlation with the nature of the food or with feeding habits (Khanna 1961; Mohsin 1962), the caeca are typically absent or much reduced in omnivorous and herbivorous species (Rust 2002). There is also no clear correlation between the number of caeca and the length of the gut, and feeding habits (Harder 1975; Hossain and Dutta 1996). Pyloric caeca have been reported to increase the surface area for digestion and absorption but do not have any role in fermentation or storage (Buddington and Diamond 1987). In salmonids, the pH of caeca and caecal intestine is 7.0 and 7.5, respectively (Ringø *et al.* 2003). Compared to the numerous studies evaluating the finfish gut microbiota (e.g. Cahill 1990; Ringø *et al.* 1995; Ringø and Gatesoupe 1998; Hansen and Olafsen 1999; Ringø and Birkbeck 1999; Austin 2006; Kim *et al.* 2007; Merrifield *et al.* 2011; Lauzon and Ringø 2012), fewer studies have investigated the microbiota of pyloric caeca (Lesel and Pointel 1979; Gildberg *et al.* 1997; Gildberg and Mikkelsen 1998; Navarrete *et al.* 2009; Zhou *et al.* 2009b).

1.5 INTESTINE

In fish, the intestine is the main organ for digestion/absorption. In addition to digesting and absorbing feedstuffs, the intestine is critical for water and electrolyte balance, endocrine regulation of digestion and metabolism, and immunity (Ringø *et al.* 2003). The intestine shows considerable variation in its length and arrangement in different species of fish (Kapoor *et al.* 1975; Fänge and Grove 1979; Stevens 1988). Some fish have a relative intestinal length (RIL = length of intestine/length of body) less than 1, while some fish species have an RIL of 10 to 20 times their body length (Suyehiro 1942; Olsen and Ringø 1997). The highest RIL generally occurs in herbivorous and detritivorous species (Figure 1.2), while the lowest is found in strictly carnivorous and predatory species (Figures 1.1 and 1.4). The intestine in Cyprinids and Loricariids exhibits a wide range of looping and coiled arrangements (Figure 1.5), while



Fig. 1.5 Alimentary tract of detritivorous mrigal (*Cirrhinus mrigala*) showing extremely coiled intestine. (Source: Arun K. Ray.) For colour detail see Plate 4.

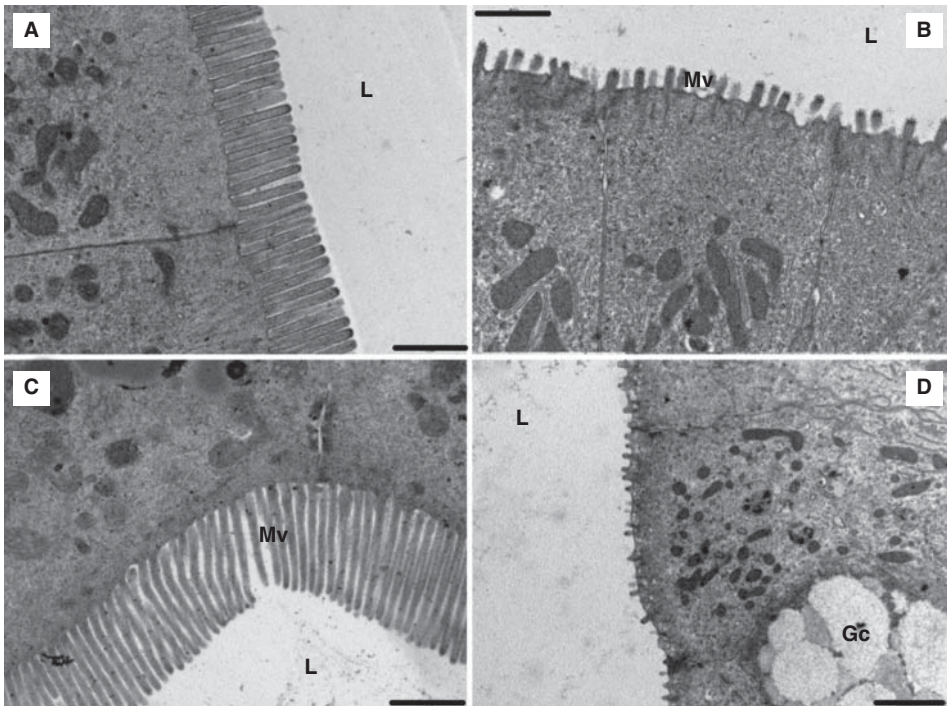


Fig. 1.6 Transmission electron microscopy images from the intestine of tilapia *Oreochromis niloticus* (A and B) and zebrafish *Danio rerio* (C and D). Images show the regional variation in the brush border formation (microvilli length and abundance) between the anterior (A and C) and posterior (B and D) intestine. Gc, goblet cell; L, lumen; M, mitochondria; Mb, cell membrane; Mv, microvilli; Tj, tight junction. Scale bar = 1 μ m. (Source: Merrifield and Harper, unpublished.)

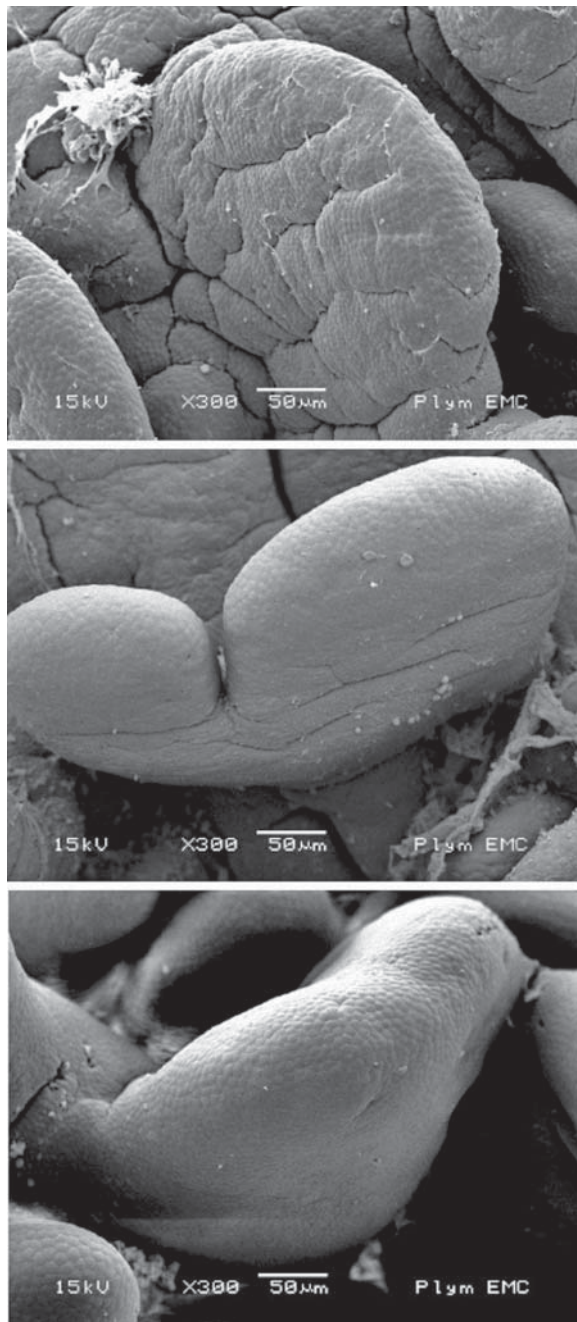


Fig. 1.7 Scanning electron microscopy images from the posterior intestine of rainbow trout *Oncorhynchus mykiss*, depicting the mucosal folds (or 'villi'). Scale bars = 50 µm. (Source: Merrifield and Dimitroglou, unpublished.)

omnivorous species show an intermediate condition. There are also differences in RIL within the same species. For example, in kalbasu (*Labeo calbasu*) the RIL of the detritivorous adult is higher (2.1 to 13.0) than that of fry feeding on zooplankton (0.5 to 1.0) (Sinha 1976). It is assumed that the long intestinal length of herbivorous compared to carnivorous fish is due to the requirement for digesting and absorbing the portion of the plant food which they normally ingest in the adult stage (Sinha and Moitra 1975). The greater length and mass of the intestine in herbivores relative to carnivores have also been thought to allow for additional processing of relatively difficult-to-digest items (Horn 1997; Clements and Raubenheimer 2005). However, it is also possible that herbivorous and/or detritivorous fish consuming plant fibres and detritus depend on extended intestines in order to increase the utilization efficiency, which is not directly related to the surface area (Olsen and Ringø 1997). On the contrary, Harder (1975) opined that there are no clear relationships between intestinal morphology and feeding type and it is not possible to draw any conclusion in this regard. Histologically, the intestine in fish contains simple, columnar absorbing epithelium lined with brush border of microvilli, which is typical of absorptive tissue (Figure 1.3C; De Silva and Anderson 1995) and goblet cells (mucus producing cells). In some fish species regional variations in the brush border formations have been observed (Figure 1.6). The numbers of goblet cells are more numerous in the posterior region than in the anterior and middle regions (Ray and Moitra 1982). The posterior part of the intestine is considered to be the main site for intestinal absorption of macromolecules in salmonids and some other fish species (for review, see Dalmo *et al.* 1997; Figure 1.7). The midgut starts immediately posterior to the pylorus and the hindgut is an extension of the midgut with gradually diminishing digestive and absorptive functions and increased level of mucus production (Ringø *et al.* 2003).

1.6 ENDOGENOUS INPUTS OF DIGESTIVE SECRETA

Different enzymes, bile acids and pancreatic enzymes are constantly secreted or leaking into the GI tract from the wall tissue and from the liver and pancreas, respectively. These fluids contain a great range of compounds that may affect the growth and composition of the intestinal microbiota. Besides macromolecules such as a great number of proteins, for example digestive enzymes and muco-polysaccharides, these fluids contain phospholipids, bile acids, antioxidants such as glutathione, minerals, waste products eliminated from the body through the faeces (e.g. bilirubins giving colour to the faeces) and bicarbonate to stabilize the pH of the luminal contents. Although our knowledge is limited for fish, it can be suggested that these fluids vary greatly in quantity as well as composition between intestinal segments and within species under different conditions. To our knowledge, no information has been reported in the scientific literature regarding quantities of water and material entering the GI tract of juvenile or adult fish. However, alterations in composition have been observed, and information is available that alterations are observed in activities of digestive enzymes within the gut contents of salmonids by incorporation of plant material in the diet (Romarheim *et al.* 2006; Gatlin III *et al.* 2007; Santigosa *et al.* 2008) as well as alterations in content of bile acids caused by dietary fibre (Romarheim *et al.* 2006). Various dietary components may serve as substrates for the gut microbes, and enzymes such as proteases and lipases, bile acid and antimicrobial components will also probably modulate the gut microbiota.

1.7 LUMINAL PH

Information on the pH of digesta along the GI tract of finfish is not well described in the scientific literature. In the stomach the pH values can be below 4, while in the pyloric region and the mid and distal intestine they are above 7 and mostly above 8. The pH of the chyme seems to be regulated within fairly narrow ranges. In the stomach, pH seem to be higher in Atlantic salmon (Gislason *et al.* 1996) compared to mammals (pH 2; Madigan and Martinko 2006). This difference in pH may be of relevance for microbial survival and colonization in the stomach. No marked decrease in the distal intestine has been observed as might have been expected if the microbial activity was high.

1.8 PASSAGE RATE AND RESIDENCE TIME

The passage rate and residence time in the various sections along the GI tract influence the microbial community and subsequently the host and the host–microbial interactions. Stomach evacuation rate and passage time through the intestine have been observed to vary with temperature, meal size, particle size, feed composition, previous nutritional history, fish size and stress (Fänge and Grove 1979; Bromley 1994). Diet is also known to affect passage time (Storebakken *et al.* 1999) and hence may affect microbial colonization in the gut. To our knowledge, no information is available on the relationship between gut microbiota colonization, gut passage rate and residence time, and this topic merits further investigations.

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2 Immune Defences of Teleost Fish

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ABSTRACT

Teleost fish have a well-established innate immune response that is vital for anti-pathogen responses in their antigen-rich environment. With the increase in scientific investigations and the availability of specific reagents, it is clear that these fish also possess pathogen/antigen-specific adaptive responses. These responses are comparable to those of higher organisms, responding to intracellular and extracellular resident pathogens, initiating cell-mediated immunity (CMI) and humoral defences, respectively. This chapter reviews the current understanding of teleost immunity, with particular emphasis on the tissues involved in immune development (thymus, head kidney and spleen) and those under most antigenic stimulation – the skin and mucosal surfaces. In this context, an understanding of teleost immune defences is important for our comprehension of the health benefits conferred by prebiotics and probiotics introduced in feed formulations.

2.1 INTRODUCTION

Teleost fish exhibit a well-developed immune system; they possess a rapid and efficient innate immune system and, in addition, display characteristics of an adaptive antigen-specific immunity which would appear to have all the traits of established mammalian systems but reacts at a much slower rate which is, in part, due to the lower local environmental temperatures. By the nature of their habitat, fish live in an antigen-rich environment and as such their immune systems are continually being challenged by both commensal and pathogenic organisms. It is thus vital that these fish can either recognize beneficial microbes and induce a state of non-responsiveness/tolerance, or activate immune anti-microbial defences to pathogens which are predominantly led by a rapid innate immune response and ideally followed by an antigen-specific adaptive response which will afford a more tailored response to a pathogen

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upon secondary exposure. The relative paucity of reliable, specific reagents to components of the teleost fish immune system has resulted in slow progress in the understanding of immune defences in these fish. Although studies have characterized the presence of immune cells and molecules, they are rarely consolidated by functional studies and, as such, some of our understanding of fish immunology has been obtained by matching observations in the fish system with inferences from more comprehensively characterized mammalian immune systems. The aim of this chapter is to review the current understanding of the immune system in teleost fish in the context of how innate, adaptive and mucosal barrier defences deal with the wide array of pathogenic organisms present in the fish's environment.

2.2 INNATE IMMUNITY

The aquatic environment of teleost fish, by its very nature, is rich in pathogens and antigenic stimuli. As a result, it is vital that the fish immune system is capable of a rapid and robust immune response capable of protecting the host from the plethora of pathogenic insults. To cope with this, teleosts have a well-developed innate immune system which is particularly strong with respect to barrier/mucosal defences. The barriers which are constantly under attack or challenge by such an antigen-rich environment include the skin and the mucous membranes of the gills and the intestinal tract. Epithelial cells are at the forefront of these barrier defences and function as both a physical barrier and an immune instructor, capable of perpetuating either immune tolerance in homeostatic conditions or immune activation in response to pathogen invasion. These cells can protect the host by innate defences which include the secretion of anti-microbial molecules such as lysozyme, cathelicidins, cathepsins and defensins. These serve to break down pathogen cell walls, hence killing them and preventing their multiplication. If this barrier defence is compromised, then the pathogen is faced with a barrage of underlying defences which include a plethora of cellular and soluble components. These innate defences include cells such as macrophages and neutrophils involved in phagocytosis, complementing production and expression of innate inflammatory cytokines; each displays different effector functions which are dependent on the immune cell being activated and by the pathogen activation molecules encountered.

The immediate lines of defence, prior to epithelial cells and those of the underlying innate immune cells, consist of a physical barrier of commensal bacteria and mucus. Commensal bacteria are integral to mucosal immunity and will be covered later in this chapter and in following chapters. In general, commensal bacteria compete with pathogenic microbes for nutrients, binding sites on epithelial cells, and can modulate the immune system to benefit the host. Mucus is also vital to host protection and is produced at mucosal surfaces such as the gills and intestinal tract as well as the external surface of the skin. Mucus exists in a state of constant translocation, being physically removed from the skin and mucosal surfaces, hence trapping and removing pathogens and preventing their attachment to and invasion of host cells (reviewed in Ellis 2001). Skin mucus acts as a lubricant, is involved in locomotion and osmoregulation, and plays a role in the prevention of colonization by pathogens such as bacteria, fungi and parasites. Its biochemical properties afford it chemical defence (Jakowska 1963). The basic components of mucus include macromolecular mucin components (mucopolysaccharides) and glycoproteins. In general, mucus contains many different types of secretory substances with a wide variety of functions. These secretory substances

include the antibacterial peptides (pleurocidin, piscidins, trypsin-like proteases, cathepsins L and B, lysozyme and β -defensins), c-reactive protein (CRP), lectins (involved in carbohydrate recognition, resulting in agglutination, opsonization and complement activation) and immunoglobulins, which confer passive immunity on newly hatched fry who mucus-feed from parental skin (Hildemann 1959; Buckley *et al.* 2010).

In addition to the barrier defence associated epithelial cells, the main effector cells of the teleost innate immune system include neutrophils, monocytes, macrophages and granulocytes such as basophils, eosinophils, mast cells/eosinophilic granule cells (MCs/EGCs) and rodlet cells. All of these play a role in inflammatory responses to acute or chronic infection. The neutrophils and macrophages function as phagocytes in the recognition and clearance of pathogenic materials, and when activated can release anti-microbials as part of the respiratory burst and cytokines to instruct other parts of the immune response (reviewed in Rombout *et al.* 2010). Rodlet cells are thought to be immature eosinophilic granulocytes that play a significant role in defence against parasites; rodlet cell numbers increase in the presence of helminths where aggregations have been observed in infected epithelia of the gills and intestinal tract. MCs/EGCs possess both acidophilic and basophilic granules; these cells are recruited and observed in high numbers in chronically inflamed tissues. Degranulation results in the secretion of acid and alkaline phosphatases, trypsin, nucleotidases, 5-HT, lysozyme and peptide antibiotics (piscidins and pleurocidin). Recruitment, activation and degranulation of these cells have been described in response to the bacterial infection of *Aeromonas salmonicida*, *Renibacterium salmoninarum* and nematode infestation of intestinal tissue (reviewed in Reite and Evensen 2006).

The first step in activation of innate immune effector functions is the recognition of pathogens and their antigens. Pathogen recognition occurs in response to a broad array of conserved pathogen-associated molecular patterns (PAMPs) expressed by the pathogen; these in turn are recognized by their innate counterparts, the pattern recognition receptors (PRRs). This array of PRRs exists as both secreted and membrane-associated recognition receptors. In general, the secreted PRRs include collectins, pentraxins, complement components and mannose binding lectin (MBL). The membrane-associated PRRs exist as either signalling receptors or binding receptors for clearance by phagocytosis. The secreted PRRs such as lectins (MBL, interlectin and pentraxins) and complement components are involved in opsonization, phagocytosis, chemotaxis, inflammation and pathogen killing. The lectin class of molecules is involved in recognition of sugar moieties expressed by pathogens and not normally expressed on host tissues. This class of molecules consists of the collectin MBL, which activates complement cascades via the lectin pathway. MBL recognizes mannose, fucose and N-acetyl-D-glucosamine, acting as an opsonin for recognition and phagocytosis as well as activating serine proteases for proteolytic breakdown (Nakao *et al.* 2006). The pentraxin molecules are composed of five identical subunits and include CRP and SAA. CRP binds phosphocholine moieties of certain bacterial and fungal cell wall lipopolysaccharides, resulting in the activation of the complement cascade via binding to C1q. Another class of lectin molecule has been identified in teleost fish, namely interlectin (reviewed in Vasta *et al.* 2011). So far, this molecule has been demonstrated at the molecular level but functional data have yet to ascertain a clear role for it in innate immunity; mammalian studies, however, have indicated that this class of lectin may be important for the recognition of and responses to bacterial cell wall galactofuranose moieties and protection against intestinal nematode infection (Tsuji *et al.* 2001; Pemberton *et al.* 2004). Finally, the last group of secreted PRRs

includes the complement components which can either act directly on the pathogen or activate membrane-bound PRRs, the complement receptors. Teleost fish produce an extensive array of complement components which include C1q, C1r, C1s, C2, C3, C4, C5, membrane attack complex (MAC) and complement receptors CR3, CR4 and C5aR. As such, complement cascades and their receptors are involved in pathogen recognition, tagging, phagocytic clearance, inflammation and chemotaxis, whereby other cells of the teleost immune system are recruited to the site of pathogenic challenge (Boshra *et al.* 2006). The natural phagocytic cells (macrophages and neutrophils) rely on recognition and phagocytosis of pathogenic material via sugar/lectin interactions, CRP and also C3 binding to LPS in bacterial cell walls, and MBL has been demonstrated to opsonize *Aeromonas salmonicida* in Atlantic salmon in the absence of complement. The recognition and phagocytosis result in release of reactive oxygen species (ROS) such as hydrogen peroxide, nitric oxide, peroxynitrite and hydroxyl radicals as part of the respiratory burst (Secombes 1996; Lamas and Ellis 1994; Ottinger *et al.* 1999). Indeed, this inducible NO response has been observed as a consequence of a faster and greatly elevated expression of iNOS in the head kidney and gill tissue of rainbow trout *Oncorhynchus mykiss* upon infection with *Renibacterium salmoninarum* (Campos-Perez *et al.* 2000).

In addition to soluble PRRs, membrane-bound PRRs are utilized by teleosts in the phagocytic clearance of pathogens (non-signalling membrane-bound PRRs) and immune inflammatory signalling resulting in cytokine expression and immune cell activation (signalling membrane-bound PRRs). Signalling PRRs recognize a broad range of PAMPs and are expressed according to location of pathogen, that is, they may be expressed on the external surfaces of cell membranes responding to extracellular pathogens or may be located on endosomal membranes in response to intracellular pathogens. Teleosts express TLRs 1, 2, 3, 4, 5, 5S, 7, 8, 9, 13, 14, 18, 19, 20, 21, 22 and 23 (reviewed in Jault *et al.* 2004; Rebl *et al.* 2010). The expression of TLRs 1, 2, 3, 4, 5, 7, 8 and 9 is conserved with humans; these PRRs recognize and respond to lipopeptides, dsRNA, LPS, flagellin, ssRNA and CpG DNA, respectively. Teleost fish express the additional receptors TLR14 (lipopeptides), TLR21 (CpG DNA), TLR22 (dsRNA) and TLR23 (PAMP ligand unknown). Thus, in general, bacteria are recognized by TLRs 1, 2, 4, 5, 9, 14 and 21 whereas viruses are recognized by TLRs 3, 7, 8, 9, 21 and 22. Of interest is the observation that TLR5 can exist as a soluble secreted form; whether it acts as a signal enhancer or competitively inhibits flagellin binding to membrane TLR5 has yet to be clarified. Teleost fish display evolutionary conserved expression of the signal adaptor molecules Myd88, Mal, TRIF, TRAM and SARM and the downstream signal molecules IRF3, IRF7 and NF κ B (Purcell *et al.* 2006). Finally, the intracellular receptors NOD1 and NOD2 are also conserved in teleost fish which is suggestive of innate responsiveness to intracellular-resident bacterial pathogens. Of particular importance is the observation that teleost fish also express endogenous negative regulators of TLR signalling such as DIGIRR (Gu *et al.* 2011) and Tollip (Rebl *et al.* 2011); control by these is important to tolerance or the switching off of innate responses to PAMP challenge. Thus, in the absence of these negative regulators, dependent on which PRRs have been activated, the immune cell involved in recognition of the PAMP can be activated and programmed towards either an anti-viral response via IRF3- and IRF7-induced type I interferon production or an inflammatory response via NF κ B-dependent induction of pro-inflammatory cytokines such as TNF α and IL-1 β .

The innate response is dependent on these cytokine signalling molecules produced by cells such as the epithelial cells, macrophages, neutrophils and eosinophils. The cytokine profile secreted determines amplitude and type of immune response to be raised against pathogens.

Cytokines which drive innate responses include TNF α , IL-1 β , IL-6, CK-1, IL-8, IL-10, IL-11, IL-12, IL-15, IL-17, IL-18, and type I and type II interferons. Such immune signals initiate inflammation, induce chemotaxis of immune cells, and regulate inflammation, respiratory burst and pathogen killing responses via induction of soluble molecules or the proliferation and activation of natural killer cells and antiviral responses, preventing viral replication (see Section 2.4).

Finally, the innate immune system, although being very robust in teleost fish, plays a role in the development of a more pathogen- and antigen-specific response upon host re-exposure to the pathogen. Thus, the cells and cytokines of the innate immune system have an important role in the adaptive immune response. The characteristic effector function of cells such as macrophages to phagocytose, process and present antigenic peptides to T cells, coupled with the ability of cytokines to direct T cell differentiation, ensure that the teleost is capable of mounting an antigen-specific adaptive immune response that effectively protects the host from specific pathogen exposure.

2.3 ANTIGEN-SPECIFIC ADAPTIVE IMMUNITY

In an aquatic environment which is highly antigenic, teleost fish are the first vertebrates to exhibit an adaptive immune system, where the response elicited is determined by type of pathogen/antigen and route of entry into the host. They display all the characteristics understood for adaptive immunity: response is antigen specific, slower to develop and displays memory for enhanced or more rapid responses upon re-exposure to the same antigen/pathogen. The teleost immune system must react to a wide array of pathogens such as bacteria, viruses, fungi, protozoa and helminths, and this defence is influenced by temperature, pH, oxygen tension and salinity; in other words the fish immune system and particularly the adaptive response must be capable of functioning under a broad spectrum of environmental conditions (Engelsma *et al.* 2003). Significant changes to these environmental parameters can result in immune deviation or even immune suppression with grave effects on the host's ability to fight off the infectious organism (reviewed in Tort *et al.* 2004). The presence of an adaptive/specific immune response is confirmed by the expression of MHC (gene products for IA, β_2m , IIA and IIB), TCR and immunoglobulin (Ig) as well as the presence of helper and cytotoxic T cells and differential subpopulations of B cells (Stet *et al.* 1996; Miller *et al.* 1998; Scapigliati *et al.* 1999; Nakanishi *et al.* 2002). The presence of an adaptive response, however, does not detract from the importance of the innate defences which, although not antigen/pathogen specific, are defence mechanisms that are mounted by the host in a matter of minutes/hours post infection. The adaptive immune system, by its very nature of developing more sophisticated antigen-specific defences, and taking into consideration the low temperature environment of these fish, is much slower to initiate and may take weeks to months to develop. This makes the innate immune system of utmost importance if a host is to survive primary stages of infection with a pathogen and be able to tailor a specific adaptive response that facilitates pathogen killing and clearance.

Our understanding of functional development and elicitation of adaptive responses in teleost fish is predominantly derived from molecular and immunohistochemical characterization of adaptive markers and by inference from the wealth of understanding of mammalian adaptive systems. With regards to characterization of effector cells of the adaptive immune system,

leukocytes were found to be abundant in the intestinal tract, in particular, in the barrier of the intestinal epithelium and in the underlying lamina propria of the mucosa (Rombout *et al.* 1989b; 1993; Abelli *et al.* 1997). Teleost fish, as a whole, display a lower level of organization of the gut-associated lymphoid tissue (GALT) than mammals; however, lymphocytes, antigen-presenting cells (APCs) and other innate immune cells are diffusely organized along the intestinal tract which is suggestive of both innate and adaptive system readiness to respond to pathogens that infiltrate the mucosal barrier defences. The lack of any comprehensive data regarding the functional role of dendritic cells (DCs) in the gut mucosa of teleost fish may suggest that the predominant APC that primes adaptive responses is the macrophage cell; however, DCs are likely to exist as the DC differentiation marker CD83 has been characterized in teleost and elasmobranch fish (Ohta *et al.* 2004). Gut macrophages have been described in a number of teleost fish (Rombout *et al.* 1986; Georgopoulou and Vernier 1986; Inami *et al.* 2009) and have been shown to increase in density of numbers upon anal/intestinal antigenic challenge and to present antigenic determinants at their surface upon processing (Rombout *et al.* 1985; 1986; Rombout and van den Berg 1989a). These antigenic determinants, like mammalian systems, are presented in the context of MHC molecules where MHC I gene products are expressed on all nucleated cells in teleost fish (Hansen *et al.* 1996); upon vaccination, MHC II was demonstrated to be highly inducible in the head kidney, spleen, hindgut and gills of the Atlantic salmon (Koppang *et al.* 1998). In addition, the abundance of both B and T cell lymphocytes in proximity to these antigen-presenting macrophages and their MHC antigen-presentation molecules infers a clear presentation to and priming of both T cells and B cells involved in MHC I-restricted T cell cytotoxic responses and MHC II-restricted T helper (Th) responses including cell-mediated immunity (CMI) and humoral responses.

2.3.1 T cells: molecular and functional characterization

The presence of conventional T cells in teleost fish was first suggested upon observation of graft rejection and antibody production. Immunohistochemical analysis of gut mucosal tissue has resulted in the characterization of several T cell populations. In addition, molecular studies have demonstrated a wide diversity and repertoire of T cell receptors expressed in teleosts (reviewed in Castro *et al.* 2011). Intestinal intraepithelial lymphocytes were found to express $\alpha\beta$ TCR, CD3, CD4 and CD8 α and even $\gamma\delta$ TCR (Hansen and Strassburger 2000; Nam *et al.* 2003; Bernard *et al.* 2006a; Shang *et al.* 2008). By inference with mammalian systems and some observations made in the fish mucosal immune system, these markers are expressed on Th, cytotoxic T cells (Tc) and $\gamma\delta$ T cells; these cells are integral to cell-mediated cytotoxic immunity, humoral immunity and homeostatic, tolerogenic regulatory responses. Functional analyses have characterized lymphocytes from teleosts to exhibit potent cytotoxic activity to allogeneic and xenogeneic target cells (Fischer *et al.* 2003; Bernard *et al.* 2006a; Toda *et al.* 2009). Thus, it would appear that functionally teleost fish have the capability to induce immune non-responsiveness and tolerate food antigens and commensal organisms of benefit to the host whilst, at the same time, maintaining the capability to positively respond both to pathogens that reside within host cells and those that reside extracellularly. The possible existence of a regulatory T cell subset has been strongly suggested by the identification of two FoxP3 genes (transcription factor associated with natural T_{reg} development) in rainbow trout (Wang *et al.* 2010a). In addition, the same group also demonstrated the sequence and expression analysis of the developmental transcription factors T-bet and GATA-3 that are associated with the development of the T helper subsets Th₁ and Th₂ (Wang *et al.* 2010b).

More recently, with the generation of monoclonal antibodies to CD8 α , capable of being used for flow cytometry, cell sorting, immunohistochemistry, western blotting and immunoprecipitation, teleost T cell subsets have been phenotypically characterized with the ensuing opportunity to characterize functionality. Takizawa and colleagues (2011) have been able to characterize T cell populations and some functionality on the basis of CD8 α expression. CD8 α was expressed in rainbow trout at high levels in the thymus and both intra- and subepithelial T cells of the gills and intestine. In contrast, CD8 α was expressed at lower levels in the pronephros, spleen and blood. The simultaneous transcript expression analysis demonstrated Ig and CD4 expression in the CD8 α ⁻ population, that is, associated with distinct B cell and T_h subsets. Interestingly, in the case of thymocytes, CD4 was co-expressed with CD8 α , suggestive of the thymocyte development stage of double-positive thymocytes prior to single positive CD4⁺ and CD8⁺ T cells. Upon cell sorting of these CD8 α ⁺ and CD8 α ⁻ populations, the CD8 α ⁺ T cells were found to express both CD28 and CTLA-4, where the expression of the immunosuppressive receptor CTLA-4, suggestive of T_{reg} maintenance of mucosal homeostasis, was highly expressed in mucosal lymphocytes and much less so in non-mucosal lymphocytes. PHA stimulation of these populations resulted in the CD8 α ⁻ cells expressing both Th₁ (IFN γ) and Th₂ (IL-4/13A) cytokines, whereas the CD8 α ⁺ cells expressed the characteristic CTL functional effector molecules, perforin and granulysin. Previously, functional alloantigen-specific cytotoxicity has been demonstrated for ginbuna crucian carp (*Carassius auratus langsdorfii*) CD8 α ⁺ lymphocytes (Toda *et al.* 2009) and the killing mechanism was perforin dependent (Toda *et al.* 2011a). Finally, although the Th₁₇-associated transcription factor ROR γ T homologue has not yet been characterized in teleost fish, the presence of such a Th₁₇ subset has been suggested by the expression of Th₁₇-associated cytokines IL-17 and IL-22, whilst the Th₁₇ differentiation cytokines have also been described (see Section 2.4).

Expanding on currently available data on transcript expression analysis, by the development of specific antibodies to T cell subsets through the recognition of subset-specific transcription factors, specific membrane molecules in combination with functional readouts such as effector cytokines, it will be possible to fully characterize teleost T cell subsets present and inducible during antigenic challenge and to investigate their functional activity involved in adaptive immune responses to pathogenic infection.

2.3.2 B cells, immunoglobulins and humoral immunity

Immunoglobulins are involved in precipitation, agglutination, neutralization, opsonization and complement activation. As a result, they are likely to function in accord with observations made for antibody function in higher animals/mammals, where immunoglobulins confer humoral immune protection involved in prevention of infection, clearance and anti-pathogen killing responses aimed at extracellular resident pathogens. Ig⁺ cells have been described in European sea bass (*Dicentrarchus labrax*), salmonids and cyprinids (Rombout *et al.* 1993; Abelli *et al.* 1997). In comparison to humans, whereby functional characteristics are associated with expression of five main antibody isotypes (IgM, IgD, IgG, IgA and IgE), until recently teleost fish were thought to generally express IgM and IgD isotype antibodies which predominate in serum and the periphery (Flajnik 2002), where IgD exhibits a restricted expression pattern in the spleen and head kidney and IgM expression is observed in renal haematopoietic tissue, spleen and thymus with lower levels in the skin and gill. Antibody responses in teleost fish are generally slow, for example antibody production takes 4 to 6 weeks in salmonids, and are of restricted diversity as a consequence of a lack of recombination by class switching

(Saunders and Magor 2004), although somatic hypermutation has been demonstrated (Flajnik 2002). This lack of isotype diversity (isotopy) is overcome by cross-linking of basic IgM antibody subunits resulting in a range of antibody complexes: at least six structural isoforms exist as a consequence of structural reorganization of basic monomer subunits into monomers, dimers, trimers and tetramers (Sanchez and Dominguez 1991; Evans *et al.* 1998; Kaattari *et al.* 1998). IgM is found in the cutaneous mucus of the Atlantic salmon, *Salmo salar*, but not in the gut mucus; indeed serum IgM, when added to gut mucus, was readily degraded by proteolytic enzymes present (Hatten *et al.* 2001). Mucosal expression of antibodies has been generally difficult to characterize. Fish do not express the classical mucosal antibody IgA; however, recently a new third teleost Ig heavy chain isotype of antibody has been observed as a specialized mucosal isotype which is secreted in mucus. These new antibodies have been termed IgZ, IgT and IgF on the basis of being first described in mucosal secretions of zebrafish *Danio rerio* (IgZ: Danilova *et al.* 2005; Flajnik 2005), rainbow trout (IgT: Hansen *et al.* 2005) and fugu (IgF: Savan *et al.* 2005). In the common carp (*Cyprinus carpio*), however, two IgZ subclasses have been described that display specific expression profiles which is suggestive of unique humoral immune functions; where IgZ₁ is expressed in systemic organs (head kidney, trunk kidney, spleen and PBLs in the blood) and IgZ₂ is expressed in the mucosa of the gills and gut; both subclasses are transcribed as a membrane form and a secreted form (Ryo *et al.* 2010). Challenges with the mucosal *Lernaea* parasite were observed to induce predominant expression of IgZ₂, whereas the blood parasite *Trypanoplasma borreli* induced IgZ₁. These challenge expression studies are suggestive of functionality whereby IgZ₁ plays a role in protection/immunity against blood parasites and IgZ₂ is protective against mucosal parasites (Ryo *et al.* 2010). In addition, Ig expression is dependent on the route of challenge by pathogens and their pathogenic antigens; studies investigating mucosal immunization of carp resulted in specific antibody-secreting plasma cells in the mucosa of the gut and gills, and no plasma cells were detected in the blood, head kidney or spleen. No such antibody-secreting plasma cells were observed upon systemic immunization. Oral immunization with *Vibrio anguillarum* antigens resulted in high numbers of plasma cells present in the gills (Rombout *et al.* 1989c; Joosten *et al.* 1997; Cain *et al.* 2000; dos Santos *et al.* 2001).

Teleost humoral responses can be further compared to human and mammalian systems on the basis of the existence of B cell subsets, responsiveness to PAMPs (generally recognized by innate responses) and the ability to passively transfer immunity to fry. As with mammalian systems, B cells and immunoglobulin production can be stimulated by both T cell-dependent and T cell-independent antigens. In addition to pathogen-specific peptide antigens presented by MHC and requiring T cell-dependent activation of B cells, the bacterial PAMP, LPS, directly stimulates fish B cell proliferation and induction of antibody responses in the absence of T cell involvement and memory (Dalmo and Seljelid 1995; Salati *et al.* 1987; Jakobsen *et al.* 1999; Aakre *et al.* 1994; and reviewed in Swain *et al.* 2008). Indeed, vaccination of carp with *Vibrio anguillarum* antigens, which are rich in LPS, induces IgM and IgZ₁ expression in the head kidney and gut tissue, respectively (Ryo *et al.* 2010). Additionally, LPS has been demonstrated to up-regulate mIgM-2, a novel membrane IgM isotype in zebrafish, widely expressed in immune-related tissues such as the intestine, kidney and skin (Hu *et al.* 2011). This T cell-independent, PAMP-activated B cell and antibody isotype-specific response is suggestive of a distinct B cell subset analogous to the human B1 subset. Finally, due to the fact that tailored adaptive responses take a relatively long time to be initiated and that development of an immune repertoire in fish is slow, it is vital that some level of adaptive immune

protection is passed on to fry as soon as possible in their development. Like mammalian systems, this early protection is afforded to the fry passively. With regard to the ontogeny of humoral responses, IgM and IgZ were detectable 1 to 4 days post fertilization, with the first B cells detected from 7 days post fertilization (Romano *et al.* 1997a); it is probable that early antibodies were of maternal origin. Indeed, parenteral immunization or passive immunity may be conferred through mucus feeding of fry, where studies have demonstrated the elevated presence of IgM-like antibodies in the skin mucus of parent fish (Buckley *et al.* 2010). Thus, the humoral response of teleost fish, although slower to initiate than that of mammals/humans, would appear to exhibit all the cellular and molecular complexities already characterized in their warm-blooded mammalian counterparts. Further vaccination/challenge studies will elucidate functional B cell/antibody phenotypes involved in tissue-specific protection against a variety of fish pathogens present in such an antigen-rich environment.

2.4 CYTOKINES DRIVE IMMUNE RESPONSIVENESS

The development, activation and functionality of immune cells such as T and B lymphocytes are characterized by specific profiles of their cytokine signalling molecules and their receptors. In the relative absence of species-specific antibodies for detection of cytokine proteins, many researchers have found mRNA expression of cytokine genes a useful tool for analysing immune responses elicited in fish and interpreting functional outcomes through comparison with the wealth of functional cytokine data available in mammalian systems. A wide array of cytokine homologues have been described as being expressed by teleost fish; many of these cytokines are likely to play an important role in differentiation, initiation, activation and regulation of both innate and adaptive immune responses (reviewed in Aoki *et al.* 2008). The homologues so far described include IL-1 β (Hong *et al.* 2004), IL-4R α (Wang *et al.* 2011a), IL-6 (Bird *et al.* 2005b; Iliev *et al.* 2007), IL-11 (Wang *et al.* 2005), IL-12 p40/p35 subunits (Nascimento *et al.* 2007), IL-13R α 1 and IL-13R α 2 (Wang *et al.* 2011a), IL-15 (Wang *et al.* 2007), IL-17 gene products (Wang *et al.* 2010b), IL-18 (Zou *et al.* 2004), IL-20 (Wang *et al.* 2010c), IL-21 (Wang *et al.* 2011b), IL-22 (Monte *et al.* 2011), IL-23 α chain (Holt *et al.* 2011), IFN γ (Zou *et al.* 2005), TNF α (Laing *et al.* 2001) and type I antiviral interferons (Robertson *et al.* 2003). Adaptive regulatory cytokines and their signalling molecules already described include TGF β , IL-2 and two IL-10 gene products (Daniels and Secombes 1999; Zou *et al.* 2003; Bird *et al.* 2005a; Diaz-Rosales *et al.* 2009; Harun *et al.* 2011) and SOCS-1-3 (Wang and Secombes 2008). By inference, IL-12, IL-18, IL-2, TNF α and IFN γ are all involved in the development and effector functions of Th₁ cells that drive CMI responses (Tc activation and delayed type hypersensitivity (DTH)) to intracellular-resident pathogens such as the bacterium *Mycobacterium marinum* and infectious pancreatic necrosis virus (IPNV). IL-4R α , IL-13R α 1 and IL-13R α 2, IL-20 and possibly IL-10 are likely to be involved in Th₂-driven humoral antibody-mediated immunity to extracellular pathogens. IL-1 β , IL-6, IL-23 and IL-17 are implicated in Th₁₇-mediated anti-fungal responses through the activation of granulocytic cells. Additionally, the expression of IL-10, TGF β and IL-2, in combination with FoxP3 characterization, is suggestive of the presence and the suppressive regulatory function of natural T_{reg}. Finally, the expression of IL-15, IL-21 and IL-22 may also indicate the format of immune response being mediated. IL-15 is a gamma-chain receptor utilizing cytokine that promotes T cell growth, in particular CD8⁺ memory T cells and IELs as well as NK cells. In addition,

IL-21 plays a role in innate and adaptive responses (both CMI and humoral) by inducing proliferation of T, B and NK cells. Both IL-17 and IL-22 are generally produced by Th₁₇ cells and induce pro-inflammatory cytokine production by epithelial cells, affecting epithelial cell turnover and mucosal barrier integrity. Thus far, little functional evidence exists in teleost fish for the role played by these cytokine homologues. Future research investigating functional aspects of these fish cytokines will further expand our understanding of cellular and molecular aspects of teleost immunity against aquatic pathogens.

The evidence presented thus far suggests the existence of many of the cellular and molecular components required to mount efficient adaptive responses to specific fish pathogens, whether they be intracellular resident or extracellular. The existence of APCs, MHC I and MHC II, CD8⁺ Tc, CD4⁺ Th subsets and B cell subsets expressing immunoglobulin points to teleost fish having the full capability of mounting humoral responses as well as CMI responses such as Tc-cytotoxicity and DTH responses. In a functional context, DTH, cell-mediated lympholysis (CML) and graft versus host reactions have been described in teleost fish (Nakanishi *et al.* 1999 and reviewed in Manning and Nakanishi 1996 and Nakanishi *et al.* 2002). These responses utilize many of the potential cellular and molecular mechanisms alluded to by the wealth of cloning and expression data that exist for teleosts. In general, there is still a long way to go in the functional characterization of teleost adaptive immune responses; however, all the basic adaptive components are present to be able to interpret immune observations in teleost fish in the context of the more comprehensive understanding that exists in human/mammalian systems. Studies investigating the interrelations between immune cell function, tissue expression and co-expression with other immune cell subsets will be vital to our overall understanding of the teleost immune system.

2.5 IMMUNE TISSUES

The fish immune system developed as a form of protection from aggressive life forms to ensure survival of the individual to reproductive age and consequently ensure survival of the species. It would seem that the driving force that led to the evolution of organized lymphoid structures in prehistoric fish was linked to the primary route of pathogen entry into the organisms, a process which in general occurs through mucosal surfaces (Matsunaga and Rahman 2001; Boehm and Bleul 2007). Looking at the modern bony fish (teleosts), the immune system was found to be compartmentalized into specialized organs and to use specialized cell types to defend against different pathogen challenges. Lymphoid organs are important regulators of lymphocyte development and immune responses and it has been suggested that they evolved to facilitate antigen-receptor gene assembly (variable-diversity-joining-type recombination) (Boehm and Bleul 2007). Teleosts have two primary lymphoid organs, the thymus and a suggested bone marrow analogue located in the head kidney, which may also serve as a secondary lymphoid organ together with the spleen. Apart from functioning as physical barriers separating host animals from the external environment, mucosae are also active immunological sites that protect fish against exposure to microbes and stressors. These tissues constitute the mucosa-associated lymphoid tissue (MALT) that, in teleosts, can be subdivided into three main immune compartments: skin-associated lymphoid tissue, gill-associated lymphoid tissue and gut-associated lymphoid tissue (GALT). In contrast to mammalian germinal centres, B cell follicles and lymph nodes have not been reported in teleost fish.

2.5.1 The thymus

About 500 million years ago, a new type of adaptive immune defence emerged in basal jawed vertebrates, accompanied by morphological innovations, including the thymus. The thymus is a primary lymphoid organ that is found in all vertebrates with the exception of jawless fish, such as the lamprey, in which, however, discrete thymus-like lympho-epithelial structures, termed thymoids, have been identified. The thymoids were found in the tips of the gill filaments and the neighbouring secondary lamellae (both within the gill basket) of lamprey larvae. Only in the thymoids the an expression of the orthologue of the gene encoding forkhead box N1 (FoxN1), a marker of the thymopoietic microenvironment in jawed vertebrates, accompanied by expression of genes encoding type A variable lymphocyte receptors (VLRA) and cytosine deaminase 1 (CDA1), found (Bajoghli *et al.* 2011).

The thymus first appears as an identifiable organ in the chondrichthyes and osteichthyes (Rasmussen and Arnason 1999) and its emergence in evolution parallels the appearance of a VDJ recombination as a novel means of somatically diversifying antigen receptors (Boehm and Bleul 2007). The thymus has the unique capacity to support the development of self-tolerant T cells expressing a diverse repertoire of antigen receptors and the production of self-restricted T cells (Manley 2000) providing the appropriate microenvironment. Generally, the thymus develops in the lamina propria of the gastrointestinal tract (GIT) in pouches located at the base of the gill arches; its development can be traced through evolution, beginning in early fish species as a thickening in the epithelium of the pharyngeal area of the GIT. In teleosts the thymus appears as a pair organ located dorsally beneath the operculum and covered by a mucosal epithelium (Zapata and Amemiya 2000; Matsunaga and Rahman 2001; Bowden *et al.* 2005; Langenau and Zon 2005). Its parenchyma consists of lymphocytes, macrophages, dendritic/interdigitating cells and myoid cells, while the stroma is composed of cells of epithelial morphology (Zapata *et al.* 1996).

Anatomical studies demonstrated that the differentiation of the thymic structure is highly variable among fish species (Bowden *et al.* 2005). In fact in many fish there are no clear zones to the thymus (Luer *et al.* 1995; Liu *et al.* 2004), while in European sea bass (Abelli *et al.* 1994), turbot *Scophthalmus maximus* (Fournier-Betz *et al.* 2000), zebrafish (Danilova *et al.* 2004), halibut *Hippoglossus hippoglossus* (Bowden *et al.* 2005), rainbow trout (Fisher *et al.* 2005), common carp (Huttenhuis *et al.* 2005) and Atlantic salmon (Koppang *et al.* 2003), studies have observed a distinction between outer (cortex) and inner (medulla) regions of the thymus, even if the lack of a corticomedullary junction persists. The thymus possesses a capsule of epithelial cells enclosing a cortex of lymphoid tissue within which are lymphocytes whose normal development is dependent upon cell interactions with thymic stroma and secreted molecules. The capsule invaginates into the organ producing trabeculae giving passage to capillaries (Manley and Blackburn 2003) and enclosing a 3D structure that is considered important for positive selection (affinity to MHC) to allow sufficient interaction with the MHC molecules of the reticular epithelium. In this respect the expression of MHC class I and II molecules found in rainbow trout thymus suggested a possible role to mediate the acquisition of a T cell repertoire by participating in the positive and negative selections of thymocytes (Fisher *et al.* 2005). In teleosts, the thymic microenvironment is the site of T cell maturation in a dynamic process that, as described in mammals, is fed by the recruitment of early thymocyte progenitors from the bloodstream and culminates in the export of mature thymocytes back into circulation (Ladi *et al.* 2006). In zebrafish, it has been well documented that early steps in T cell (thymocyte) development and thymic organogenesis are similar in

fish and higher vertebrates, as judged by histology and gene expression patterns employing mutants and several immune-relevant genes such as RAGs, TCRs, Ikaros (which encodes a family of haematopoietic-specific zinc finger proteins), *lck* etc. (reviewed in Langenau and Zon 2005). Although evidence of intrathymic apoptosis has been obtained in the thymus of European sea bass and common carp, indicating the occurrence of negative selection of T cells in fish (Abelli *et al.* 1998; Romano *et al.* 1999), little is known about the late stages of T cell development within fish thymus, particularly from double negative (DN, $CD4^-CD8^-$) to single positive (SP, $CD4^+CD8^-$ and $CD4^-CD8^+$) thymocytes. In this context a new hypothesis of positive selection and differentiation of T cells has been proposed in European sea bass juveniles by Picchiatti *et al.* (2008; 2009), providing new evidence about the functional compartmentalization of the teleost thymus, with a specific role in thymocyte differentiation pathways. The authors identified $CD4^+$ and $CD8\alpha^+$ thymocytes, in each adult thymic lobe, that largely overlapped and filled the cortex, while in the medulla $CD4^+$ thymocytes were isolated and $CD8\alpha^+$ were mainly arranged in cords. These data suggested that intrathymic T cell development in fish is similar to that in mammals, where $\alpha\beta$ T cells mature through a $CD4/CD8$ double-positive stage giving rise to further selection in the medulla to become single positive thymocytes. More recently, Takizawa and colleagues (2011) found high ratios of $CD8\alpha^+$ cells in trout thymus and considerable $CD4$ expression in $CD8\alpha^+$ thymocytes, reminiscent of the $CD4^+/CD8^+$ double-positive cortical thymocytes of mammals (Ellmeier *et al.* 1999; Laky *et al.* 2006). In addition, rainbow trout extrathymic $CD8\alpha^+$ lymphocytes strongly expressed $CD8\alpha$ and $-\beta$ and very low amounts of both $CD4-1$ and -2 , while extrathymic $CD8\alpha^-$ lymphocytes expressed $CD4s$ but not $CD8s$. Such an expression pattern was in accordance with the presence of both $CD8^+CD4^-$ and $CD4^+CD8^-$ T cell subsets (Takizawa *et al.* 2011). Similarly, Toda *et al.* (2011a; 2011b) confirmed the presence of teleost $CD4^+$ and $CD8\alpha^+$ T cell subsets with morphology, tissue distribution and gene expression in clonal ginbuna crucian carp similar to those of mammalian $CD4^+$ and $CD8^+$ T lymphocytes. The authors defined that more than 15% of the cells in the ginbuna thymus were $CD4/CD8$ DP T cells, while negligible numbers of DP cells were present in other tissues, suggesting that the thymus is the site of T cell development, although the precise differentiation process awaits further investigation.

2.5.2 The head kidney and spleen

The kidney is a paired organ that has been described as having various anatomical and functional compartments. As in all vertebrates, the kidney is located retroperitoneally, exterior to the dorsal wall or the body cavity. The foremost part of the teleost kidney lacks excretory tissue and is often referred to as the head kidney or pronephros. In teleosts, the head kidney is an important haematopoietic organ (Fänge 1986). In fact, studies performed by Kobayashi *et al.* (2006) showed that haematopoietic stem cells, having repopulation and multilineage potential, reside in the ginbuna kidney, particularly in the trunk kidney. The head kidney is the site of antibody production (Rombout and Joosten 1998b; Press and Evensen 1999) and the melanomacrophage accumulations of the parenchyma are able to retain antigens for long periods of time after administration or vaccination (Lamers and de Haas 1985; Herraez and Zapata 1986; Tsujii and Seno 1990; Brattgjerd and Evensen 1996), which possibly has a role in immunological memory (Press *et al.* 1996).

It has been suggested that the head kidney is the bone marrow analogue, being the major source of B cells (Meseguer *et al.* 1995; Zapata *et al.* 2006; Koppang *et al.* 2003; Zwollo *et al.* 2005). In rainbow trout this is suggested by a high expression of Ikaros, TdT, RAG 1

and RAG 2 (Hansen and Kaattari 1995; 1996; Hansen *et al.* 1997; Hansen 1997). In addition, multiple forms of germline IGI transcripts are expressed in the head kidney of both rainbow trout and Atlantic cod *Gadus morhua* (Daggfeldt *et al.* 1993; Partula *et al.* 1996). As germline transcription correlates with accessibility to the V(D)J recombination machinery (Gorman and Alt 1998), this feature suggests an ongoing development of B cells. Maturation of B cells in the head kidney appears to be a continuous process during the development of rainbow trout (Hansen and Zapata 1998) and it has been speculated that developing B cells can mature in the head kidney and then migrate to the spleen and the mid kidney for activation (Zwollo *et al.* 2005). In this context subsequent analysis of the entire rainbow trout kidney, divided into five contiguous segments K1–K5, reveals a complex pattern of early developing B cells, late developing B cells, and IgM-secreting cells. Patterns in anterior kidney segment K1 were most similar to those of mouse bone marrow, while the most posterior part of the kidney, K5, had many IgM-secreting cells, but lacked early developing B cells. A potential second B lymphopoiesis site was uncovered in segment K4 of the kidney (Zwollo *et al.* 2010). Toda *et al.* (2011a; 2011b) found the absence of CD4/CD8 double-positive T cells in the ginbuna kidney and in the other lymphoid organs with the exception of the thymus, implying that T cell development may not occur in the teleost kidney; then pre-T cells (lymphoid progenitors) residing particularly in the cyprinid trunk kidney could migrate via the head kidney or directly to the thymus for differentiation and functional maturation. Flow cytometric analysis of rainbow trout cells showed low percentages of CD8 α^+ cells in splenocytes (approximately 2%) and pronephrocytes (approximately 4%). Accordingly, tissue sections revealed many CD8 α^+ cells in the thymus, intestine and gill, with only a few scattered CD8 α^+ cells in the spleen and pronephros (Takizawa *et al.* 2011). At the same time it has been demonstrated that the head kidney serves as a secondary lymphoid organ important in the induction and elaboration of immune responses (Kaattari and Irwin 1985) and the spleen, like the head kidney, is another important source of B cells in adult teleosts. It may also serve as a secondary lymphoid tissue where mature lymphocytes are activated by antigens and differentiate into antibody-secreting cells (Kaattari and Irwin 1985; Zapata and Cooper 1990; Bromage *et al.* 2004; Zwollo *et al.* 2005). The spleen has a fibrous capsule and small trabeculae extend into the parenchyma, which can be divided into red and white pulp. The red pulp, which may occupy the majority of the organ (Grace and Manning 1980; Secombes and Manning 1980), consists of a reticular cell network supporting blood-filled sinusoids that hold diverse cell populations including macrophages and lymphocytes. The white pulp is often poorly developed but may be divided into two compartments: the melanomacrophage accumulations and the ellipsoids, which appear to have a specialized function for plasma filtration and the trapping of blood-borne substances, particularly immune complexes (Secombes and Manning 1980; Espenes *et al.* 1995; Solem and Stenvik 2006). In Atlantic salmon scattered CD3 ϵ^+ cells were found throughout the spleen with a majority of cells detected in the white pulp surrounding ellipsoids of the organ (Koppang *et al.* 2010). This distribution of T cells in the white pulp was similar to the periaarteriolar lymphoid sheet as described in mammals (Ruddle and Akirav 2009), whose white pulp has been described as an organized lymphoid structure (Ruddle and Akirav 2009) and this seems to apply in salmonids. Recently Barr *et al.* (2011) defined four major trout B cell subsets present during terminal B cell differentiation in rainbow trout spleen using the transcription factor XbpI, concluding that trout B cells follow a highly conserved activation pathway, albeit slower than that observed in mammalian species. In addition it has been demonstrated that the teleost spleen possesses abundant IgM $^+$ mature B cells (Kaattari

and Irwin 1985; Irwin and Kaattari 1986; Zapata and Cooper 1990; Zwollo *et al.* 2005; 2008) and IgM-secreting cells are generated in LPS-activated cultures derived from splenic B cells (Bromage *et al.* 2004). In this context, analysis of Ig isotype expression demonstrated differential expression patterns between the spleen and the head kidney. In particular the head kidney of Atlantic salmon (Tadiso *et al.* 2011) showed the highest expression of IgM, IgT and IgD followed by the spleen, while in the thymus all Ig transcript levels were much lower in accordance with previous studies (Stenvik and Jorgensen 2000; Stenvik *et al.* 2001; Hirono *et al.* 2003; Hansen *et al.* 2005; Saha *et al.* 2005; Tian *et al.* 2009). In particular, IgM transcripts were most abundant followed by IgT, especially in the head kidney and spleen, as compared to IgD, indicating that IgT is the dominant Ig next to IgM (Tadiso *et al.* 2011). The expression and tissue distribution patterns of IgT, reported simultaneously as the orthologous molecule in zebrafish, named IgZ (Danilova *et al.* 2005; Hansen *et al.* 2005), however, can exhibit considerable variation in different species. In fact, in Atlantic salmon (Tadiso *et al.* 2011) and rainbow trout (Hansen *et al.* 2005), IgT is expressed in various tissues, especially in the spleen and head kidney; in adult zebrafish, however, the expression of IgZ is limited to primary lymphoid organs, including the thymus (Danilova *et al.* 2005). In contrast, transcripts of a newly discovered zebrafish IgZ-2 isotype are widely expressed in both primary and secondary lymphoid organs (Hu *et al.* 2010).

2.5.3 The skin-associated lymphoid tissue

Skin is a metabolically active tissue (Bullock and Roberts 1974) that has been thought to play a rather passive role in protective immunity, serving as an anatomical and physiological barrier against the external environment. In addition to being a mechanical barrier, the fish skin has other roles including the secretion of mucus, which contains innate immune factors such as proteases, antibacterial agents and other immune molecules (Suzuki *et al.* 2003; Easy and Ross 2009), and it has been shown as having an active immunological role against parasitic infection (Lindenstrom *et al.* 2004; Singh *et al.* 2004a; 2004b; Gonzalez *et al.* 2007a; 2007b; Forlenza *et al.* 2008). Its importance as a vital immune organ within the mucosal immune system was demonstrated when transcript analysis of common carp revealed 82 orthologues of immune-relevant genes previously described in other organisms (Gonzalez *et al.* 2007a). Likewise, microarray analysis of Atlantic salmon skin after infection with the louse *Lepeophtheirus salmonis* showed changes in the expression of genes belonging to immune response, oxidative stress, protein folding and cytoskeletal/structural proteins (Skugor *et al.* 2008). In addition a recent study performed by Caipang *et al.* (2011) evaluated the transcription profiles of selected genes involved in the cutaneous immune defence of Atlantic cod. Specifically, the expression levels of genes related to antibacterial activity, antiviral response, cytokine production, glucose transport, stress response and anti-apoptotic activity were quantified in the dorsal and ventral regions of the skin of Atlantic cod and, in general, the highest expression was found at the latter side. Analysis of skin mucosal proteomic mapping ascertained that skin mucosa in Atlantic cod is a veritable source of several important proteins, many of them considered immune competent molecules (galectin-1, mannan binding lectin, serpins, cystatin B, cyclophilin A, FK-506 binding protein, proteasome subunits alpha-3 and -7, ubiquitin, and g-type lysozyme) (Rajan *et al.* 2011).

Adaptive and innate immune elements are detectable in teleost skin; in fact in addition to secretory cells, leukocytes such as granulocytes, macrophages and lymphocytes (Iger *et al.* 1988; Peleteiro and Richards 1990; Davidson *et al.* 1993; Herbolme *et al.* 2001) have been

observed in the skin of different teleost species. In particular, B cells were found in common carp (Rombout *et al.* 1993), rainbow trout (St Louis-Cormier *et al.* 1984), spotted wolffish *Anarhichas minor* Olafsen (Grøntvedt and Espelid 2003) and channel catfish *Ictalurus punctatus* skin (Zhao *et al.* 2008). In this latter species the numbers of IgM-secreting cells increased 20-fold following immunization with the protozoan parasite *Ichthyophthirius multifiliis*, showing that it plays a critical role in host defence against surface infection (Zhao *et al.* 2008). In addition the ciliate parasite *Cryptocaryon irritans* induced specific IgM responses in the skin of grouper (*Epinephelus coioides*) after immunization by surface exposure or intraperitoneal injection (Luo *et al.* 2007). Functional studies on the skin secretory system, however, are nevertheless scarce and always refer only to IgM levels; how sIgs interact with antigens at a mucosal surface, which is constantly exposed to water and swimming forces, is completely unknown. Only recently has the expression of pIgR on common carp (Rombout *et al.* 2008) and fugu (Hamuro *et al.* 2007) skin been discovered. In particular, biochemical analysis of fugu skin mucus showed that the pIgR is associated with fugu IgM (Hamuro *et al.* 2007), while in carp it was concluded that skin epithelial cells have IgM at their surface and it was suggested that pIgR is at least one or even the major Ig receptor playing a significant role in mucosal immunity (Rombout *et al.* 2008).

2.5.4 The gill-associated lymphoid tissue

Teleost gills are large mucosal surfaces that comprise the main respiratory surfaces of the fish, and in addition with the intestine are considered important immune organs capable of mounting robust immune responses (Paulsen *et al.* 2001; Penissi *et al.* 2003; Smith and Fernandes 2009). The gills are also considered as portals of entry for fish pathogens (Ellis 2001; Holzer *et al.* 2003; Grove *et al.* 2006; Mulero *et al.* 2008a) and resistance to infection and recovery is facilitated in part by innate non-specific immunity consisting of a plethora of constitutively expressed elements as well as induced components of the inflammatory response (for review, see Dickerson 2009). The gills of modern bony fish consist of four paired arches, each containing two rows of posteriolaterally oriented filaments with lamellae covered by respiratory epithelium. The filaments are supported along the proximal third of their length by an interbranchial septum of connective and muscle tissue (Wilson and Laurent 2002). Recent morphological studies on Nile tilapia (*Oreochromis niloticus*) have described the fine structure of the branchial filaments, suggesting that the filament's superficial layer is involved in gill osmoregulation whereas the deep layer, through immune and neuroendocrine systems, acts in the regeneration and defence of the tissue (Monteiro *et al.* 2010). Small and large lymphocytes (Lin *et al.* 1998; Grove *et al.* 2006), macrophages (Lin *et al.* 1998; Mulero *et al.* 2008b), neutrophils (Lin *et al.* 1998), eosinophilic granulocytes (Barnett *et al.* 1996; Lin *et al.* 1998; Mulero *et al.* 2007) and antibody-secreting cells (Davidson *et al.* 1997; dos Santos *et al.* 2001) have been observed in the gill-associated lymphoid tissue of different fish species. However, in contrast to the intestinal epithelium, the function of the various types of leukocytes in the gills has been poorly investigated. It has been suggested that their roles are presumably to capture foreign substances and kill infectious agents that gain entry from the water, and in particular it has been proposed that gill phagocytes participate in the clearance of foreign substances from the blood.

To date, very few studies have examined the distribution and role of B cells in the gill arch and filaments of teleost fish (Davidson *et al.* 1997; Grøntvedt and Espelid 2003; Savan *et al.* 2005; Grove *et al.* 2006), however the presence of IgM⁺ cells in the gill mucosa of different

fish species is conflicting. In fact, IgM⁺ cells have been detected in the gill filament epithelium of halibut and in spotted wolffish (Grøntvedt and Espelid 2003), while in cod IgM⁺ cells were detected in connective tissue and blood plasma, similar to the findings in Atlantic salmon (Schröder *et al.* 1998; Haugarvoll *et al.* 2008). Moreover, both IgM⁺ and IgZ⁺ B cells were described along the gill filaments of mandarin fish *Siniperca chuatsi* (Tian *et al.* 2009), while IgZ⁺ cells were also detected in the gill of fugu (Savan *et al.* 2005). Interestingly, in salmonid interbranchial lymphoid tissue (Haugarvoll *et al.* 2008; Koppang *et al.* 2010), very few IgM⁺ cells were detected while abundant T cells were embedded in a meshwork of epithelial cells, showing an organization that has no resemblance to that previously described in lymphoid tissues (Koppang *et al.* 2010). Takizawa *et al.* (2011) found that in contrast to leukocytes from blood and secondary lymphoid organs of rainbow trout, CD8α⁺ cells were abundant in the mucosal respiratory (gill) tissue. In the gill, CD8α⁺ cells were present not only in the epithelium of secondary lamellae but also in lymphoid structures located at the surface of gill arches and primary lamellae, as well as at the basal junction between primary lamellae, which is akin to the situation in healthy Atlantic salmon (Hetland *et al.* 2010) and the organization of salmonid interbranchial lymphoid tissue (Haugarvoll *et al.* 2008; Koppang *et al.* 2010). The high abundance of T lymphocytes in the teleost gill can be explained by considering that the gill provides an efficient gas exchange with the aqueous environment but in fact possesses a thin gill epithelium and the protecting mucus layer is relatively sharp, thus increasing the risk of pathogen entry.

2.5.5 The gut-associated lymphoid tissue

The immune system of the gut is referred to as gut-associated lymphoid tissue (GALT); the piscine gut immune system is quite different from that of mammals. In mammals, the GALT consists of both organized lymphoid tissues, such as mesenteric lymph nodes (MLN) and Peyer's patches (PP), and more diffusely scattered lymphocytes in the intestinal lamina propria (LP) and epithelium including large numbers of IgA⁺ plasmablasts (Forchielli and Walker 2005). Essential to mucosal function in the follicle-associated epithelium are the M cells, which are specialized enterocytes facilitating antigen transport through the epithelium (Beier and Gebert 1998; Owen 1999; Kyd and Cripps 2008). M cells promote adherence and uptake of foreign macromolecules, particles and microorganisms (Neutra *et al.* 2001) that are actively transported to the underlying lymphoid tissue, resulting in IgA class switching (Cerutti 2008) and secretion of high amounts of dimeric IgA (Brandtzaeg and Pabst 2004; Brandtzaeg *et al.* 2008). The secreted IgA is bound by the polymeric Ig receptor (pIgR) and transcytosed to the intestinal lumen (or to the bile in the liver) to coat and protect mucosal surfaces against harmful microbial invasion. In contrast to mammals, the intestinal mucosal surfaces of teleost fish are arranged in folds rather than as villi and do not have crypts of Lieberkühn (Ng *et al.* 2005; Wallace *et al.* 2005) (Figure 2.1). Although teleost fish possess an adaptive immune system, characterized by the presence of key antigen receptors of the immunoglobulin superfamily (Cooper and Alder 2006), they lack lymph nodes and Peyer's patches. They do, however, have epithelial cells that share morphological similarities with immature mammalian M cells, whose phenotype may represent evolutionary early antigen-sampling enterocytes (Fuglem *et al.* 2010). As far as teleost research extends, dendritic cells (DC) are not known to be well established in the mucosae. Such cells in mammals directly take up luminal antigen (Brandtzaeg and Pabst 2004; Brandtzaeg *et al.* 2008); however, cells with dendritic appearance (MHC II⁺) have been identified in the second gut segment of Atlantic salmon (Koppang *et al.* 2003).

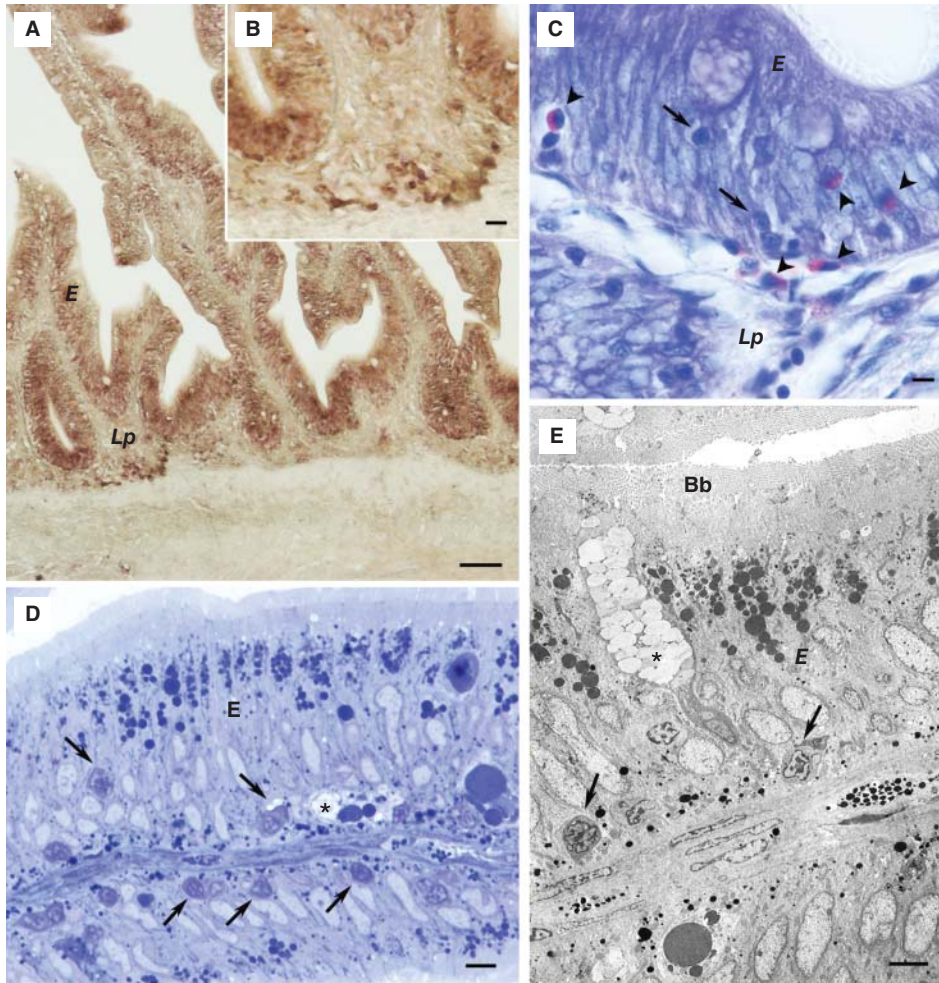


Fig. 2.1 Histological characterization of intestinal mucosal immune cells. Immune cells of the teleost intestinal mucosa are less well organized and more diffusely arranged than in mammals. This figure shows the distribution of some of these immune cells in the mucosa and the lamina propria of bony fish in a series of five images. (A/B) Predominance of CD8 α -positive cells in both the epithelium and the lamina propria of the European sea bass intestine (CD8 α RNA *in situ* hybridization). Scale bar = 50 μ m. The CD8 α ⁺ cells aggregated in the lamina propria are shown at higher resolution in the insert (B), where the scale bar = 10 μ m. (C) May-Grünwald-Giemsa staining of the gilthead sea bream (*Sparus aurata*) intestinal mucosa, showing lymphoid cells (arrows) and acidophilic granulocytes (arrowheads). Scale bar = 4 μ m. (D) Semi-thin section of the gilthead sea bream intestinal mucosa housing numerous basolateral lymphocytes (arrows) and an intraepithelial macrophage (star). Scale bar = 5 μ m. (E) Transmission electron microscopy of the gilthead sea bream intestinal mucosa showing a goblet cell (star) amongst enterocytes and basolateral lymphocytes (arrows). Scale bar = 3 μ m. Key: Ep, epithelium; Lp, lamina propria; Bb, brush border. For colour detail see Plate 5.

It is generally accepted that fish lack an organized GALT and thus contain not follicular lymphoid structures but rather a more diffusely distributed gut-associated lymphoid tissue containing many lymphoid cells, macrophages, eosinophilic and neutrophilic granulocytes (Zapata and Amemiya 2000; Bernard *et al.* 2006a; Huttenhuis *et al.* 2006). Mucosal T lymphocytes represent the major leukocyte population within the teleost gut observed in European sea bass (Abelli *et al.* 1997; Picchiatti *et al.* 1997; Scapigliati *et al.* 2002) and common carp (Rombout *et al.* 1998a) intestine. In addition an anti-human CD3e antibody demonstrated an abundance of T cells in the epithelium and lamina propria of common carp (Urán *et al.* 2008) and Atlantic salmon (Bakke-McKellep *et al.* 2007). *In situ* hybridization studies confirmed the wide distribution of T cells throughout the gut of European sea bass (Picchiatti *et al.* 2011) which exhibited cytotoxic function. In fact, lymphocytes purified from intestinal mucosa exhibited significant cytotoxic activity against xenogeneic and allogeneic cell targets (Picchiatti *et al.* 2011). The results corroborated previous data showing intraepithelial lymphocytes (IEL) isolated from rainbow trout intestine to be spontaneously cytotoxic against a mouse tumour cell line (McMillan and Secombes 1997). In addition in European sea bass intestine, TCR β and CD8 α expressing cells exceeded CD4 $^{+}$ cells, indicating the predominance of CD8 α^{+} cells in the gut, whose transcripts significantly increased in the posterior segment. These data agree with previous findings demonstrating an increase of mucosal T cells along the European sea bass intestine towards the anus (Abelli *et al.* 1997; Picchiatti *et al.* 1997; Romano *et al.* 1997b), reflecting regional immune specialization as previously reported in other teleost species (Rombout *et al.* 1989b; 1989c; Vigneulle and Baudin-Laurencin 1991).

In addition to the major sources of B lymphocytes (head kidney and spleen), the teleost GALT also contains B cells, although little is known with regard to their origin, their activation, their differentiation into plasmablasts and plasma cells, and the distribution of Ig $^{+}$ cells in different regions of the GIT. The proportion of B cells in isolated gut cell suspensions has generally been reported to be low and variable among teleost species. In European sea bass (dos Santos *et al.* 1997; Romano *et al.* 1997a; dos Santos *et al.* 2000), common carp (Rombout *et al.* 1998b) and rainbow trout (Zhang *et al.* 2010) approximately 2–12% of the isolated leukocytes were surface IgM-positive and these IgM $^{+}$ cells were detected mainly in the lamina propria, although in Atlantic halibut IgM $^{+}$ cells were distributed more commonly within the epithelium (Grove *et al.* 2006). In a recent report, the gene sequences of three different Ig heavy chains, namely IgM, IgD and IgZ, were cloned from mandarin fish and the distributions of these three kinds of Ig-producing cells were investigated in the intestine by *in situ* hybridization. The data confirmed that IgM-producing cells could only be detected at the submucosa and lamina propria of the posterior intestine, while no IgZ or IgD positive cells were found in the gut (Tian *et al.* 2009). IgT, an immunoglobulin isotype of unknown function, was described as acting like a mucosal antibody in rainbow trout. This IgT was claimed to be specialized in mucosal immunity and, in addition, IgT responses to a gut parasite were restricted to the intestine (Zhang *et al.* 2010). In this species both IgM $^{+}$ and IgT $^{+}$ cells were detected in the lamina propria and to a lesser degree within the epithelium (Zhang *et al.* 2010), while in fugu IgZ $^{+}$ cells could also be detected in the intestinal epithelium (Savan *et al.* 2005).

In contrast to T and B cells, few studies are dedicated to the intestinal macrophages of fish, probably because of the lack of suitable markers. Although gut macrophages are morphologically described in a variety of teleosts (Georgopoulou and Vernier 1986; Temkin and McMillan 1986; Rombout *et al.* 1986; Inami *et al.* 2009) their number, size and Ig-binding capacity vary among species. In particular, cyprinid fish have many macrophage-like cells in and under the

intestinal epithelium and their number is increased after anal intubation with antigens (Davina *et al.* 1980; Rombout *et al.* 1985; 1989a). Due to their Ig-binding capacity, these cells are strongly Ig⁺ (Rombout *et al.* 1993; Koumans-van Diepen *et al.* 1994) and can be loaded with luminal antigens such that, after degradation, antigenic determinants can subsequently be presented at their surface (Rombout *et al.* 1986; 1989b). Granulocytes are also described in the teleost intestinal mucosa. Although intermediate cells are sometimes described as separate subpopulations, two main types predominate: neutrophils and eosinophils, the latter strongly resembling mammalian mast cells (Rombout *et al.* 1989c; Urán *et al.* 2009) and named mast cells or eosinophilic granule cells (Reite 1998). These eosinophils release tryptase (Dobson *et al.* 2008), antimicrobial peptides such as lysozyme (Sveinbjörnsson *et al.* 1996; Urán *et al.* 2009), piscidin (Silphaduang and Noga 2001) and pleurocidin (Murray *et al.* 2007). They are abundant in mucosae, and can react strongly upon inflammation via migration and granule release (Urán *et al.* 2009). Fish neutrophils are phagocytic and abundant in the circulation, which are rapidly recruited from the blood to sites of inflammation (Ellis 1977; Hamdani *et al.* 1998). In the healthy, homeostatic status, fish intestine in general contains fewer neutrophils than eosinophils/basophils; however their number strongly increases under danger or stress conditions and they frequently penetrate the intestinal epithelium (Sundh 2009).

2.6 MUCOSAL IMMUNITY

These mucosal tissues, such as the gills and gut, are under constant challenge as a consequence of the antigenic nature of the aquatic environment; thus mucosal immunity is vital to the well-being of the fish. These host mucosal defences can be thought of as existing in several layers; these layers to the mucosal immune system include (1) mucus, (2) epithelial lining and (3) underlying lamina propria/mucosal tissue. In addition to these layers of host mucosal defence, an additional non-host defence layer can be considered – that of the commensal microflora that populate the real external surface (for more information, refer to *Chapters 4–6* focusing on fish gut microbiota). The commensal defence layer consists of commensal non-harmful beneficial microbes that line the gut; they play an important role in protection against pathogen invasion by utilizing several mechanisms. Commensals and indeed probiotic organisms that are obtained through fish feeds (or the rearing water) facilitate host defences by competing with pathogens for nutrients, binding sites on the epithelial cell surface, initiating production of defensin and other anti-microbial peptides from the epithelial cells, modulating epithelial cell growth and apoptosis turnover, increasing epithelial barrier strength by modulating tight junction proteins, and advancing epithelial cell mucus production and modulation of underlying innate and adaptive immune components present in the lamina propria (reviewed in Merrifield *et al.* 2010) (also refer to later chapters in this book). The second layer encountered in mucosal defence is the mucus. This acts as a viscous barrier, preventing pathogen invasion of mucosal tissue and facilitating the removal of potentially pathogenic antigenic material; this mechanism may be aided by trapping of antigenic material via binding to mucosal immunoglobulin which is itself bound to mucus by mucin binding sites. It is possible that the teleost mucosal isotype IgZ₂ (Ryo *et al.* 2010) may function in this way – facilitating neutralization of pathogen invasion and clearance.

In addition to their capability in mounting non-specific innate responses, antigen sampling by cells in the teleost intestinal epithelium was thought to be undertaken by specialized

epithelial cells and macrophages, acting as conventional APCs that process and present antigenic peptides in an MHC-restricted manner, capable of mounting specific adaptive immune responses. The intestinal mucosa is indeed a first-line defence system which involves the recognition and processing of antigen. This mucosal surface exists in folds rather than villi, as this tissue lacks the lacteals characteristic of villi and, in addition, does not have a lymphatic system, lymph nodes or Payer's patches or lymphoid follicles. As a result, the GALT is rather diffusely distributed in the intestinal mucosa and it is likely that antigenic priming of responses takes place at the site of invasion. Thus, all the important cells of the immune system are generally present in the tissue rather than relying on transport of antigen or cells in and out of the infected site (Zapata and Amemiya 2000; Bernard *et al.* 2006a; Huttenhuis *et al.* 2006). The relatively high expression of MHC II in the gills and in the second segment of the mid intestine is suggestive that these are the major mucosal sites for antigen processing and presentation to prime adaptive responses. In addition, these sites have been described to include both dendritic-cell-like APCs and lymphocytes (Koppang *et al.* 1998; 2003). A recent study by Fuglem *et al.* (2010) sought to further define antigen-sampling cells in the second segment of the mid intestine. Due to the relatively low glycocalyx level in salmonids, they investigated the uptake of gold-labelled bovine serum albumin (gold-BSA) by the gut of Atlantic salmon and rainbow trout. The uptake of gold-BSA was restricted to a small number of specialized epithelial cells which were located in the mucosal folds of the second segment of the mid intestine and to dendritic-like cells which were shown to project dendrites through the tight junction between epithelial cells and to extend into the lumen enabling the cell to sample luminal contents. In addition, morphological studies coupled with lectin binding demonstrated that the specialized epithelial cells resembled the phenotype of immature mammalian M cells. This study is of great value as it demonstrated that teleost fish possess both antigen sampling cells and sampling mechanisms similar to those understood for the systems in mammals/humans. By inference from our understanding of these immune systems, it is reasonable to suggest that these antigen-sampling cells play similar roles in immune tolerance versus immune activation decisions as those in their higher counterparts. These immune decisions are made by the presence or absence of danger signals (signals derived from pathogens or from host tissue breakdown) detected by their cognate pattern recognition receptors, predominantly TLRs (see Section 2.2). As a consequence of recognition of danger signals, co-stimulatory molecules are likely to be up-regulated on APCs. Indeed, the co-stimulatory molecule B7, and its T cell counterparts CD28 and CTLA-4, have been described as expressed in teleost fish (Hansen *et al.* 2009; Bernard *et al.* 2006b) and, like the human immune system, B7 has several homologous forms which may both positively and negatively regulate T cell activation through ligation of the CD28 receptor or CTLA-4.

Integral to the efficient functioning of gut mucosa is the ability to discriminate between self, beneficial non-self and harmful non-self. The gut is required to gain nutrients from an environment that is full of food-derived antigens, commensal organisms and pathogenic organisms; inappropriate immune activation to beneficial non-self would result in inflammation of the gut, a failure to efficiently digest food and absorb required nutrients, and hence a failure to thrive. The gut has developed sophisticated mechanisms capable of responding to harmful pathogenic non-self, while maintaining the capability to switch off or suppress potential immune responses directed against beneficial non-self antigens. Oral tolerance is a mechanism characteristic of intestinal immunology where repeated mucosal immunization and antigenic challenge result in immune hyporesponsiveness or suppression. It must be noted, however,

that conditions stressful to the fish, such as environmental changes (temperature, pH etc.) and predation, can result in breaking of tolerance and the elicitation of inappropriate inflammatory immune responses that are potentially harmful to the gut tissue and hence digestive processes. Mucosal tolerance has been described in teleost fish (Davidson *et al.* 1994; Piganelli *et al.* 1994) where repeated challenge by bacterial antigens resulted in suppression of antibacterial antibody responses. In addition to mucosal oral tolerance induced by mucosal alloantigen challenge being demonstrated for immunoglobulin production, tolerance induction has been observed for suppression of cell cytotoxicity which was reversible upon systemic administration of the same alloantigenic challenge (Sato and Okamoto 2007). Thus, mucosal/oral tolerance mechanisms characterized in teleost fish display antigen specificity and result in the suppression of both $CD8^+ T_c$ and $CD4^+ T_h$ involved in CMI and humoral responses. These mechanisms of suppression, like the human immune system, may involve clonal deletion by apoptosis and regulation by T_{reg} ; both of these mechanisms are determined by the nature and concentration of antigen being presented.

Mucosal tolerance arises not only as a consequence of T cell suppression (either by clonal deletion or by suppression by T_{reg}) but also through regulatory mechanisms involving innate immunity. Such mechanisms may involve TLR down-regulation on epithelial cells and macrophages and induction of negative regulators of TLR signalling such as Tollip and splice variants of adaptor proteins; such receptors and their adaptors have been described in teleost fish (Jault *et al.* 2004; Rebl *et al.* 2008; and reviewed in Purcell *et al.* 2006 and Aoki *et al.* 2008). In addition, it is possible that peptidoglycan recognition by teleost fish homologues of NOD2 may also regulate innate immune responses (Stein *et al.* 2007). Such modulatory responses would suppress or modulate both cytokine and co-stimulatory (B7) molecule expression by APCs. Thus, mucosal tolerance mechanisms of the innate immune system will also impact on adaptive immune tolerance. As a result of regulation of both innate and adaptive responses, mucosal tolerance is likely to be initiated in both an antigen-dependent and an antigen-independent manner to specific pathogens, or indeed a broad range of pathogenic microbes. The full characterization of such potential regulatory mechanisms in teleost fish is likely to elucidate the use of pathogen-specific vaccines and microbial PAMPs and commensal organisms in protection against aquatic pathogens and their resulting pathological consequences.

The mucosal response is dominated by tolerance in healthy homeostatic conditions which allow the fish to gain benefit from commensal organisms and dietary non-self. It is vital, though, that non-tolerogenic adaptive responses are initiated to harmful or pathogenic non-self. In the presence of such pathogens, the host is capable of modulating mucosal tolerance mechanisms to those of humoral and cell-mediated adaptive responses that protect teleosts from extracellular- and intracellular-resident pathogens respectively. With respect to extracellular and surface protective immunity, mucosal antibodies secreted into mucus are different compared to systemic immunoglobulin. Analysis of cutaneous mucosal immunoglobulin revealed the isotype to be IgM-like and showed that variation in antibody types in mucus resulted by oligomerization of IgM forms rather than isotypes (Wilson and Warr 1992; Hatten *et al.* 2001; Morrison and Nowak 2002). Due to the proteolytic environment present in the gut and the potential for antibody degradation, it is not clear as to whether IgM is expressed in gut mucus. Integral to mucosal immune function, fish express pIgR in mucosal tissues. This receptor is expressed by mucosal epithelial cells which allows for the binding of mucosal immunoglobulin

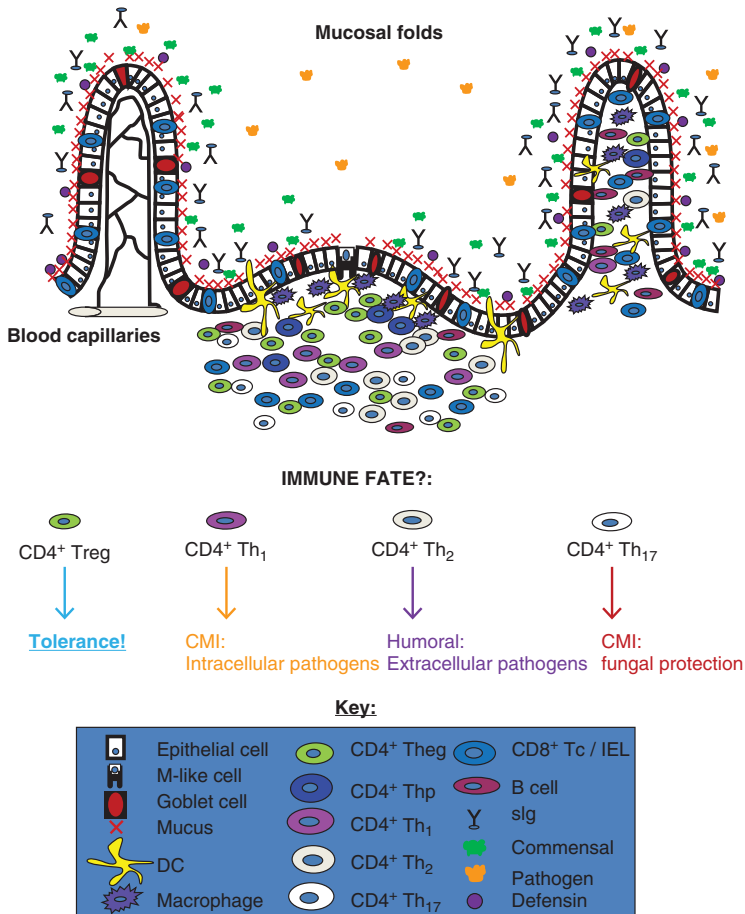


Fig. 2.2 Mucosal immune system of the gut. This figure shows the current understanding that exists for the gut mucosal immune system of the teleost fish. This tissue exists in folds, but cannot be described as villi due to the lack of lacteal/lymphatics. The immune defences exist at many levels of the layers of the mucosal tissue, namely commensal organisms, mucus, epithelial cell layer and sub-epithelial mucosa. Unlike the mammalian GALT, teleosts do not exhibit Payer's patches or lymphoid follicles. Instead, their lymphoid tissue is rather diffuse but contains all the immune cells which give the teleost GALT the capability to either tolerize immune mechanisms (T_{reg}) or initiate immune mechanisms directed at the clearance of intracellular-resident (CD4⁺ Th₁, CD4⁺ Th₁₇ and CD8⁺ T_c) and extracellular-resident pathogens (CD4⁺ Th₂ and B cells). One area of controversy in this diagram is the existence of dendritic cells (DCs); it is not understood whether DCs, specialized macrophages or specialized M-cell-like epithelial cells capable of antigen transcytosis act as APCs to contextualize MHC-restricted T-cell-driven adaptive immune responses. Broad-spectrum pathogen defence is associated with barrier function (commensal organisms, mucus secreted by goblet cells and trapping of slg-bound pathogens, intact tight junctions and epithelial cell secretion of anti-microbial products such as defensins) and innate immune responses (macrophage and granulocyte activity mediated by cytokines and anti-microbial compounds). Through the induction of expression of teleost cytokine homologues, immune responses can be directed towards the desired effector response to pathogens and non-pathogens. Such teleost cytokines include TGFβ and IL-10 (T_{reg}), IL-12 (Th₁), IL-4 (Th₂) and IL-23 (Th₁₇), thus directing mechanisms of mucosal tolerance, cell-mediated immunity, humoral immunity and anti-fungal responses, respectively. For colour detail see Plate 6.

on the basolateral surface, transcytosis through the epithelial cell and secretion into the luminal compartment of the gut. In doing so, the extracellular domain of the receptor is cleaved off with the antibody and serves as a proteolytic cleavage protective secretory component. This polymeric immunoglobulin receptor pIgR has been described in teleost fish including fugu, common carp, grouper and rainbow trout (Hamuro *et al.* 2007; Rombout *et al.* 2008; Feng *et al.* 2009) and has been shown to bind IgM and IgT (Zhang *et al.* 2010). With the recent characterization of the mucosal isotype IgZ₂ (Ryo *et al.* 2010), it is likely that this pIgR-mediated mechanism of antibody transcytosis also exists for this isotype, making it readily available in secretions at the mucosal membranes and relatively resistant to proteolytic cleavage.

T cells are the predominant leukocyte population present in the gut mucosa with a wide distribution throughout the intestine and with CD8⁺ T cells outnumbering CD4⁺ T cells. The gut, however, exhibits regional immune functional specialization (Rombout *et al.* 1989c; Vigneulle and Baudin-Laurencin 1991), as CD8⁺ T cells were found to be particularly highly prominent in the posterior region of the intestine whereas CD4 and MHC II expression was relatively low in this region. In contrast, CD4 and MHC II β were expressed at higher levels in the anterior and middle regions of the gut. MHC I α gene transcripts were generally constantly expressed along the length of the gut, independent of CD8 α expression (Picchietti *et al.* 2011). Thus, it is clear that the intestinal mucosa can be segregated according to T cell populations and hence functionality. The posterior intestine gut segment is dominated by cytotoxic CD8⁺ T_c and potentially IELs whereas the anterior and middle segments are dominated by CD4⁺ T_h cells; this is possibly reflective of a more tolerant T_{reg}-driven function during homeostasis and T_h-driven CMI and humoral responses upon breakage of tolerance to pathogen invasion of the mucosa.

From the increasing wealth of both expression and functionality studies focused on the gut mucosa of teleost fish, it is evident that these fish possess all the immune machinery capable of responding to pathogenic non-self, beneficial non-self and self. Anti-pathogen responses can be mediated by aggressive non-specific innate immune defences initiated by macrophages, neutrophils and MCs/EGCs whereas more antigen-specific responses are mediated by Th₁, Th₂, Th₁₇, Tc-driven humoral and CMI in response to intracellular and extracellular pathogens, respectively. The selective sampling of non-danger signals in luminal contents by DCs/M cells results in tolerance/regulation of the gut mucosa. This mechanism suppresses anti-pathogen responses and mechanisms potentially harmful to host tissue. The complexity of and interplay between all of the components of the mucosal immune system thus determine the desired response (Figure 2.2). The teleost fish mounts immune defences against harmful pathogens and tolerates non-harmful, beneficial commensal microbes and probiotics, while at the same time tolerating food components that benefit the development of the host.

2.7 COMMON PATHOGENS INFECTING TELEOSTS: WHAT IMMUNE RESPONSES ARE REQUIRED?

Relatively little is known with regard to the functional immune mechanisms that must be induced in teleost fish to be able to recognize and eradicate defined pathogens. As a consequence of a stronger innate immune system and the abundance of specific reagents aimed at identifying functional cells and molecules of innate immune responses, it has become relatively simple to identify innate responses, but these by their very nature are pathogen and

antigen non-specific and do not involve memory. It is very much a future goal to identify specific responses to pathogens and to harness these mechanisms for the benefit of immune protection to the host, conferring clear health benefits to the host living in this antigen-rich environment. To be able to harness this specific immunity, it is essential that fish pathogens and the resulting pathological mechanisms are clearly understood. There are several issues that must be considered which include: types of pathogens challenging teleost fish (parasites, bacteria, viruses), size of pathogen, route of entry into host, location once inside the host, and pathogen escape mechanisms.

The aquatic environment is a pathogen-rich environment through which the teleost fish must navigate. They are exposed to a wide range of pathogenic organisms which include bacteria, viruses, fungi, protozoa and helminths. The predominant routes by which they infect fish are via the mucosal surfaces of the skin, gills, eyes and the GIT. The immune battles that ensue are determined by the route of entry, the molecules expressed by the pathogen, and the relative size of the pathogen compared to host cells. The first immune battles initiated will be non-specific innate defences which are likely triggered through recognition of PAMPs by their respective PRRs, initiating destructive inflammatory responses mediated via a range of cells (basophils, eosinophils, macrophages) and a wide array of their effector molecules (chemokines, pro-inflammatory cytokines, proteases, antimicrobial peptides, lysozyme, complement factors) (see Section 2.2). The second-line defences are generally slower to develop but involve antigen-specific adaptive responses and develop memory to secondary exposure with the specific pathogen. The effectiveness of this later specific response is, however, dependent on the size and location of the pathogen. In general, large pathogens, that is larger than host cells (helminth worms, fungi and some bacteria), reside in an extracellular environment, open to a plethora of immune cells and their secreted effector proteins; the relevant humoral immune response to these pathogens utilizes antibodies and other secreted effectors. In an attempt to avoid these defences, smaller pathogens (such as viruses and some bacteria) will shelter from these humoral effectors and will escape inside the host's cells. The appropriate host defence to these pathogens would be to kill the host cell in which these infective microbes reside; this response requires the killing capability of the cell-mediated immunity arm of the host's defences (Tc and DTH).

There are a multitude of infectious pathogens causing a wide range of pathologies in teleost fish. Such pathologies include: (1) bacterial pathologies, including furunculosis caused by the bacterial pathogen *Aeromonas salmonicida*, enteric redmouth by *Yersinia ruckeri*, emphysematous putrefactive disease by *Edwardsiella tarda*, and mycobacteriosis by *Mycobacterium marinum*; (2) viral pathologies, including infectious pancreatic necrosis by IPNV, viral haematopoietic septicaemia by VHSV, and infectious haematopoietic necrosis by IHNV; (3) fungal pathologies (reviewed in Ramaiah 2006), including dermatocystidiosis by *Dermatocystidium*; (4) protozoal pathologies, including ichthyobodosis by *Ichthyophthirius multifiliis* and trichodinosis by *Trichodina* sp.; and (5) helminth pathologies, including intestinal nodules by *Pomphorhynchus laevis*. (For an extensive review of bacterial fish pathogens, particularly of the intestinal tract, refer to *Chapter 3*.)

Intracellular-resident pathogens such as IHNV and the bacterium *Mycobacterium marinum* are barely visible to humoral defences as they evade these responses by living inside host cells. It is vital that the teleost immune system is capable of detecting these pathogens inside host cells. As discussed earlier, the innate immune system is capable of recognizing

bacterial PAMPs via TLRs 1, 2, 4, 9 and NOD1 and NOD2, whereas TLRs 3, 7, 8 and 9 recognize viral PAMPs. These innate responses to intracellular pathogens facilitate activation and programming of the slower adaptive cell-mediated immune responses. CMI is involved in defence against intracellular pathogens by mounting cytotoxic responses. APCs present antigen in the context of both MHC I and MHC II: antigenic peptide presented by MHC I restricts T_C-mediated responses which kill infected host cells via perforin and granulolysin, whereas MHC II antigen activates Th₁ cells that induce either a DTH response through cytotoxic activity of activated macrophages or T_C activation through Th₁-specific effector cytokines. In addition, these pathogens may initiate Th₁₇ activity via IL-23 etc., resulting in activation of neutrophil/granulocytic cytotoxic cells. Extracellular-resident pathogens such as the intestinal helminth *Pomphorhynchus laevis* and fungi do not hide inside host cells and are thus generally visible to immune defences. The innate immune system recognizes these PAMPs via an array of receptors which include MBL, dectin-1, TLRs 2, 4, 5 and NLRs; these are capable of mounting cytotoxic, inflammatory, phagocytic and humoral responses. Such humoral factors involved in these responses to extracellular pathogens include immunoglobulin production. MHC II-restricted antigen recognition in the context of IL-4/IL-5/IL-13 primes for a Th₂-mediated B cell activation and antibody production. These antibodies are likely to be involved in a range of functions which include neutralization, tagging for phagocytotic clearance, complement activation and antibody-dependent cell cytotoxicity (ADCC).

Dependent on the immune response elicited to a defined pathogenic infection, the host fish may clear the pathogen and return to a state of well-being, or can deteriorate, either as a consequence of over-growth of the pathogen or as a result of inappropriate or over-exuberant immune responses leading to host-driven immunopathology. This over-exuberant response is likely to result in a tissue-destructive hypersensitivity or inflammatory response, ultimately manifesting as deep ulcerations and tissue/organ failure with consequences for the ability of the fish to thrive. Such an allergic/hypersensitivity response, for example, may result from the release of helminth worms from cysts; the immediate massive antigenic challenge resulting from release of massive numbers of worms present in a cyst induces a massive Th₂-driven allergic response which is more harmful to the host. The ensuing degranulation responses create a proteolytic and inflammatory environment which has devastating consequences for the fish host tissue. The efficient mounting of an immune response to a pathogen which presents early and in controllable numbers is desirable but unrealistic. The pathogen itself would rather hide or employ mechanisms of immune evasion, until such time that it is powerful enough, or present in sufficient numbers, that it can expose itself to host immune reactions during the process of translocation and reinfection of fresh host tissue.

The immune fight which ensues between host teleost fish and pathogen is a real dynamic battle. It is not merely a matter of the host mounting an anti-pathogen immune response, capable of recognition, killing and clearance. To understand any battle between host protection and pathogen infection, it is necessary to think of this situation as a bidirectional fight between host and pathogen. Let battle commence! In addition to the immune response, integral to the outcome of the fight is the ability of the pathogen to counteract the host's immune response; this is achieved by specialist and often pathogen-specific mechanisms that can either deviate, suppress or evade the host's responses. These evasion techniques (or pathogen escape mechanisms) are varied; any successful fish pathogen may have one or a collection of immune-evasion responses. These evasion responses are likely to include hiding from host

immunity such as intracellular habitation, and escape from intracellular processing pathways such as residence in phagosomes by prevention of phagolysosomal fusion or through escape from the phagosome to the cytosolic compartment. Pathogens may employ immune deviation through the expression of cytokine homologues which may change an efficient clearing immune response to an inappropriate response resulting in pathogen retention. In addition, many pathogens have been described as expressing superantigens, capable of inducing immune suppression via oligo-clonal deletion of a wide array of TCR V β sharing T cells, and finally perhaps inducing suppression via immunoregulatory mechanisms employing T_{reg} and regulatory cytokines such as IL-10 and TGF β . Thus far, these escape mechanisms are suggested in mammalian systems but await full characterization in teleost fish. Only with the full characterization of both host-derived responses and pathogen counter-responses will scientists be in a position to successfully design pathogen-specific treatments resulting in total eradication of the infectious pathogen.

2.8 FUTURE CONSIDERATIONS

Pathogens and microbes may be used to modulate or divert immune responses to other pathologies! The recognition of PAMPs derived from fish pathogens may indeed be harnessed in the context of immune activation. It is well established that PAMPs such as LPS, PGN, LTA etc. induce the expression of co-stimulatory molecules such as B7; any such up-regulation of B7 is likely to play a role in microbial adjuvanticity to initiate more antigen-specific adaptive responses tailored towards vaccine development against specific pathogenic infections. To initiate the most appropriate immune responses – CMI towards intracellular pathogens and humoral responses to extracellular pathogens – it is essential that scientists fully understand the adjuvanticity role of innate response activators and whether the co-stimulatory molecules up-regulated display T cell subset specificity. Teleost fish express several homologues of the co-stimulator B7; it is possible that these isoforms will differentially regulate immune responses, namely immune suppression/regulation and the activation of specific T cell subsets. In addition, characterization of antigen/pathogen processing and presentation in the context of immunodominant peptide mapping will uncover the best way to develop pathogen-specific vaccines which mediate their effect through antigen-specific adaptive responses. It should not be ignored, however, that the teleost fish immune system is predominated by innate immune responses. In this antigen-rich environment of the teleosts, would it be more appropriate to focus our attention on enhancing innate defences to a broad range of pathogens, rather than rely on antigen-specific adaptive responses to a specific pathogen?

The overall strengthening of teleost immune responses may be enhanced or modulated by nutrients, supplements and probiotics. Of particular interest at the moment is the role of probiotic bacteria in modulating the teleost mucosal immune responses of the intestines. Extensive research has demonstrated that probiotics modulate immune defences in a similar manner as that of human mucosal immunity. Probiotic bacteria used in fish feed have been shown to influence mucosal barrier integrity, promote commensal bacterial defences and modulate mucosal immunity by immune deviation, activation or suppression (reviewed in Merrifield *et al.* 2010). Thus, the use of probiotics in the feed of teleost fish can facilitate appropriate immune responsiveness to pathogenic organisms or the induction of mucosal tolerance, whereby teleost fish tolerate non-pathogenic commensal organisms and food-derived antigens through immune

regulation/suppression but at the same time maintain the capability to respond positively in response to pathogenic challenge.

2.9 CONCLUSION

Teleost fish have a well-developed, fast, potent innate immune response which results in an efficient first-line defence against pathogens which are encountered in the aquatic environment. This constitutive immunity responds to a broad spectrum of pathogens and their PAMPs in a variety of ways which involve innate cytokines, reactive oxygen and nitrogen intermediates, complement factors, lysozyme and proteolytic anti-bacterial factors, shared with the immune systems of higher animals. The mechanisms involving cells and molecules of innate responses are relatively well characterized; the adaptive response, on the other hand, is less well understood. This paucity of mechanistic information regarding adaptive responses is as a result of the lower dominance of adaptive responses in poikilothermic fish and the scarcity of reagents, such as specific antibodies to immune molecules that facilitate characterization of adaptive mechanisms. The characterizations of gene homologues of human immune molecules such as cytokines, T cell markers and transcription factors have been important observations which, by inference from the human immune system, suggest teleost adaptive responses to be similar to those in humans. Teleost fish adaptive responses include CMI driven by Th₁, Th₁₇ and Tc, whereas Th₂ cells drive humoral responses, and these are all likely to be controlled by the suppressive activities of T_{reg}. The involvement of these components of both the innate and the adaptive immune responses in the mucosal tissues of teleost fish suggest that the mucosa are integral to immune activation versus regulation decisions made by the teleost immune system as a consequence of exposure to harmful non-self pathogens, non-harmful non-self commensals, or self. The comprehensive investigation of teleost immune responses elicited to pathogenic challenge will facilitate the manipulation of fish immunity to the advantage of the host. Finally, these responses characterized will introduce new and improved future prophylactic and therapeutic interventions through the use of probiotics, nutrient supplementation and vaccination protocols, resulting in improved aquaculture and fish productivity for years to come!

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3 Gastrointestinal Pathogenesis in Aquatic Animals

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ABSTRACT

The mucosal surfaces are the first barrier against systemic infection of bacteria. Bacteria may find their way to and through the skin, gills, eye and gastrointestinal (GI) tract. Some bacteria may induce disease symptoms in the mucosal surfaces, while others form 'disease foci' in internal organs and tissues. In this review emphasis has been put on GI tract infections as this organ represents one of the most important portals of entry of numerous bacterial species. Infection through the GI tract is multifactorial and requires several stages: potential pathogens must be able to survive gastric transit, outcompete commensal gut microbiota, survive and colonize the intestinal mucus, and finally colonize and breach the intestinal brush border. The precise mechanisms underpinning these processes for a number of pathogens are partly described. Although we are becoming increasingly informed about the mucosal immune responses of fish, there remains a paucity of information regarding production of cytokines or expression of cytokine mRNAs in the GI tract during natural pathogen outbreaks. In response to experimental bath challenges however it is evident that immune responses may be stimulated, resulting in increased expression of certain pro-inflammatory cytokines (e.g. IL-1 β and TNF- α) in the oral mucosae. The use of prebiotics, and probiotics, offers a means to fortify the gut microbiota, potentiate the intestinal immune response and reduce intestinal pathogenesis.

3.1 INTRODUCTION

The main boundary between teleost fish tissues and the surrounding water is the mucus layer which covers the epidermis, the gills and the GI tract (see *Chapter 2*). Attachment of bacterial pathogens to one or more of these surfaces is important for initiation of infection. But the mode of transmission and the route of infection of fish bacterial pathogens are still debated.

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Table 3.1 Diseases and aetiological agents that infect, or translocate through, the GI tract of fish.

Pathogen	Disease	Species affected
<i>Vibrio (Listonella) anguillarum</i>	Vibriosis	Cold water marine fish
<i>Vibrio ordalii</i>	Vibriosis	Cold water marine fish
<i>Vibrio (Aliivibrio) salmonicida</i>	Cold water vibriosis	Salmonids
<i>Vibrio vulnificus</i>		Various
<i>Vibrio ichthyenteri</i>		Larval Japanese flounder
<i>Vibrio harveyi/Vibrio carchariae</i>	Infectious gastroenteritis	Various marine fish
<i>Aeromonas salmonicida</i>	Furunculosis	Various
<i>Aeromonas hydrophila</i>		Various (stressed)
<i>Yersinia ruckeri</i>	Yersiniosis/enteric redmouth	Salmonids
<i>Edwardsiella ictaluri</i>	Enteric septicaemia of catfish	Catfish
<i>Edwardsiella tarda</i>	Edwardsiellosis	Various
<i>Piscirickettsia salmonis</i>	Piscirickettsiosis	Various
<i>Pseudomonas anguilliseptica</i>		Various
<i>Photobacterium damsela</i> subsp. <i>piscicida</i> (form. <i>Pasteurella piscicida</i>)	Pasteurellosis (pseudotuberculosis)	Various
<i>Enterococcus/Streptococcus</i> /various	Streptococcosis	Warm water species
<i>Mycobacterium</i> spp.	Mycobacteriosis	Various
' <i>Candidatus arthromitus</i> '	Summer enteric syndrome	Rainbow trout

The present chapter aims to give an overview of binding, growth and translocation of bacteria in the digestive tract of teleosts (Table 3.1). (Translocation is migration from the intestinal lumen through the epithelial mucosa to infect otherwise sterile tissues: Berg 1999.) In addition, the ability of the bacteria to induce GI inflammation in terms of cytokine expression is described.

3.2 *VIBRIO* spp.

Members of the *Vibrio* genus are Gram-negative curve-shaped rods often isolated from marine environments and marine animals. Species from this genus, including *Vibrio anguillarum* and *Vibrio ordalii*, are the causative agents of the clinical disease vibriosis. Vibriosis is a haemorrhagic septicaemic disease which affects both farmed and wild fish, shellfish and bivalves. Outbreaks of disease generally occur at temperatures above 10 °C. These bacteria are considered to be part of the environmental and normal bacterial microbiota from apparently healthy fish (Nayak 2010). At present, *Vibrio anguillarum* is serotyped into 23 groups (Pedersen *et al.* 1999). It has been demonstrated that the two serogroups O1 and O2 are overall the most important as fish pathogens of Atlantic salmon (*Salmo salar* L.), rainbow trout (*Oncorhynchus mykiss*) and Atlantic cod (*Gadus morhua* L.), while O3 has frequently been isolated from diseased European eels (*Anguilla anguilla*). Strains belonging to the remaining serogroups are seldom isolated from diseased fish (Pedersen *et al.* 1999). Other *Vibrio* spp. capable of inducing disease of several fish species, such as cultured gilthead sea bream (*Sparus aurata*), include *Vibrio harveyi* (*Vibrio carchariae*), *Vibrio fischeri*, *Vibrio alginolyticus*, *Vibrio splendidus* and *Vibrio ichthyenteri* (García-Rosado *et al.* 2007). *Vibrio salmonicida* is also a reported disease-causing agent in groupers and Atlantic salmon.

3.2.1 *Vibrio anguillarum*

Vibrio anguillarum, often named *Listonella anguillarum* (MacDonnell and Colwell 1985), and *Vibrio ordalii* are important pathogens of salmonids, eels and marine fish (e.g. European sea bass, *Dicentrarchus labrax* L.; sea bream; and turbot, *Scophthalmus maximus*), and are the major causative agents of vibriosis (Pedersen *et al.* 1999). The mode of transmission of the bacterium has been studied in several species indicating that *V. anguillarum* can invade fish epithelia at more than one site including the skin and the intestinal tract (Birkbeck and Ringø 2005; Toranzo *et al.* 2005).

Grisez *et al.* (1996) investigated the infection route of *V. anguillarum* after oral challenge, with live feed, to juvenile turbot. The technique was established by Chair *et al.* (1994) by bioencapsulation of *V. anguillarum* in *Artemia* nauplii. High concentrations of bacteria could be incorporated in this way (5×10^8 *V. anguillarum* cells per 6000 *Artemia*), and after feeding turbot for 12 days a mortality rate of 61% was recorded. The bacteria were released from the *Artemia* mainly in the anterior part of the intestine (the section from the end of the pyloric portion of the stomach to the beginning of the posterior intestine). Thereafter, the bacteria were transported through the intestinal epithelium by endocytosis and released to the lamina propria (Grisez *et al.* 1996). From the lamina propria the bacteria were transported via the blood to the liver. In another study spotted wolffish (*Anarhichas minor* Olafsen) fry were bath challenged with *V. anguillarum* (Ringø *et al.* 2006). As a result of infection, detachment and loss of enterocytes were observed in both the foregut and the hindgut (Figure 3.1). Loss of epithelial integrity allowed translocation of bacteria to the vasculature of the lamina propria. Several studies have induced experimental infection of rainbow trout. Baudin *et al.* (1987) challenged rainbow trout by dipping in a suspension of *V. anguillarum* (10^5 bacteria ml^{-1}) and reported that the anterior and posterior intestines were both infected a few hours after exposure, demonstrating contamination by both oral and anal routes. The adherence of *V. anguillarum* to the gut of rainbow trout has also been studied (Horne and Baxendale 1983). Three distinct regions of gut – the anterior region with pyloric caeca, the posterior intestine and the intervening section – were cut longitudinally and incubated with bacteria. Bacteria adhered to the immersed gut sections. Bacteria consistently adhered in greater numbers to the anterior and midgut regions followed by the oesophagus, stomach and lower gut. Interestingly these differences were not found *in vivo* (Horne and Baxendale 1983) which may be related to the different physicochemical environment *in vivo*. In a study by Sandlund *et al.* (2010) the comparative susceptibility of turbot, halibut (*Hippoglossus hippoglossus* L.) and Atlantic cod yolk-sac larvae to challenges with *Vibrio* spp. was investigated. The larvae were bath challenged with *V. anguillarum* serotypes O1, O2a and O2b. The O2a serotype was pathogenic to all three fish species, while the O1 serotype was only pathogenic to halibut and cod. The O1 serotype produced more severe infections and necrosis and bacteria were present in a series of tissues including the dermis, GI tract, brain and eye, while larvae exposed to the O2a serotype had bacteria solely in the GI tract. In Japan, vibriosis was a huge problem for ayu (*Plecoglossus altivelis* Temminck and Schlegel) aquaculture in the 1980s (Muroga and De La Cruz 1987). Experimental infection of ayu by immersion in *V. anguillarum* resulted in bacterial populations present in skin, thereafter in muscle, spleen and liver and then in the gills and intestine. Ransom *et al.* (1984) performed a comparative study regarding localization and histopathology on waterborne infection of three species of Pacific salmon, the chum (*Oncorhynchus keta* Walbaum), coho (*Oncorhynchus kisutch* Walbaum) and Chinook (*Oncorhynchus tshawytscha* Walbaum) salmon, with *V. anguillarum* and *V. ordalii*. This study also presented data from

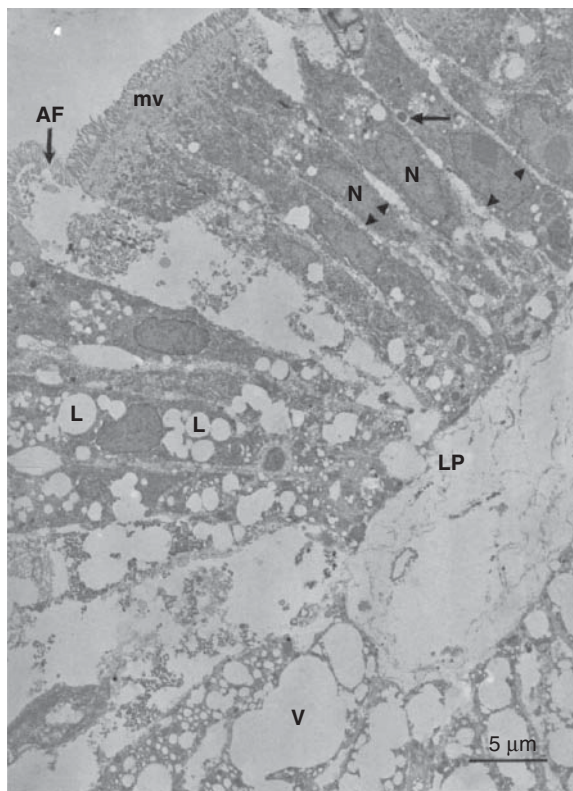


Fig. 3.1 Transmission electron microscopy of enterocytes in the hindgut of spotted wolffish fry exposed to *V. anguillarum*. Notice the severe necrosis-like damage in lamina propria (LP). Key: mv, microvilli; AF, apical microvillous brush border fragment; L, lipid droplet; N, nuclei; V, vacuole; arrow, bacteria-like profile. Scale bar = 5 μm. (Source: Ringø *et al.* 2006. Reproduced with permission of John Wiley & Sons.)

chum salmon *naturally infected* with *V. anguillarum* and *V. ordalii*. The naturally infected fish showed haemorrhages in muscle, gill and digestive tract. Additionally, necrosis of the mucosa and muscularis was observed; the posterior region was more severely affected than the anterior region. *V. ordalii* were commonly revealed in pyloric caeca. Experimentally *V. ordalii* infected chum salmon showed histopathological changes similar to those observed in naturally infected fish, and a similar pattern was seen with experimentally infected coho and Chinook salmon (Ransom *et al.* 1984). The authors suggested that the infection began in the rectum and descending intestine. In another study on ayu, fish were challenged by immersion, direct contact, cohabitation patch contact (filter paper soaked in bacterial suspension) and intubation (Kanno *et al.* 1989). From this study it was concluded that waterborne infection was the primary mode of transmission of the disease although infection was also established by oral and anal intubations. Olsson *et al.* (1992) investigated the entry and colonization of *V. anguillarum* in turbot and a closely related fish species, the dab (*Limanda limanda* L.). All of the isolates investigated (intestinal isolate 14 from dab, intestinal isolate 4:45 from turbot, skin mucus isolate 7:12 from turbot and *V. anguillarum* HI 11345) grew in and adhered to turbot

intestinal mucus. This fact strongly suggests that the turbot alimentary tract may serve as a site of amplification of *V. anguillarum* (Olsson *et al.* 1992). In another study by the same group, turbot were infected by intragastric or rectal intubations. Both methods revealed translocation of viable bacteria into the spleen, and it was proposed that the intestinal tract of juvenile turbot is a portal of entry for *V. anguillarum* (Olsson *et al.* 1996).

In a study by Spanggaard *et al.* (2000) rainbow trout were bath infected by *V. anguillarum*. The onset of mortality occurred 48 h after infection. The skin contained significantly higher numbers of bacteria than all other sample sites, including the GI tract. *V. anguillarum* was detected in just a few intestinal samples and not until an advanced stage of infection, indicating that the GI tract is of minor importance in rainbow trout. Recently a new method, *in vivo* bioluminescent imaging (BLI), was used to analyse the colonization of *V. anguillarum* in rainbow trout (Weber *et al.* 2010). The fish were challenged by immersion in bioluminescent bacteria. At 24 and 48 h post infection fish were analysed for bioluminescent bacteria. *V. anguillarum* colonized the skin in 95% and 100% of fish 24 and 48 h post infection, respectively. To visualize bacteria in the intestine, the faecal content was stripped. *V. anguillarum* was detected in 80% and 95% of the intestines 24 and 48 h post infection, respectively. The bacterial number in the intestine did not reach the same level as on the skin.

The initial stage of bacterial infection of the GI tract is likely to include a chemotactic response to, adherence to and growth within intestinal mucus before bacteria can interact with epithelial cells. Indeed, O'Toole *et al.* (1999) investigated the chemotactic motility of *V. anguillarum* to isolated rainbow trout skin mucus and intestinal mucus. The main conclusion from this study was that *V. anguillarum* moves towards both types of mucus, with a higher chemotactic response for intestinal mucus (O'Toole *et al.* 1999). Mucus is rich in nutrients that bacteria may utilize for growth. This subject was the focus of a study by Garcia *et al.* (1997), which investigated the growth of *V. anguillarum* in Atlantic salmon intestinal mucus, and showed that the bacterium was capable of rapid growth in the intestinal mucus. In another study, Larsen *et al.* (2001) observed that *V. anguillarum* was significantly more chemotactic to mucus from the skin and intestine of rainbow trout than to the gill mucus. It has been observed that *V. anguillarum* is able to bind to a neutral glycosphingolipid (glucosylceramide) receptor on the epithelial cell surface of rainbow trout intestine (Irie *et al.* 2004) which may explain some of the mechanisms of attachment observed in previous studies. In a previous study, Chen and Hanna (1992) recognized that *V. anguillarum*, *V. ordalii* and *V. parahaemolyticus* were able to attach to cultured rainbow trout cells of gonads, smears of gills, intestine, buccal mucosa and skin and cryostat sections of whole fish. Among the *V. anguillarum* strains, serotypes O1 and O2 showed the greatest attachment. Numerous virulence factors (Toranzo and Barja 1993) have been identified in *V. anguillarum* strains and the mechanisms for intestinal enterocyte destruction and translocation are inferred from experiments using other host cell types. Once in close association with host cells *V. anguillarum* strains produce a wide range of cytotoxins, including those from the repeat-in-toxin family (RTX; RtxABCHDE) and haemolysins (Vah1–5), which in combination with other extracellular products increase membrane permeability and up-regulate apoptosis (Li *et al.* 2011). A number of *ex vivo* studies, inoculating *V. anguillarum* cells into the lumen of intestinal sacs, have depicted the detrimental effects of *V. anguillarum* cytotoxins and other extracellular products on the intestinal epithelium of rainbow trout (Figure 3.2; Harper *et al.* 2011), Atlantic salmon (Figure 3.3; Ringø *et al.* 2007) and European sea bass (Figure 3.4). Effects at the brush border level include disorganized microvilli and necrotic enterocytes.

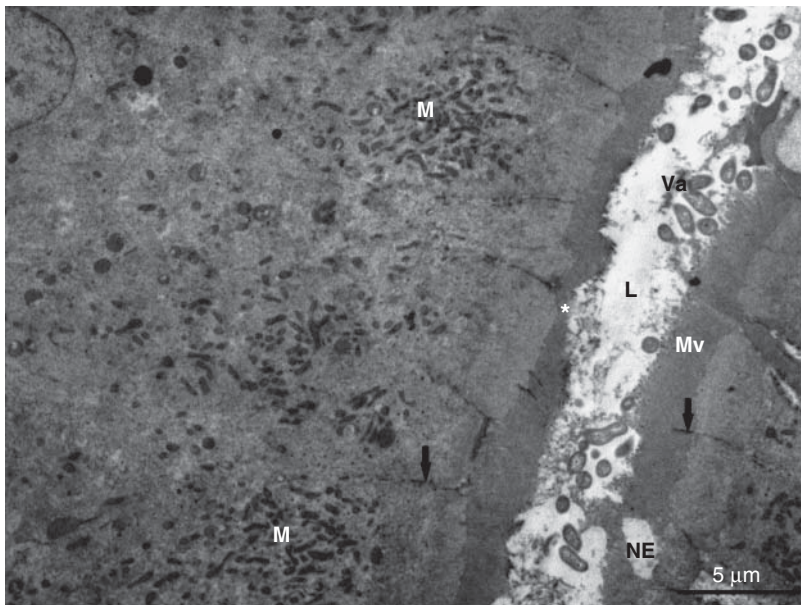


Fig. 3.2 Transmission electron microscopy micrograph of the anterior intestine (midgut) of rainbow trout exposed to *V. anguillarum* (Va) in an *ex vivo* challenge. Clear signs of tissue damage are characterized by necrotic enterocytes (NE) and disorganized microvilli (star). Key: L, lumen; M, mitochondria; Mv, microvilli; arrow, tight junctional complexes. Scale bar = 5 μ m. (Source: Harper *et al.* 2011.)

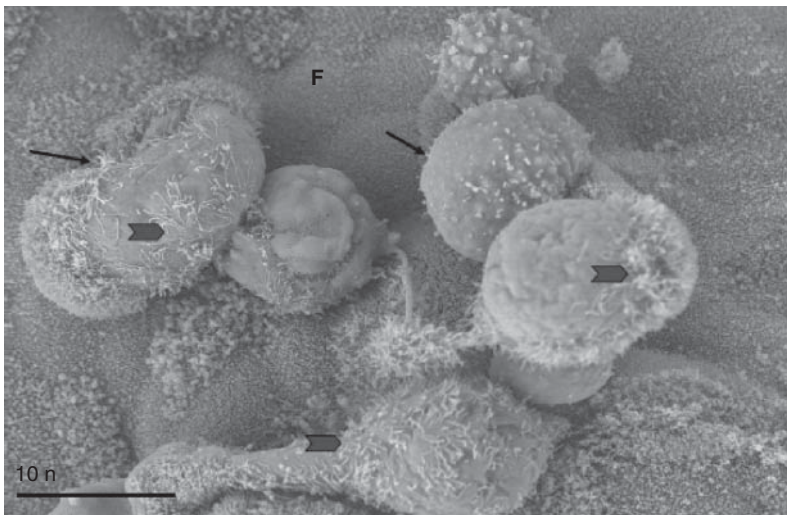


Fig. 3.3 Scanning electron microscopy of the proximal intestine of Atlantic salmon exposed to *V. anguillarum*. Note several detached or detaching enterocytes (arrows) lacking uniform microvilli at the epithelial surface. Scale bar = 10 μ m. (Source: Ringø *et al.* 2007.)

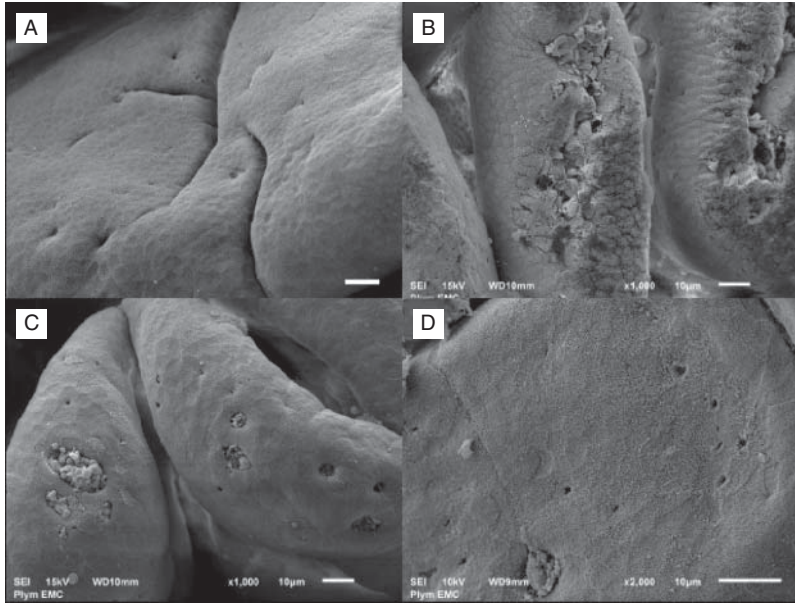


Fig. 3.4 Scanning electron microscopy images of ex vivo European sea bass intestinal *Vibrio* challenge. Posterior intestinal sacs exposed to (A) PBS (as a control), (B) *V. anguillarum*, (C) *V. harveyi* and (D) *V. alginolyticus*. Bacteria were inoculated at $\log 7 \text{ CFU ml}^{-1}$ for 1 h and incubated at 20°C . From a qualitative perspective it is evident that *V. anguillarum* and *V. harveyi* caused more brush border damage, characterized by necrotic enterocytes and disorganized microvilli, than *V. alginolyticus*, which seemed to have a negligible effect. Scale bars = $10 \mu\text{m}$. (Source: Peggs *et al.*, unpublished.)

3.2.2 *Vibrio salmonicida*

Cold water vibriosis is a bacterial infection caused by *Vibrio salmonicida* (Egidius *et al.* 1986), which has been reclassified as *Aliivibrio salmonicida* (Urbanczyk *et al.* 2007). The most susceptible species is Atlantic salmon in Norway, Scotland and the Shetland Islands, but the disease has also been recorded in rainbow trout and Atlantic cod (Bruno *et al.* 1986; Press and Lillehaug 1995). Pathological changes in experimentally infected Atlantic salmon include pale liver, haemorrhagic swim bladder and intestine, and bloody fluid in the peritoneal cavity. The bacterium is spread through the vascular system. In the early stages of the disease the bacteria were noticed exclusively inside the vascular system. The most severe damage was revealed in the heart, red muscle fibres and the lamina propria of the intestine (Totland *et al.* 1988). However, the intestinal epithelium showed no structural changes, suggesting that the digestive tract is not the main infection route.

3.2.3 *Vibrio vulnificus*

Vibrio vulnificus is a bacterial pathogen virulent with respect to humans, eels, tilapia and shrimp. The strains virulent for European eel are classified as biotype 2 (Fouz and Amaro 2003). The disease caused by these bacteria affects eels cultured in brackish (serovar E) and fresh water (serovar A) (Fouz *et al.* 2006; 2010). Results obtained by Fouz *et al.*

(2010) demonstrated that both serovars spread through water and infect eels – serovar A entering mainly by the anus and serovar E by the gills. Also, both serovars were pathogenic for tilapia (*Oreochromis* spp.), European sea bass and rainbow trout, but not for gilthead sea bream.

3.2.4 *Vibrio ichthyenteri*

Since 1971 a bacterial disease characterized by intestinal necrosis and high rates of mortality has been observed in larval Japanese flounder (*Paralichthys olivaceus* Temminck and Schlegel). It should be noted that the disease only occurs in the intestine of larval stages without a functional stomach (Muroga *et al.* 1990; Ishimaru *et al.* 1996; Kim *et al.* 2004; Montes *et al.* 2006). The disease is one of the most serious diseases in Korean and Japanese hatcheries and is caused by *Vibrio ichthyenteri* (Ishimaru *et al.* 1996), previously described as *Vibrio* species INFL (intestinal necrosis of flounder larvae) (Masumura *et al.* 1989). Little is known about the pathogenicity of this species.

3.2.5 *Vibrio harveyi* (*Vibrio carchariae*)

Although *Vibrio harveyi* (which includes *Vibrio carchariae* as a junior synonym) is often found in a free-living state in aquatic environments and is often reported to be present in the GI tract of apparently healthy fish and shellfish, a number of reports have described *V. harveyi* as the aetiological agent of infectious gastroenteritis in a number of fish species (Lee *et al.* 2002; Austin and Zhang 2006; Cano-Gomez *et al.* 2009). Liu *et al.* (2004) described outbreaks of disease with serious mortalities in cultured cobia (*Rachycentron canadum* L.) displaying swollen intestines containing transparent yellow fluid. All moribund/dead fish exhibited gastroenteritis and *V. harveyi* was shown as the causative agent. A similar syndrome has also been observed in summer flounder (*Paralichthys dentatus* L.), a commercially important species in coastal waters of the eastern United States (Soffientino *et al.* 1999; Gauger *et al.* 2006). The pathogen was identified as *V. harveyi/carchariae*. Histopathological examination revealed lesions to the peritoneum and posterior intestine. Large numbers of bacteria were observed in the peritoneum and at the serosal surface of the posterior intestine. The tissue was associated with necrosis, fibrin deposition, haemorrhaging and inflammatory cell infiltration. *V. harveyi/carchariae* has also been isolated from moribund grouper (*Epinephelus coioides*) (Yi *et al.* 1997) and red drum (*Sciaenops ocellatus*) (Liu *et al.* 2003) displaying a swollen intestine with transparent yellow fluid. *V. harveyi* has also been isolated from diseased common dentex (*Dentex dentex* L.) and was dominant in samples from diseased animals (Company *et al.* 1999). Necropsy of the diseased fish revealed haemorrhagic liver, empty digestive tract, enlarged gall bladder and trunk kidney inflammation. Histopathology revealed the presence of bacteria around the gill lamellae and in the gut lumen and in some samples the intestinal epithelium appeared highly necrotic.

A recent *ex vivo* study assessed the impact of *V. harveyi* on the intestinal brush border of European sea bass; *V. harveyi* cells were inoculated into lumen the of posterior intestinal sacs (*in vitro*) at log 7 CFU g⁻¹ for 1 h at 20 °C (Peggs, Browdy, Davies and Merrifield unpublished results). After the incubation the brush borders from control samples (exposed to PBS) were intact and apparently healthy, whereas the intestinal tissue exposed to *V. harveyi* showed regions with morphological changes, including irregular-shaped enterocytes, disorganized microvilli and necrotic enterocytes which can ultimately lead to breaching of the epithelial

barrier (Figure 3.4). The importance of this intestinal damage should be assessed in future *in vivo* studies and potential bacterial translocation through the intestinal epithelium should be assessed.

3.3 *AEROMONAS* spp.

Aeromonas spp. are Gram-negative facultative anaerobic rods which are ubiquitous in fresh and brackish water. With the exception of *Aeromonas salmonicida*, aeromonads are motile. *A. salmonicida*, an obligate pathogen, is the aetiological agent of furunculosis which affects salmonid fish and a number of other marine fish species. Of the motile group of aeromonads, *Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas sobria* are the most important with relevance to fish diseases.

3.3.1 *Aeromonas salmonicida*

Aeromonas salmonicida subsp. *salmonicida* is the causative agent of 'typical' furunculosis. The bacterium is a non-motile Gram-negative biochemically, antigenically and genetically homogeneous bacterium (Birkbeck and Ringø 2005; Toranzo *et al.* 2005). *A. salmonicida* subsp. *salmonicida* produces many extracellular proteases and toxins which have cytolytic and cytotoxic effects on various fish cell types (for a review see Ellis 1991). Typical furunculosis affects both salmonids and non-salmonid fish (Bergh *et al.* 1997; Toranzo *et al.* 2005) and although the route of entry of this bacterium is still debated, there are some reports describing intestinal presence (O'Brien *et al.* 1994; Hiney *et al.* 1994; Lødemel *et al.* 2001). 'Atypical' furunculosis forms a very heterogeneous group of bacteria affecting both non-salmonids as well as salmonids (Wiklund and Dalsgaard 1998). Examples include *A. salmonicida* subsp. *achromogenes*, subsp. *masoucida*, subsp. *pectinolytica* and subsp. *smithia*. Atypical strains demonstrate weak, slow or non-pigment production, catalase and oxidase negativity, and slow growth, and are pathogenic to fish other than salmonids (Austin 2011). Very often atypical strains give rise to superficial skin ulcerations.

Since 1994 a probe has been used for detection of *A. salmonicida* in effluent, water, faecal and sediment samples from Atlantic salmon hatcheries (O'Brien *et al.* 1994). By this method O'Brien *et al.* (1994) detected a positive correlation between pathogen detection and clinical disease. Standard bacteriology and enzyme-linked immunosorbent assay (ELISA) were employed to detect *A. salmonicida* in samples of kidney, intestine and mucus from Atlantic salmon with stress-inducible infections (Hiney *et al.* 1994). These authors suggest that the intestine may be a primary location of *A. salmonicida* in addition to mucus, fins and gills (Figure 3.5).

In a study on Arctic charr (*Salvelinus alpinus* L.), cohabitants with intraperitoneally infected fish showed variable mortality (Lødemel *et al.* 2001). Infected fish were analysed for pathology, detection of *A. salmonicida* bacteria and antigens by immunohistochemistry and immunogold labelling. The midgut epithelium of infected fish had a higher number of goblet cells (mucus producing cells), and bacteria were seen in close association with microvilli. In addition, *A. salmonicida* antigens were detected in the submucosa of pyloric caeca, midgut and hindgut (Lødemel *et al.* 2001). The conclusion drawn was that the GI tract could be an infection route of *A. salmonicida* in Arctic charr. In another study the infectivity routes were investigated in Atlantic salmon after bath and intragastric challenge with *A. salmonicida* (Rose *et al.* 1989).

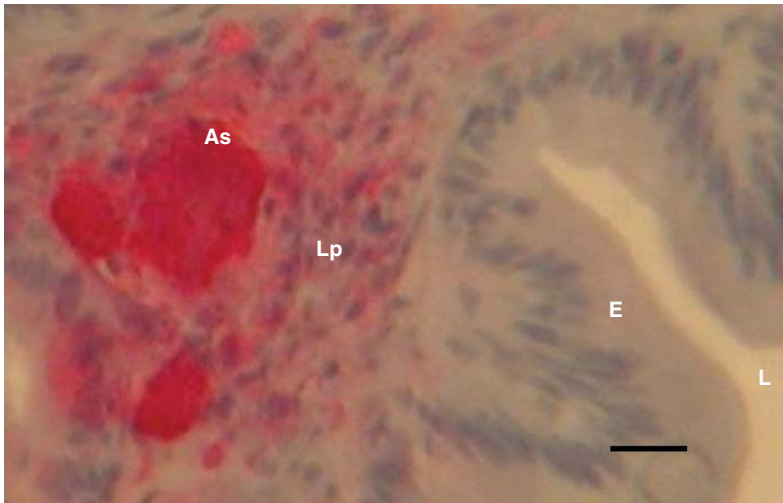


Fig. 3.5 Immunohistochemical staining, using rabbit anti-*A. salmonicida* LPS antiserum diluted 1:1000, biotinylated goat anti-rabbit Ig and streptavidin biotinylated alkaline phosphatase, and Fast red/Naphthol AS-Mx as the substrate, in analysis of the spotted wolffish intestine from fish infected with waterborne atypical *A. salmonicida*. Large numbers of bacteria (As) were observed in the lamina propria (Lp). Sections obtained from non-infected controls were negative for bacteria. Key: E, epithelium; L, lumen. Scale bar = 10 μ m. (Source: Dag Vollstad.) For colour detail see Plate 7.

The results of this study showed that *A. salmonicida* infection may occur both by ingestion of infected fish and after epidermal contact. Jutfelt and colleagues studied the translocation of viable *A. salmonicida* across the intestine of rainbow trout (Jutfelt *et al.* 2006; 2008). An ‘Ussing chamber’ was used in this study and the results demonstrated translocation of the bacteria across the intestinal epithelium, suggesting the intestine as a possible route of infection. The bacterium seemed to use several mechanisms for this translocation including surface proteins and secreted proteins (Jutfelt *et al.* 2008; Dacanay *et al.* 2010). Ringø *et al.* (2004) observed substantial cell damage in Atlantic salmon after *A. salmonicida* infection. Enterocytes were clearly detached from the anterior intestinal region while less damage was observed in the posterior intestinal region.

In a study by Hodgkinson *et al.* (1987), rainbow trout were immersed in a suspension of *A. salmonicida* for 2 min. The pathogen was present on the skin/mucus, on the gills and in the mouth for up to 24 h after challenge. Small numbers of bacteria were also recovered from the upper and lower regions of the intestine and in faecal samples. By immersion of the tail region, *A. salmonicida* were recovered from the hindgut for up to 24 h. It is likely, however, that the uptake of the pathogen occurred through all natural openings and/or the skin and gills. It has been reported that *A. salmonicida* are capable of inducing the expression of pro-inflammatory cytokines in the rainbow trout intestine (Mulder *et al.* 2007). Modulated IL-1 β , TNF- α , TGF- β and IFN- α mRNA expression was reported following experimental bath challenge. In another study, isolated intraepithelial cells (IEC) responded to live *A. salmonicida* by increased expression of the pro-inflammatory cytokines TNF- α 2 and IL-1 β (Komatsu *et al.* 2009).

3.3.2 *Aeromonas hydrophila*

Aeromonas hydrophila is a motile Gram-negative bacterium that may cause diseases of both warm water and cold water fish. In general, *A. hydrophila* does not cause disease in healthy well-conditioned fish. The bacterium has been reported to be a secondary pathogen or a primary pathogen of fish under stressful situations such as low oxygen levels, thermal fluctuations and spawning (Harikrishnan and Balasundaram 2005). As reported by Hazen *et al.* (1982), isolates of *A. hydrophila* from fish lesions had a significantly higher chemotactic index to surface mucus than isolates of *A. hydrophila* from water. In another recent study the chemotaxis towards, adhesion to, and growth in common carp (*Cyprinus carpio*) gut mucus of two *A. hydrophila* strains was examined (Van der Marel *et al.* 2008). Both strains did not move towards the mucus but the more pathogenic strain of the two showed 13 times higher adhesion to mucus and a higher growth rate in the mucus. As discussed by the authors, a faster multiplication of bacteria in mucus may increase the pathogenicity since the flushing of bacteria due to mucus erosion and turnover is reduced (Van der Marel *et al.* 2008). Despite these interesting data, it has been suggested that the gills and skin are the main portals of entries of *A. hydrophila* in carp (Chu and Lu 2008). Future studies on the pathogenicity of *A. hydrophila* are recommended.

3.4 *YERSINIA RUCKERI*

Yersinia ruckeri is the causative agent of yersiniosis or enteric redmouth (ERM) disease (Furones *et al.* 1993). The disease has been reported in salmonids, predominantly in rainbow trout in North America, Europe, Australia and South Africa (Tobback *et al.* 2007). *Y. ruckeri* belongs to the Enterobacteriaceae family and is a Gram-negative rod of variable motility. The rods spread between fish by direct contact with infected animals or carriers. As many as 25% of fish in a rainbow trout population were recognized to carry the bacterium in their lower intestines (the intestinal region exhibiting an absorptive epithelium but with a degree of mucosal folding lower than that of the upper gut) (Busch and Lingg 1975). The portal of entry of *Y. ruckeri* was studied by Tobback *et al.* (2009) after experimental infection of rainbow trout. These studies demonstrated that the gills may be an important portal of entry. In addition the bacterium may gain access to the body by a variety of routes including the skin and gut (Busch and Lingg 1975; Tobback *et al.* 2009). In a recent study the interactions of *Y. ruckeri* with the gills and gut of rainbow trout were studied by perfusion techniques (Tobback *et al.* 2010). Five *Y. ruckeri* strains were tested, two virulent and three avirulent. All the strains adhered to both gill and gut tissues and there were no significant differences in adhesion and invasion of the strains between the tissue types. Additionally, there were no significant differences between virulent and avirulent *Y. ruckeri* strains with regard to adherence to the tissues.

3.5 *EDWARDSIELLA* spp.

Members of the *Edwardsiella* genus are Gram-negative, facultatively anaerobic rod-shaped bacteria. There are three species within the genus and two of them are pathogenic to fish: *Edwardsiella ictaluri* and *Edwardsiella tarda*.

3.5.1 *Edwardsiella ictaluri*

Edwardsiella ictaluri is the aetiological agent of enteric septicaemia of catfish (ESC) which can result in high mortalities (Thune *et al.* 1993; Birkbeck and Ringø 2005). The pathogenesis of ESC was previously studied by experimental infections of channel catfish (*Ictalurus punctatus* Rafinesque) via the gut and water (Shotts *et al.* 1986). The gut-exposed fish showed signs of a systemic infection within 2 weeks, suffering from enteritis, hepatitis, interstitial nephritis and myositis. Some of the non-injected cohabitants developed systemic infections beginning in the intestine and nostrils. In another study the pathogenesis of ESC was investigated by intragastric intubation (Baldwin and Newton 1993). Within 15–30 minutes of exposure *E. ictaluri* was observed in contact with intestinal brush border and the trunk kidney was positive for bacteria, indicating a rapid trans-mucosal passage. Additionally, bacteria were observed in phagocytes of the intestinal mucosa, and later it was documented that *E. ictaluri* is able to invade, survive and replicate in catfish macrophages (Booth *et al.* 2006).

3.5.2 *Edwardsiella tarda*

Edwardsiella tarda is a pathogen of several fish species including common carp, mullet (*Mugil cephalus* L.), tilapia (*Tilapia mossambica* Peters), Japanese eel (*Anguilla japonica* Temminck and Schlegel), blue gourami (*Trichogaster trichopterus* Pallas), channel catfish and Japanese flounder (Thune *et al.* 1993). The pathogenesis of experimental edwardsiellosis was studied in Japanese flounder by intraperitoneal injection, immersion and oral intubation (Rashid *et al.* 1997). The viable counts of *E. tarda* in the intestine, liver and kidney tissues were always higher than those in the blood. Histopathological examinations demonstrated massive necrosis in liver and kidney, and sporadic necrosis in epithelia and lamina propria. Intraphagocytic multiplication of *E. tarda* was confirmed in these three tissues. In another study the entry of *E. tarda* in the blue gourami was investigated by immersion challenge (Ling *et al.* 2001); the bacteria were detected in all parts of the fish one day after exposure with the highest bacterial concentration in the anterior section of the intestine. At day seven bacterial numbers were insignificant in all organs studied except for the intestine.

3.6 *PISCIRICKETTSIA SALMONIS*

Piscirickettsiosis is caused by a Gram-negative intracellular pathogen, *Piscirickettsia salmonis*, and was first isolated from marine netpen-reared coho salmon in southern Chile (Fryer *et al.* 1990; Garcés *et al.* 1991; Branson and Diaz-Munoz 1991; Fryer *et al.* 1992). The disease was also observed in other aquacultured salmonids such as Chinook salmon, rainbow trout, masu salmon (*Oncorhynchus masou* Brevoort) and Atlantic salmon (Fryer and Lannan 1996; Almendras *et al.* 1997; Smith *et al.* 1999). The disease has also been reported in Canada, Norway and Ireland (Marshall *et al.* 1998). Piscirickettsia-like bacteria have also been associated with diseases in non-salmonids. Examples are white sea bass (*Atactoscion nobilis* Ayres), black sea bass (*Dicentrarchus* sp.), tilapia (*Oreochromis*, *Tilapia* and *Sarotherodon* spp.) and blue-eyed plecostomus (*Panaque suttoni* Eigenmann and Eigenmann) (Mauel and Miller 2002).

Coho salmon and Atlantic salmon have previously been tested for their susceptibility to infection by a rickettsia isolated from cell cultures from diseased coho salmon (Garcés *et al.*

1991). Mortalities ranged from 88% to 100% in both species. Moribund fish were anaemic, the gills and liver were pale, the spleen was enlarged, and many fish had petechial haemorrhages on the pyloric caeca. The digestive tract was fluid filled and devoid of food. Horizontal transmission did not occur between injected and non-injected individuals (Cvitanich *et al.* 1991). Experimental infection with *P. salmonis* via the intraperitoneal, oral and gill routes were compared. The results revealed that the relative risks of mortalities by intraperitoneal and gill infections were significantly higher than fish challenged by the oral route. After oral challenge the pattern of mortality was delayed relative to the intraperitoneal and gill routes, which indicates that infection via the GI tract may occur but the GI is not as important an infection route as the gills.

3.7 PSEUDOMONAS ANGUILLISEPTICA

Pseudomonas anguilliseptica is an opportunistic Gram-negative fish pathogen first described in Japanese eels suffering from haemorrhagic septicaemia. In Europe numerous fish species have suffered from this disease: gilthead sea bream, black sea bream (*Acanthopagrus schlegelii* Bleeker), European sea bass and turbot (Doménech *et al.* 1997). *P. anguilliseptica* was also isolated from diseased salmonids in Finland (Wiklund and Bylund 1990). Turbot have been shown to be sensitive to intraperitoneal infection by a virulent strain from diseased turbot, while bath exposure gave no mortalities (Magi *et al.* 2009). Clinical signs of naturally infected gilthead sea bream were the presence of ascitic fluid in the peritoneal cavity, haemorrhages on the kidney and liver, and a congestive intestine containing yellowish exudate (Doménech *et al.* 1997). Seven days after intraperitoneal challenge of turbot with *P. anguilliseptica* an enlarged spleen, a friable liver and a congestive and dilated gut with yellow exudate were observed (Magi *et al.* 2009). Immunohistochemically positive bacteria were detected in the spleen, gut, liver, kidney, muscle and brain. Histopathological changes were seen in the gut four days after injection including hypertrophy of epithelial cells, and seven days after infection necrosis and oedema of the gut mucosa were observed.

3.8 PHOTOBACTERIUM DAMSELA subsp. PISCICIDA (PASTEURELLA PISCICIDA)

Photobacterium damsela subsp. *piscicida*, formerly *Pasteurella piscicida*, is a non-motile pleomorphic Gram-negative bacterium and is the aetiological agent of pasteurellosis. The disease causes the septicaemia known as 'pseudotuberculosis' due to the fact that diseased fish show whitish tubercles in internal organs. Examples of affected species are European sea bass, sea bream, turbot, yellowtail (*Seriola quinqueradiata* Temminck and Schlegel) and Japanese flounder (Magarinos *et al.* 1996a). Serologically the bacterium constitutes a highly homogeneous group. Reports suggest that *Photobacterium damsela* subsp. *piscicida* can be transmitted orally, since the bacterium was isolated from the freshwater fish snakehead (*Channa maculate* Lacépède) fed infected marine trash fish. Following gastric intubation of high doses of *Photobacterium* the authors reported the presence of this bacterium in the peripheral blood within 72 h (Wakabayashi *et al.* 1977; Tung *et al.* 1985). Also, it was found that *P. damsela* subsp. *piscicida* infected yellowtail either through the gills or via the GI tract

by feeding contaminated fish (Kawahara and Kusuda 1987). Sea bass exposed to *P. damsela* subsp. *piscicida* bacteria in dialysis tubing, surgically implanted in the abdominal cavity, revealed necrosis of intestinal cells in the lamina propria and submucosa with infiltration of inflammatory cells (Poulos *et al.* 2004). Strong adhesion of the bacteria to intestines of sea bream, European sea bass and turbot was demonstrated by Magarinos *et al.* (1996b). Furthermore, infection with *P. damsela* induced intestinal inflammatory responses as shown by modulated expression of TNF- α and IL- β mRNA (Montero *et al.* 2010).

3.9 STREPTOCOCCOSIS

Streptococcosis is a septicæmic disease that affects both farmed and wild fresh and marine water fish. The disease has been reported in turbot, yellowtail, Japanese eel, striped bass (*Morone saxatilis* Walbaum), striped mullet (*Mugil cephalus* L.) and menhaden (*Brevoortia patronus* Goode) (Kusuda and Salati 1993; Romalde *et al.* 1996). The causative agents of streptococcosis include *Lactococcus garvieae*, *Lactococcus piscium*, *Streptococcus iniae*, *Streptococcus agalactiae*, *Streptococcus parauberis*, *Enterococcus* spp. and *Vagococcus salmoninarum* (Toranzo *et al.* 2005). Possible routes of infection have been investigated by bath exposure and intragastric inoculations with food and faeces contaminated with an *Enterococcus* strain isolated from diseased turbot (Romalde *et al.* 1996). The invasive capacity of the bacterium was limited after bath challenge. However, intragastric inoculations with contaminated food or faeces established an infective state that resulted in mortalities 16 to 20 days after inoculation (Romalde *et al.* 1996).

3.10 'CANDIDATUS ARTHROMITUS'

'*Candidatus arthromitus*' is the name of a Gram-positive endospore-forming segmented filamentous bacterium (SFB), the causative agent of rainbow trout gastroenteritis (RTGE) occurring predominantly in the summer season (Michel *et al.* 2002). No formal taxonomic criteria are applicable due to the unculturability of the bacterium (Michel *et al.* 2002; Manzano *et al.* 2010). The disease was first described in France in 1992 and then in Spain, Italy, the United Kingdom and Croatia (Del-Pozo *et al.* 2009; 2010a). Autopsies of RTGE infected fish reveal acute enteritis with a haemorrhagic and oedematous intestinal mucosa. Pathological changes also include multifocal detachment of the mucosal layer resulting in a direct exposure of the lamina propria to the digestive lumen (Del-Pozo *et al.* 2010b). The whole digestive tract including the stomach and pyloric caeca may be filled with a mucoid material (Michel *et al.* 2002). Most affected fish contained segmented filamentous bacteria in the distal intestine and pyloric caeca. These bacteria were also observed in some apparently healthy fish although in lower numbers than in fish affected with RTGE (Del-Pozo *et al.* 2010b; 2010c).

3.11 MYCOBACTERIUM spp.

Mycobacteriosis is a common disease in both wild and captive fish. Over 167 species of fish have been documented to be susceptible to mycobacteriosis (Jacobs *et al.* 2009).

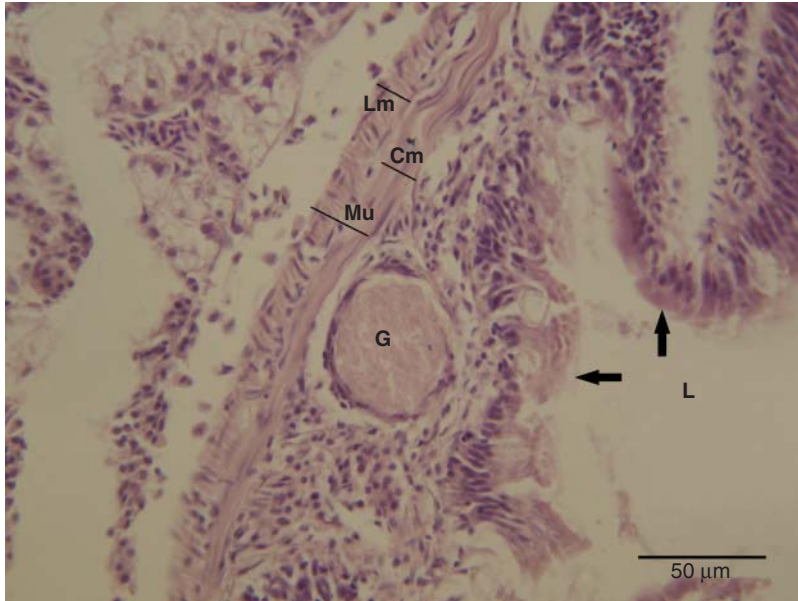


Fig. 3.6 Light microscopy (H&E staining) transverse section of the intestine of zebrafish with clinical signs of chronic mycobacteriosis. A lesion (granuloma, G) in the submucosa is indicated by the formation of fibrous connective tissue around a small (~50 μm) focus of acellular material. Key: arrow, epithelium; Cm, circular muscle; L, lumen; Lm, longitudinal muscle; Mu, muscularis. Scale bar = 50 μm. (Source: Kuehlwein *et al.*, unpublished.) For colour detail see Plate 8.

Mycobacterium spp. can survive and replicate within macrophages which allows them to evade the host immune system. Mycobacteriosis causes ulcerations of the skin, emaciation, and granulomatous lesions (organized collections of macrophages). Internally, grey-white granulomas develop in the liver, kidney, spleen, heart, muscles and intestine (Figure 3.6). There is presently no cure for mycobacteriosis. Mycobacterial infections of fish are zoonotic, which means the organisms can cause disease in humans. Infected fish release the organism from skin and gill lesions, and shed them from the GI tract. Infection can be spread when fish have direct contact with infective material or ingest infected tissue. Examples of this are from the early history of the salmon industry where fish were fed unpasteurized fish waste which subsequently caused a serious problem with mycobacteriosis. There has been a dramatic increase in the use of small fish in research, especially zebrafish *Danio rerio*. One of the most common diseases of zebrafish is mycobacteriosis. Watral and Kent (2007) conducted a study with intraperitoneal injection of various *Mycobacterium* spp. and recorded mortalities and conducted histological appraisal. *Mycobacterium marinum* isolates caused 100% infection and between 30% and 100% mortality. None of the other *Mycobacterium* spp. (*Mycobacterium peregrinum* and *Mycobacterium chelonae*) caused significant mortalities. Harrieff *et al.* (2007) established infection in zebrafish with both *M. peregrinum* and *M. marinum*, using both bath and oral challenge, and concluded that the intestine was the primary infection route (with high mycobacterium isolation from the intestine) and that *M. marinum* caused a higher number of clinical infections than *M. peregrinum*.

Mycobacterium haemophilum, a recognized pathogen of children, has also been reported as a pathogen in fish (Kent *et al.* 2004). Two severe outbreaks from different zebrafish facilities on different continents (Europe and North America) occurred with mortalities over a period of several weeks. Zebrafish appear to be particularly vulnerable to *M. haemophilum* and a recent study showed that experimentally infected (intraperitoneal injection of *M. haemophilum*) fish exhibited severe mycobacteriosis characterized by multiple granulomas and diffuse chronic inflammation (Whipps *et al.* 2007). Essentially all organs were involved: the kidney, spleen, liver, pancreas, heart, muscle and intestine exhibited particularly heavy infections in most fish.

Mycobacterium infections have also been studied in wild fish associated with sea cages. Bacterial transmission may occur between wild and caged fish if local wild fish communities gain access to cages, if wild animals escape from sea cages into the surrounding ecosystem, or if there is contamination through the water column. Diamant *et al.* (2000) studied the infection patterns of *M. marinum* in wild rabbitfish (*Siganus rivulatus* Forsskål) associated with commercial mariculture cages along the Israeli Red Sea coastline. Two *M. marinum* isolates from rabbitfish captured at Eilat harbour and the coral reef were shown to be identical to isolates from rabbitfish trapped inside the mariculture cages as well as isolates from locally cultured European sea bass. Further study of the transmission pathways is clearly warranted.

While there is scarce information on mucosal immune response following *M. marinum* infection it has been shown that *M. marinum* infections induce expression of intestinal ISG15 (Interferon Stimulated Gene 15) which may indicate an ongoing cellular defence process (Liu *et al.* 2002). Some further information is available at the transcriptomic level – especially in zebrafish (Meijer *et al.* 2005). Of particular interest is the down-regulation of MHC class I genes that may be deleterious since *M. marinum* is an intracellular pathogen that would better be eradicated by effective MHC class I antigen presentation and CD8+ activation.

3.12 CONCLUSION

The bacterial pathogens of major importance for disease in the GI tract of fish have been summarized in this chapter. As shown, the pathogens are a diverse group, of which many are Gram-negative. Some of the bacteria may live for a long period outside the host, and some may live and multiply within their host without causing disease. Some are intracellular and so the bacterium's survival and spread depend on the ability to replicate inside eukaryotic cells and on the host immune responses. There are a growing number of studies in the literature dealing with the mechanism of GI tract infections, particularly with respect to *V. anguillarum* and *A. salmonicida*, but still the mechanisms of establishment, cytotoxicity and translocation of GI tract infections are debated and only partly described. This should be a topic of future focus, as well as comprehensive studies of the endogenous gut microbiome and the applications of probiotics and prebiotics in order to stabilize beneficial bacterial populations and reduce the presence of, and incidence of infection caused by, GI based pathogens.

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4 The Gut Microbiota of Fish

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ABSTRACT

Animals harbour a complex microbial community, consisting of bacteria, yeast, viruses, archaeans and protozoans, in their gastrointestinal (GI) tract. These microbes influence various host functions including development, digestion, nutrition, disease resistance and immunity. One important aim of GI microbiota studies therefore is to give a scientific basis for developing effective strategies for manipulating GI microbial communities to promote the host health and improve productivity. This chapter reviews the current knowledge on the microbiota composition in several fish species, emphasizing the compilation of results reported regarding the most frequently observed bacterial genera and phyla in marine and freshwater species. This also includes descriptions of the microbiota in early stages of development, the influence of environmental and host factors on the establishment of the bacterial populations that become part of the gut microbiota, and the importance of these microbial communities on host health, development and nutrition.

4.1 INTRODUCTION

In the classic description, the complex community of microorganisms inhabiting body sites in which surfaces and cavities are open to the environment is termed the microbiota; previously this was called the microflora or microbial biota. Moreover, the epithelial surfaces of fish and all other vertebrates are colonized at birth by large numbers of microorganisms (microbiota) that form commensal or mutual relationships with their hosts (Spor *et al.* 2011). The majority of these microbes reside in the digestive tract, where they influence a broad range of host biological processes. The vertebrate gut harbours a coevolved consortium of microbes that play critical roles in the development and health of this organ. This microbial community can be subcategorized into two major groups. One group simply passes through the lumen with food

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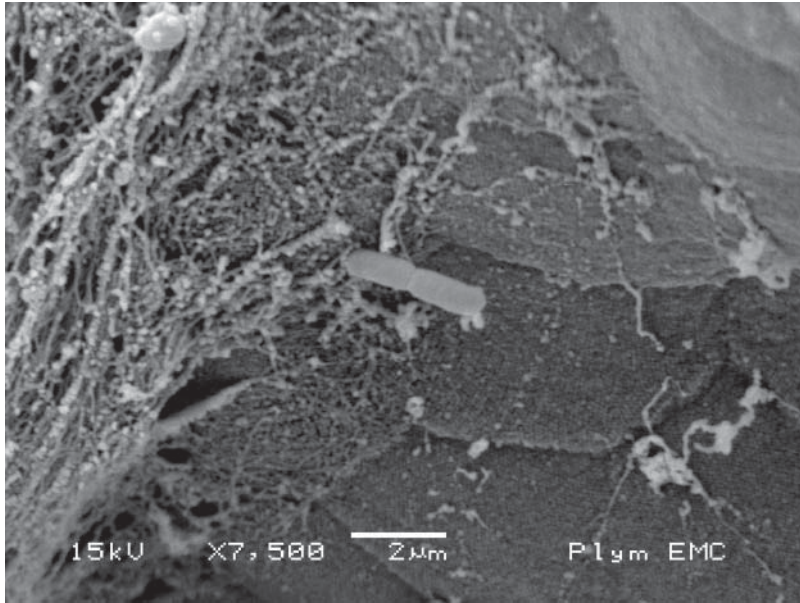


Fig. 4.1 Scanning electron micrograph of the anterior intestinal mucosa of rainbow trout; a pair of autochthonous bacterial rods is present in close association with the mucosal brush border. Scale bar = 2 μ m. [Source: Merrifield *et al.* 2009. Reproduced with permission of John Wiley & Sons.]

or digesta (the allochthonous microbiota), whereas the other group is potentially resident and intimately associated with host tissues (the autochthonous microbiota; Figure 4.1) (Ringø and Birkbeck 1999). The *normal* microbiota has also been defined as the community of microbes present in most individuals of a population or a species that, despite continual contact with different tissues, cause no harm to the host (Berg 1996).

In previous investigations to study the microbiota of the GI tract of fishes, the general approach has been the use of conventional culture based methods (Cahill 1990; Ringø and Birkbeck 1999). However, it has been reported that these methods present several disadvantages since the number and species of bacteria detected are affected mainly by the culture conditions and the culture media used, particularly certain fastidious and obligate anaerobes (Spanggaard *et al.* 2000). These conventional methods are time consuming and lack accuracy in isolate identification. Early in the 1990s, Cahill (1990) reviewed the current knowledge concerning the bacterial communities in fishes, at that time mostly based on culture-dependent observations. The description provided in that review was mainly based on biochemical identification of the microorganisms, which has restricted discrimination power and may lack proper definitions of relationships between aquatic-environmental microorganisms and fish microbiota. The lack of cultivability of the majority of the indigenous bacteria in many aquatic environments, including the GI tract of aquatic animals, is becoming increasingly apparent (Amann *et al.* 1995; as discussed in Chapter 5). For example, in Atlantic salmon (*Salmo salar* L.), coho salmon (*Oncorhynchus kisutch*) or yellowtail (*Seriola lalandi*), cultivable bacteria (using tryptic soy agar (TSA) incubated at 10 days at 17 °C) represent $\leq 1\%$ of the total bacteria (Romero and Navarrete 2006; Navarrete *et al.* 2009; Aguilera *et al.* 2013). To

study such environmental samples, several culture-independent molecular techniques have been developed. These methods have allowed the identification of microorganisms without isolation and the determination of the phylogenetic affiliation of community members, revealing the enormous extent of microbial diversity. The analysis of DNA extracted directly from a complex environmental sample provides a powerful and relatively bias-free alternative approach towards characterizing a microbial community (Nayak 2010). Typically, fragments of 16S or 18S ribosomal genes are selectively amplified by PCR to provide information on prokaryotic and eukaryotic communities, respectively (Navarrete *et al.* 2010a). Patterns of diversity and relative abundance of amplified DNA fragments can then be assayed using several strategies (see *Chapter 5*). Massive sequencing technologies have revolutionized this field by allowing direct sequencing of millions of DNA molecules from a single sample (Qin *et al.* 2010). It is thus now possible to obtain unbiased qualitative and quantitative reconstructions of complete microbial communities – including both cultivable and uncultivable representatives – within reasonable time frames and at affordable cost. Molecular techniques have been successfully applied in dozens of studies profiling the GI microbial communities of fish. Several attempts have been made to describe the microbiota in a number of important aquacultured fish species. Molecular methods based on PCR amplification of DNA extracted from frozen samples have typically been the favoured approach and have proven to be efficient in studying the GI bacterial community of fishes (Griffiths *et al.* 2001; Jensen *et al.* 2004; Romero and Navarrete 2006; Hovda *et al.* 2007; Kim *et al.* 2007). Recently, studies have begun to analyse the fish gut microbiota using massive sequencing strategies (e.g. van Kessel *et al.* 2011; Roeselers *et al.* 2011; Desai *et al.* 2012; Wu *et al.* 2012). It is anticipated that as this technology becomes more accessible it will significantly improve our knowledge of the fish gut microbiota, enabling identification of the rare biosphere and community metabolic pathways.

The ultimate goal of these studies is to provide a scientific basis for developing effective strategies for manipulating gut microbial communities to promote animal health and improve productivity. To achieve this goal, the principles governing microbiota composition (assembly) and maintenance within the intestine must be understood. The vast majority of these studies have focused on the bacterial communities and to a lesser extent yeast; very little information is available for the viral, archaean and protozoan populations in the GI tract of fish.

4.1.1 Current knowledge of the gut microbiota in fish

Our current knowledge of the microbiota composition is derived from a compilation of information in numerous reports; most of them focused on farmed fish, and among these the salmonids have received much attention. Figure 4.2 summarizes the most commonly reported bacterial phyla in salmonids, based on the review of Nayak (2010). Proteobacteria and Firmicutes are the most frequently reported phyla in the salmonid gut microbiota, suggesting that members of these bacterial classes are especially well adapted to conditions in the fish intestine or their surrounding aquatic environment. Interestingly, studies in salmonids show that some particular bacterial genera can be predominant in the microbiota composition; for example, *Pseudomonas* can represent more than 60% of the community when ribosomal amplicons are cloned and sequenced (Navarrete *et al.* 2009). The dominance of a particular bacterial group has been observed in salmonid guts using similar culture-independent methods. Holben *et al.* (2002) reported that some genera were highly abundant in reared Atlantic salmon from two different locations: in a Scottish hatchery, *Mycoplasma* corresponded to 81% of clones retrieved, whereas in a Norwegian hatchery, *Acinetobacter* accounted for 55%.

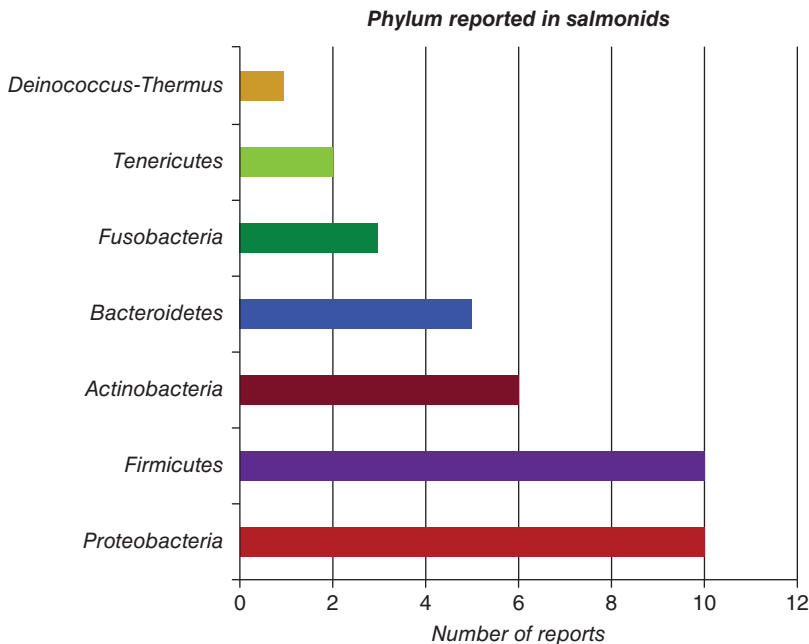


Fig. 4.2 Bacterial phyla observed in the gut microbiota of salmonids. [Source: Nayak 2010. Reproduced with permission of John Wiley & Sons.] For colour detail see Plate 9.

Although other genera were also present, their abundance was closer to 2%. Interestingly, in wild salmon (entirely carnivorous), the abundance of *Mycoplasma* was 96% of the clones analysed. Similarly, Pond *et al.* (2006) described the intestinal microbiota of rainbow trout (*Oncorhynchus mykiss* Walbaum) by using a cloning approach. They reported only two major groups among 200 clones analysed, which corresponded to *Clostridium* and *Aeromonas*. Furthermore, Kim *et al.* (2007) reported that *Clostridium* dominated the gut microbiota in rainbow trout analysed by denaturing gradient gel electrophoresis (DGGE). The carnivorous diet of salmon may explain in part the low number of taxa observed, since a recent study indicated that diet influences the bacterial diversity of the digestive tract. In this report, a more comprehensive analysis of vertebrate gut microbiota (albeit mostly mammalian) indicates that bacterial diversity increases from carnivore to omnivore to herbivore (Ley *et al.* 2008). This has recently been observed in Antarctic fish, among which the omnivorous *Notothenia coriiceps* (yellowbelly rockcod or bullhead notothen) exhibits greater diversity than the exclusively carnivorous *Chaenocephalus aceratus* (blackfin or Scotia Arc icefish) (Ward *et al.* 2009). This may indicate that increasing herbivory in fish leads to gut microbiota diversification, as observed in mammals.

Descriptions of microbiota from wild fish have also been reported, especially in herbivorous fish and also in some habitats of ecological interest. Recently, the microbiota of three fish from a coral reef was reported (Smriga *et al.* 2010). The studied fish corresponded to different diets representing two trophic levels: *Chlorurus sordidus* (parrotfish) is a herbivore that consumes primarily endolithic and epilithic algae; *Lutjanus bohar* (two-spot red snapper) is a top predator that consumes fishes and crustaceans; and *Acanthurus nigricans* (whitecheek surgeonfish)

Table 4.1 Summary of descriptions of yeast isolated from the GI tract of fish.

Fish species	Yeast species	References
Topsmelt (<i>Atherinopsis affinis littoralis</i>)	<i>Metschnikowia zobelii</i> and <i>Kloeckera apiculata</i>	Van Uden and Castello-Branco (1963)
Rainbow trout (<i>Oncorhynchus mykiss</i>)	<i>Candida</i> sp., <i>Saccharomyces cerevisiae</i> , <i>Debaryomyces hansenii</i> , <i>Cryptococcus</i> sp., <i>Leucosporidium</i> sp., <i>Trichosporon</i> sp. <i>Rhodotorula rubra</i> and <i>R. glutinis</i>	Sakata <i>et al.</i> (1993), Andlid <i>et al.</i> (1995), Aubin <i>et al.</i> (2005), Gatesoupe (2007), Waché <i>et al.</i> (2006)
European plaice (<i>Pluronectes platessa</i>)	<i>Rhodotorula</i> sp.	Andlid <i>et al.</i> (1995)
European flounder (<i>Platichthys flesus</i>)	<i>Rhodotorula</i> sp.	Andlid <i>et al.</i> (1995)
Bluefish (<i>Pomatomus saltatrix</i>)	<i>Rhodotorula</i> sp.	Newman <i>et al.</i> (1972)
Turbot (<i>Scophthalmus maximus</i>)	<i>Candida zeylanoides</i>	Toranzo <i>et al.</i> (1993), Vázquez-Juárez <i>et al.</i> (1997)
Pacific jack mackerel (<i>Tachurus symmetricus</i>)	<i>Metschnikowia zobelii</i> and <i>Debaryomyces</i> sp.	Van Uden and Castello-Branco (1963)

is a herbivore that consumes filamentous algae and detritus. Proteobacteria represented the largest portion of the total classifiable sequences in all three fish species, although the portion was smaller in *A. nigricans*. The percentage of the total library (138 clones) classified as Vibrionaceae (Gammaproteobacteria) was 75% in *L. bohar*, 70% in *C. sordidus*, and 10% in *A. nigricans*. As minor components, the *C. sordidus* microbiota also contained sequences corresponding to the phyla Bacteroidetes, Fusobacteria and Planctomycetes, and *L. bohar* contained Fusobacteria and Firmicutes. In contrast *A. nigricans* contained numerous non-*Vibrio* Proteobacteria, Bacteroidetes, Firmicutes and Spirochaetes sequences as well as representatives from other unclassified phyla. The authors suggested that the observed differences among fishes may reflect gut microbiota and/or bacterial assemblages associated with different ingested prey. Smriga *et al.* (2010) also reported that PCR amplification attempts using Archaea-specific primers produced no products from any of the three fish species. Fidopiastis *et al.* (2006) also reported negative PCR amplification for Archaea in the herbivorous fish *Hermosilla azurea*. In contrast, van der Maarel *et al.* (1999) detected archaeal ribosomal sequences in the digestive tract and faecal samples of flounder (*Platichthys flesus*) and grey mullet (*Mugil cephalus*). Yeast and protozoa have been proposed as other putative contributors to the ecology of the GI tract in fish. Several examples of yeast descriptions in different fish are summarized in Table 4.1 and reviewed by Gatesoupe (2007). Protozoa have been described in a number of fish species (Grim *et al.* 2002; Li *et al.* 2009; Merrifield *et al.* 2011a). New species of *Balantidium* and *Paracichlidotherus* were described as inhabiting the intestines of surgeonfish; however, further research is necessary to determine the contribution of these protozoan to fish health and nutrition.

The microbiota of herbivorous fish has been studied with interest because components of the microbiota in some fish use fermentation to convert carbohydrates into short-chain fatty acids (SCFAs) that can be absorbed by fish gut epithelial cells (Stevens and Hume 1998; Clements *et al.* 2009). It has been claimed that SCFAs in the gut may represent the contribution of

microbial fermentation to the total energy requirements of herbivorous fishes. Mountfort *et al.* (2002) reported SCFAs turnover rates comparable to those of ruminants for three herbivorous species, indicating that hindgut processes are an important contributor to the energy needs of the host fishes. With regard to microbiota composition, culturable bacteria may be dominated by *Vibrio* spp. in some fishes (Clements and Bellwood 1988; Clements *et al.* 1989). Meanwhile, 'giant' *Epulopiscium* spp. have only been observed in surgeonfish guts, which suggests that some fish gut-associated bacterial phylotypes are specific symbionts (Angert *et al.* 1993). However, a more diverse microbiota has been described in the digestive tracts of numerous species of herbivorous fishes representing several mainly tropical families (Fidopiastis *et al.* 2006). The high-nutrient assimilation efficiency and high levels of fermentation end products in the gut of kyphosids (Choat and Clements 1998) suggest that microbial fermentation may play an important role in the digestion of algal polysaccharides in these herbivores. Using a molecular approach based on 16S rDNA cloning, Fidopiastis *et al.* (2006) described the microbiota of the zebrafish (*Hermosilla azurea*), which has a strictly macroalgae diet and a relatively long digestive tract with an enlarged hindgut and an associated blind caecum (HC). These authors reported that bacterial counts and also the SCFAs concentration were significantly higher in HC contents compared to anterior gut regions. In the HC section, the microbiota composition was dominated by the Proteobacteria *Enterovibrio* and *Desulfovibrio*; other minor components were *Bacteroides* and *Faecalibacterium* from the phyla Bacteroidetes and Firmicutes, respectively. Contrasting results were described by Moran *et al.* (2005) about the microbiota of the herbivorous *Kyphosus sydneyanus*, a species from the same family as *Hermosilla azurea*. Phylogenetic analysis of sequences retrieved showed that most formed a clade within the genus *Clostridium* (Firmicutes), with one clone associated with the parasitic mycoplasmas. In subsequent studies (Skea *et al.* 2005; 2007; Clements *et al.* 2007) the microbiota of three temperate marine herbivorous fish species (*Kyphosus sydneyanus*, *Odax pullus* and *Aplodactylus arctidens*) was investigated using molecular cloning. In all of these herbivores, close to 50% of the cloned sequences corresponded to Clostridia, including different taxonomic members of this bacterial group. Clostridia are mostly polymer degraders, using polysaccharides and proteins as substrates and yielding alcohols and SCFAs as fermentation products. A bacterial community dominated by clostridial species is therefore consistent with the ratios of SCFAs previously reported in *K. sydneyanus*, *O. pullus* and *A. arctidens* (Mountfort *et al.* 2002).

Data on the composition of microbiota in fish intestines are controversial. According to some authors, the composition is similar to that of integuments and gills, and most intestinal bacteria are aerobic or facultative anaerobic (Cahill 1990). On the other hand, there are data showing that the intestines of fishes (especially herbivorous species) contain both facultative and obligate anaerobes (Clements 1997). Interpretation and comparison of relevant results obtained by different authors are complicated by the fact that a wide variety of differing techniques have been used and some of them distinguished between allochthonous bacteria and bacteria closely associated with the intestinal mucosa (autochthonous) (e.g. Hansen and Olafsen 1999; Ringø *et al.* 2006; Bakke-McKellep *et al.* 2007; Olsen *et al.* 2008; Ringø *et al.* 2008; Zhou *et al.* 2011; Hartviksen *et al.* 2014; Ringø *et al.* 2014). However, some general remarks about the bacterial composition of the microbiota of fish can be made using current information. Based on the review of Izvekova *et al.* (2007), bacteria observed in different fish were grouped using several criteria (structural and metabolism) to obtain graphical representation of the most commonly reported microbes in marine and freshwater fish. Figure 4.3 shows the distribution of aerobic microbes grouped into Gram-negatives (A) and

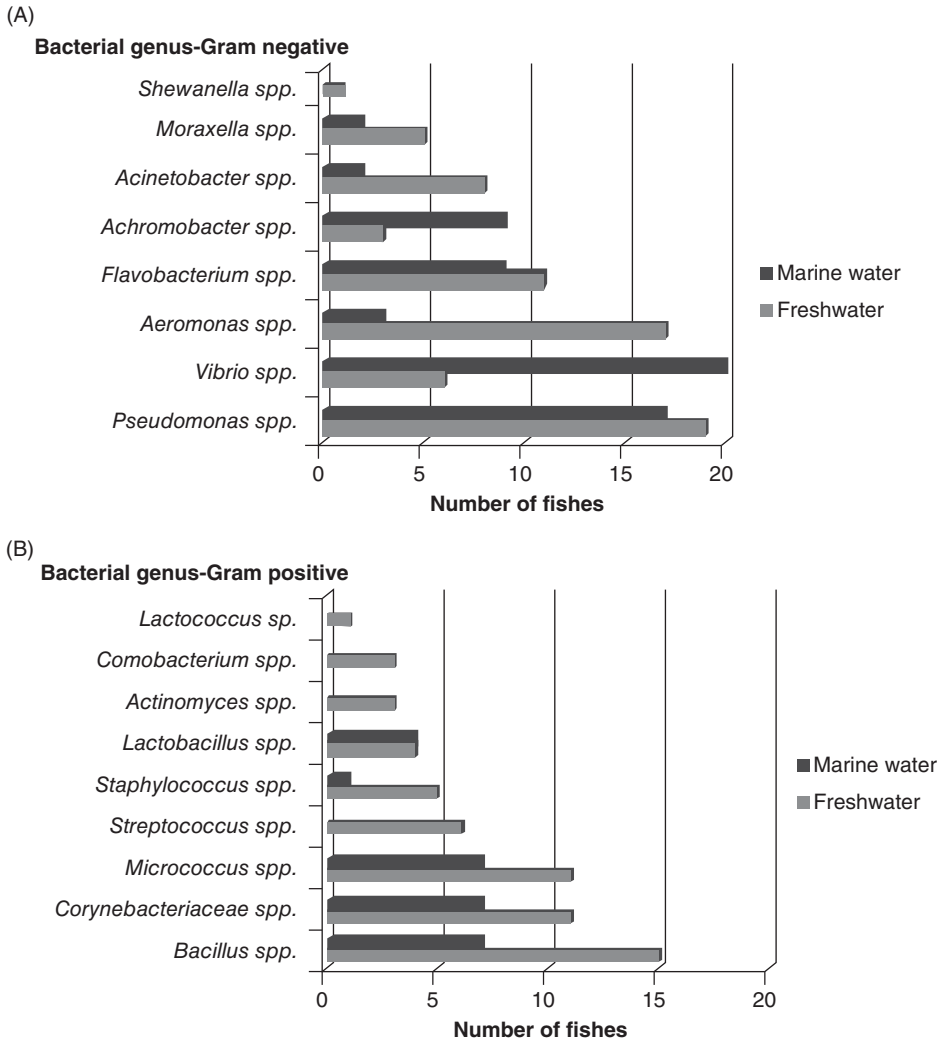


Fig. 4.3 Aerobic Gram-negative (A) and Gram-positive (B) bacterial species reported in the GI tract of marine and freshwater fish. (Source: Data from Izvekova 2007.)

Gram-positives (B) as they have been observed in the GI tract of marine or freshwater fish. It is interesting that some bacterial genera such as *Vibrio*, *Pseudomonas*, *Bacillus* and *Micrococcus* spp. are more frequently observed. It is also observed that some bacteria showed a tendency or preference for a particular environment (marine or freshwater). The anaerobic microbes reported in the gut microbiota of fish are graphically represented in Figure 4.4; *Clostridium* and *Bacteroides* seem to be the most frequent anaerobic bacterial genera in fish intestines.

A graphical representation of the phyla observed in different fish is shown in Figure 4.5. This figure indicates that the predominant microbiota present belong to five phyla: Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes and Fusobacteria. These different phyla

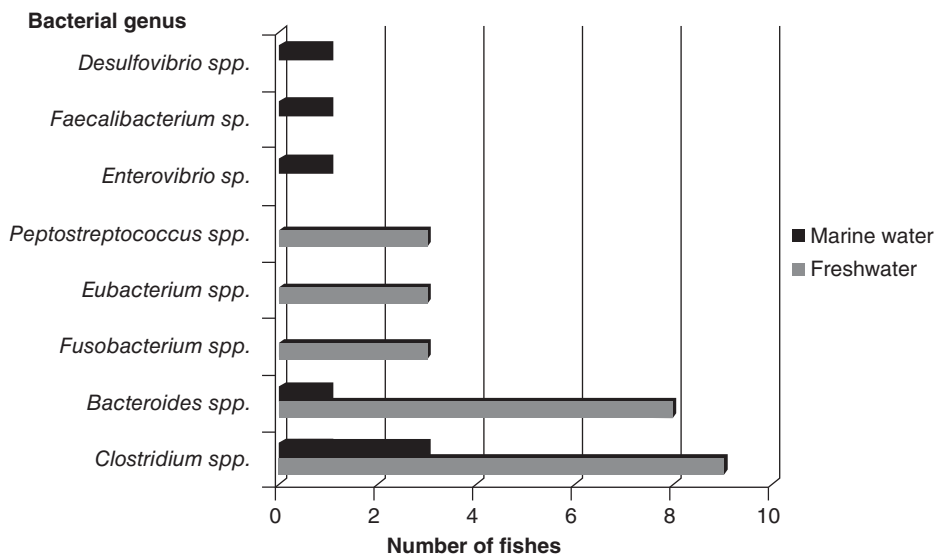


Fig. 4.4 Anaerobic bacteria reported in the GI tract of marine and freshwater fish. (Source: Data from Izvekova 2007.)

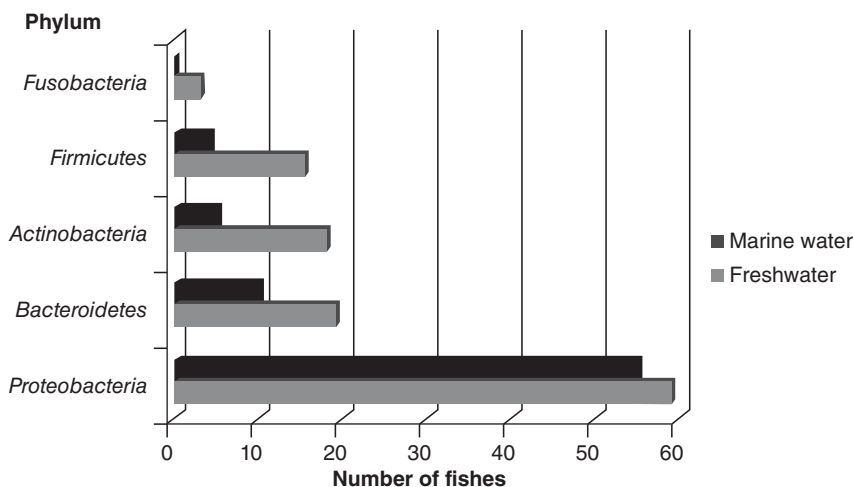


Fig. 4.5 The bacteria phyla reported in the GI tract of marine and freshwater fish. (Source: Data from Izvekova 2007.)

observed may contribute with different activities to the host inner environment. Bacteria belonging to the Proteobacteria phylum, which were present in a high percentage in all families, are known to induce important responses in the host (Rawls *et al.* 2004; 2007; Bates *et al.* 2006). Also, members of this phylum can exploit environmental reservoirs outside their hosts to proliferate and persist in aqueous environments, which helps to explain the relative high prevalence of these bacteria in the GI tract of fish (Rawls *et al.* 2006).

Lactic acid bacteria (LAB) constitute an interesting bacterial group that has been investigated in several animal hosts. It has also been reported that LAB are present in fish intestines (Ringø and Gatesoupe 1998; Ringø *et al.* 2000; Ringø 2004; Ringø *et al.* 2005; Vazquez *et al.* 2005; Gatesoupe 2008; Navarrete *et al.* 2010a; Lauzon and Ringø 2012; *Chapter 6*). A number of endogenous LAB strains have been tested for their probiotic potential (Merrifield *et al.* 2010). The presence of LAB and their relevance to their aquatic animal hosts is further discussed in *Chapter 6*.

4.1.2 Viruses

The digestive tract of fish is also an environment available for bacteriophages and the microbial composition, particularly bacterial, might be influenced by the effect of bacteriophages. Lysis by bacteriophages has been claimed as an important selective pressure participating in the complex regulation of bacterial populations in the microbiota of animals. The estimated 1200 viral genotypes in human faeces suggest that phage attack is a powerful shaper of the gut's microbial landscape (Backhed *et al.* 2005). The number of free bacteriophage particles in coastal seawater may exceed 10^8 particles per ml (Berg *et al.* 1989) and bacterial mortality due to viral lysis has been estimated as 30% to 60%. Bacteriophages have been described in the majority of bacterial genera, among which are various pathogenic bacteria such as *Vibrio* spp. and *Aeromonas salmonicida* (Hansen and Olafsen 1999). Recently, Bastías *et al.* (2010) isolated bacteriophages from digestive tracts of fish commonly found in Mexico and Chile and after characterization they showed that some bacteriophages might correspond to a cosmopolitan phage group widely spread in separated geographical locations. The determination of the types, numbers and importance of phages in the fish GI tract is a topic which should be explored comprehensively in future studies.

4.1.3 Yeast

To our knowledge, the first study isolating yeast from fish – topsmelt (*Atherinopsis affinis littoralis*) and Pacific jack mackerel (*Trachurus symmetricus*) – was demonstrated by van Uden and Castelo Branco (1963). Since then yeasts have been identified as part of the normal microbiota of fish; sometimes high population densities are observed in healthy fish, but the data are variable in terms of colony counts and taxonomical diversity (Gatesoupe 2007). However, the literature on the role of yeast in fish health and nutrition is scarce. Yeasts are widely distributed in several natural environments including freshwater and seawater. Marine yeast participate in several ecological processes in the sea, especially in estuarine and near-shore environments, such as decomposition of plant substrates, nutrient recycling, biodegradation of oil and recalcitrant compounds, and as part of the microbiota of marine animals (Kutty and Philip 2008). This is due in part to the fact that yeast have an extraordinary metabolic potential available for exploitation (Kutty and Philip 2008; Song *et al.* 2010). Notably, the vast majority of this potential has yet to be discovered. Several compounds that are produced by yeast have huge biological value as reagents, cell proteins, vitamins, pigments, immunostimulants and enzymes (Chi *et al.* 2009) and thus the presence of yeast in the GI tract of fish is likely to be of importance to the host.

The yeast load in the fish gut is variable and can fluctuate from non-detectable levels to up to 10^7 CFU g^{-1} of intestinal content (Gatesoupe 2007). It is important to note that yeast cells can be a hundred times bigger than bacterial cells, which may explain the fact that the introduction

of a low yeast population (10^4 CFU g⁻¹) through feed can induce beneficial effects in the host (Tovar *et al.* 2002; Tovar-Ramírez *et al.* 2004). Indeed, the volume of a yeast cell may be larger than those of bacteria (200–300 μm^3 for brewer's yeast versus 1 μm^3 for *Pseudomonas*; Gatesoupe 2007) and therefore yeasts may be of physiological importance even when accounting for less than 1% of the total microbial isolates. Therefore, an apparently low yeast load may correspond to a population size sufficient to act upon the host.

Yeast identified from the fish intestine belong to two phyla: Ascomycota, among which Saccharomycetaceae are probably the most important family, and Basidiomycota, which include the genus *Rhodotorula* (red yeast commonly detected in the microbiota of both marine and freshwater fish) (Newman *et al.* 1972; Andlid *et al.* 1995). The Ascomycota yeast *Metschnikowia zobellii* and *Candida tropicalis* and the Basidiomycota yeast *Trichosporon cutaneum* are dominant in some marine fish. The Ascomycota *Debaryomyces hansenii*, *Candida* spp., *Saccharomyces cerevisiae*, and the Basidiomycota *Leucosporidium* sp. have been frequently isolated as dominant yeast from the rainbow trout intestine. *Cryptococcus*, *Pichia*, and Saccharomycodaceae have also been occasionally isolated (Gatesoupe 2007). A summary of descriptions is shown in Table 4.1.

It has been reported that yeast isolated from the intestine of rainbow trout may adhere to and grow in intestinal mucus (Andlid *et al.* 1998). Some yeast can colonize the intestine of fish when introduced through feed (Waché *et al.* 2006). This colonization ability may be related to cell surface hydrophobicity (Vázquez-Juárez *et al.* 1997) and the ability of the strains to grow on mucus (Andlid *et al.* 1998). Furthermore, yeast have immunostimulatory properties due to components such as β -glucan, mannoproteins, chitin (as a minor component) and nucleic acids (Ortuño *et al.* 2002). Studies have shown the beneficial effect of *S. cerevisiae* when added to fish feed. Fish diets supplemented with this yeast act as immunostimulants that enhance the growth, feed efficiency, blood biochemistry, survival rate, and non-specific immune response in several fish species (Welker *et al.* 2007; Chiu *et al.* 2010; Harikrishnan *et al.* 2010; Tukmechi *et al.* 2011). Recently, Hoseinifar *et al.* (2011) reported that dietary inactive brewer's yeast might affect microbiota composition; although the total intestinal bacterial counts were not affected, the levels of LAB were significantly elevated in fish fed dietary yeast. Most published studies have been performed with *Saccharomyces cerevisiae*; however, promising results have also been obtained with *Debaryomyces hansenii*, which has been assessed in grouper and gilt-head sea bream (Reyes-Becerril *et al.* 2008a; 2008b).

4.2 THE IMPORTANCE OF THE MICROBIOTA

It is generally recognized that the GI microbiota of animals serves several functions, namely aiding digestion and the development of the mucosal system, angiogenesis, and as a protective barrier against disease (Rawls *et al.* 2004; Ringø *et al.* 2007). An important study by Rawls *et al.* (2004) showed that the GI microbiota can regulate the expression of 212 genes in the digestive tract of zebrafish, some of them related to the stimulation of epithelial proliferation and promotion of nutrient metabolism and innate immune response. Studies performed in model vertebrates – mice and zebrafish – also provide insights into the microbial–host molecular dialogues that impact on several functions of the host, including nutrition, immunity and development (Rawls *et al.* 2006; Round and Mazmanian 2009). An important aspect of these results was the specificity of the host response, which depends on the bacterial species

that colonize the digestive tract (Rawls *et al.* 2004; 2007). Hence, it is relevant to know the composition of this microbiota in fish.

Using germ-free zebrafish, Rawls *et al.* (2007) investigated microbiota–zebrafish interactions. *Pseudomonas*, as a common bacterial genus described in fish microbiota, were investigated in comparison with defective *Pseudomonas* mutants. *Pseudomonas* lacking flagella were unable to interact with host, and non-motile mutants expressing flagella showed very limited interaction. Hence, *Pseudomonas* spp. also require motility to stimulate inflammatory signals in zebrafish. These authors suggested that flagella-dependent swimming motility promotes physical interaction between *Pseudomonas* and the host epithelium, where the presence of surface-attached antigens (including the flagellum itself) and other bacterial products can be detected and monitored by the host.

Other studies using germ-free zebrafish reveal the importance of gut microbes on host digestive tract development and function. Bates and colleagues (2006) observed that the differentiation of the GI tract was arrested in the absence of the microbiota, as was illustrated by a lack of brush border intestinal alkaline phosphatase activity (an enzyme associated with mucosal tolerance with respect to detoxifying bacterial lipopolysaccharide endotoxins), immature patterns of glycan expression, and a reduction of goblet cells (mucus producing) and enteroendocrine cells which ultimately leads to the failure to uptake protein macromolecules in the distal intestine. Interestingly, however, the reintroduction of microbiota can reverse these phenotypic changes in the GI tract.

Beyond the developmental stages, the microbiota continues to be involved in nutritional functions. Smriga *et al.* (2010) suggested that members of Proteobacteria, Bacteroidetes, Firmicutes and Fusobacteria phyla may contribute to the digestive process by providing a variety of enzymes in fish such as parrotfish, snapper or surgeons. Members of the phylum Fusobacteria, which are known to colonize the gut of zebrafish (Roeselers *et al.* 2011), can excrete butyrate (Kapatral *et al.* 2003) or synthesize vitamins (Roeselers *et al.* 2011) which may exert a positive effect on fish health. The phylum Actinobacteria represents one of the largest taxonomic units among the 18 major lineages currently recognized within the domain Bacteria. Members of this phylum exhibit diverse physiological and metabolic properties, such as the production of extracellular enzymes and the formation of a wide variety of secondary metabolites (Ventura *et al.* 2007).

A particularly interesting case is that of *Cetobacterium somerae* (previously named *Bacteroides* type A), a microaerotolerant bacterium detected in many different fish species: long-jawed mudsucker (Bano *et al.* 2007), rainbow trout (Kim *et al.* 2007), common carp (*Cyprinus carpio*) (Omar *et al.* 2012), tilapia (*Oreochromis niloticus*) (Tsuchiya *et al.* 2008), zebrafish (Roeselers *et al.* 2011) and goldfish (Silva *et al.* 2011). As *Cetobacterium somerae* produces large quantities of vitamin B₁₂ (cobalamin) and is present in high numbers, it has been suggested that this species provides a source of vitamin B₁₂ for some freshwater fish species (Sugita *et al.* 1991; Tsuchiya *et al.* 2008; NRC 2011). Indeed, it is interesting to note that some fish species such as tilapia and carp, where *C. somerae* has often been reported to be a constituent of the GI microbiota, have no dietary vitamin B₁₂ requirements, whereas other species such as channel catfish (*Ictalurus punctatus*) and Japanese eel (*Anguilla japonica*), where *Cetobacterium somerae* is not a common component of the GI microbiota, have a requirement for dietary vitamin B₁₂ (NRC 2011). However, it is important to consider that since the application of molecular methods has allowed the identification and classification of *Cetobacterium* as a separate genus from *Bacteroides* it may be the case that some of the earlier

studies which have identified isolates as *Bacteroides* by biochemical and physicochemical means may in fact have been *Cetobacterium* spp. Further research should be dedicated to understanding the distribution of *Cetobacterium* spp. in the gut of aquatic animals and quantifying their vitamin contributions to the host.

Some authors have proposed a possible contribution of the fish gut microbiota to host nutrition by providing enzymatic activities complementary to the host (Ray *et al.* 2012). It has been suggested that fish gut microbiota might have positive effects for the digestive processes of fish and indeed an extensive range of enzyme-producing microbiota have been isolated and identified in the GI tract of fish. In addition to *Bacillus*, *Microbacterium*, *Micrococcus*, unidentified anaerobes and yeast are also suggested to be possible contributors. Nonetheless, in contrast to endothermic animals, it is difficult to conclude the exact contribution of the GI microbiota because of the complexity and variable ecology of the digestive tract of different fish species, the presence (or absence) of a stomach and pyloric caeca, and the relative intestinal length. Readers with an interest in this topic are referred to the comprehensive review of Ray *et al.* (2012) which reports numerous examples of amylase-, protease-, lipase-, chitinase-, cellulase- and phytase-producing bacteria isolated from the GI tract of fish.

While it is difficult to estimate the contribution of specific bacteria to the function of the whole gut ecosystem, it is reasonable to expect that the overall microenvironment would be strongly influenced by the predominant populations of organisms. It is expected that modern molecular approaches and new sequencing technologies will significantly improve our knowledge of the fish gut microbiota and the factors that influence its composition and its effects on the host.

4.3 COMPOSITION OF THE MICROBIOTA IN EARLY LIFE STAGES

The early developmental stages of fish and other vertebrates typically occur within the chorion, a germ-free environment (Roeselers *et al.* 2011). After eclosion, vertebrates are exposed to the microorganisms present in their respective local environment. The external surfaces of the vertebrate body are subsequently colonized with microbes, with the majority of these microbial residents assembling into dense communities, particularly in the GI tract.

The early studies of Strøm and Ringø (1993), Berg *et al.* (1994), Munro *et al.* (1994), Berg (1995), Ringø *et al.* (1996) and Ringø and Vadstein (1998) revealed colonization in larval gut after hatching and the bacterial colonization seems to follow a two-step pattern. However, little is known about its stability, especially after dietary changes (live food, artificial food) or treatment with antibiotics or disinfectants, which are routine practices in larval aquaculture. Understanding some aspects of microbial ecology in aquaculture systems, such as knowing the types, numbers and sources of bacteria commonly associated with different developmental stages, could be useful for manipulating microbiota as a strategy to prevent pathogenic infection or to improve nutrition, especially in early life stages.

Some investigations have reported that bacteria present in the hatchery environment may influence the composition of GI microbiota (Cahill 1990; Ringø and Birkbeck 1999). Based on a culture-dependent approach, these results suggest that bacteria present in the GI tract generally seem to be those from water or the diet, and which can survive and multiply (Olafsen 2001). Furthermore, larvae may ingest substantial amounts of bacteria by grazing on suspended

particles and egg debris (Olafsen 2001). Hence, it is tempting to suppose that egg microbiota would also affect the primary colonization of the fish larvae. It has been suggested that the glycoproteinaceous nature of the egg surface is well suited for adhesion and colonization by bacteria (Hansen and Olafsen 1999). Furthermore, the fish embryo will secrete low molecular weight organic compounds which diffuse through the chorion and establish a gradient, attracting microbes able to utilize the secreted compounds. These elements may contribute to the establishment of a primary microbial biota on the egg surface (epibiota), which may participate in the later colonization of the larvae. Bacterial growth on fish egg surfaces may cause problems for the rearing of several marine fish species, especially in intensive production. Demanding systems may promote bacterial overgrowth on eggs and other surfaces or may affect the relationship between the innocuous-beneficial microbes and opportunists, and subsequently hamper egg development and larval health. This topic is further discussed in *Chapter 16*.

Using molecular tools to describe microbiota composition, Romero and Navarrete (2006) showed very simple bacterial communities in the early stages of coho salmon including eggs and the GI tract of first-feeding fries and juveniles, and described changes in bacterial community during growth. Based on the observation of molecular profiles (DGGE), the authors describe two or four dominant bacteria per stage analysed. In eggs, the dominant bacteria belonged to Betaproteobacteria (*Janthinobacterium* and *Rhodospirillum*). During the first feeding stage, the most abundant bacteria in the GI tract clustered with Gammaproteobacteria (*Shewanella* and *Aeromonas*). In juveniles weighing more than 2 g, prevailing bacteria were *Pseudomonas* and *Aeromonas* spp. To determine the putative origin of dominant *Pseudomonas* and *Aeromonas* reported in juvenile GI tracts, specific primers for these groups were designed based on sequences retrieved from molecular profiles (DGGE). Then samples of the water influent, pelletized feed, and eggs were analysed by specific PCR and sequencing; only the eggs and the water influent samples showed identical sequences to *Pseudomonas* and *Aeromonas* observed in the gut molecular profiles.

Similar transitions in the GI-associated bacteria have been reported by different authors in early stages of Atlantic halibut (*Hippoglossus hippoglossus*) and haddock (*Melanogrammus aeglefinus*) development (Verner-Jeffreys *et al.* 2003; Jensen *et al.* 2004; Plante *et al.* 2007). Using a culture-independent approach, Plante *et al.* (2007) observed a complex microbial community in unfed haddock yolk sac larvae and first feed larvae. This situation rapidly changed resulting in a restricted diversity between 5 and 9 days post hatching (dph), when some bacterial genera became dominant, first *Pseudoalteromonas* and *Vibrio*, followed by *Cytophaga* and *Marinomonas* at 22 dph. The microbiota composition seemed to stabilize at 29 dph, when *Sulfitobacter* was identified as the dominant component of the larval microbiota, and this dominance continued over the rest of the sampling period (to 56 dph). The rise of *Sulfitobacter* coincided with the transition in live food from rotifer to *Artemia*, and it was not altered by the introduction of dry feed. Using a similar molecular approach, comparable results were reported by Jensen *et al.* (2004) in the analysis of bacterial communities in Atlantic halibut larvae. The molecular profiles revealed simple communities after hatching and bacterial succession following growth. Molecular identification indicated that aerobic heterotrophs related to groups of *Pseudomonas*, *Janthinobacterium* and *Marinomonas* were the initial colonizers of the halibut larvae. After the onset of feeding, fermentative species (*Vibrio*) were detected as well. Altogether these results suggested that a stable microbiota could be established after the first feeding stages and its major components could be derived from water, egg epibiota or live feed.

4.4 FACTORS THAT INFLUENCE MICROBIOTA COMPOSITION

4.4.1 Host factors

The relationship between marine or aquatic eukaryotes and specific microbial populations has been reported in several examples using molecular approaches: sponges, shrimps, molluscs and finfish (Griffiths *et al.* 2001; Hentschel *et al.* 2002; Lau *et al.* 2002; Holben *et al.* 2002; Rawls *et al.* 2004; Mansfield *et al.* 2010). Furthermore, subsets of the marine microbiota seem to be associated with specific organisms, suggesting particular functions or roles for this interaction. For example, Gammaproteobacteria are well known to colonize particles and animals in the sea; however, not Gamma- but rather Alphaproteobacteria were described on diatoms and algae (Schäfer *et al.* 2002; Jensen *et al.* 2004). This could be extrapolated to the aquatic animals used in aquaculture systems, with particular considerations. Some of these animals have been domesticated and their reproduction and rearing are processes performed in enclosed facilities; therefore, they have no contact with their respective natural habitat, the place where their wild ancestors were formerly collected. The microbial colonization in these animals and its consequences for the health status of the host will be strongly dependent on the management of environmental and sanitary conditions. Nevertheless, some recent investigations have revealed that host factors are very important in the definition of the fish gut microbiota, and some evidence for the existence of a core microbiota in fish has been presented (Roeselers *et al.* 2011).

Roeselers *et al.* (2011) addressed the influence of the host in the selection of the microbial community that inhabits the gut. These authors argued two possibilities: gut microbial communities are shaped by the composition of the microbial community present in the local environment; or gut microbial community composition is shaped by selective pressures that occur within the host gut habitat. In the first case, temporal and spatial separation of reared animals from their natural habitat could result in major differences in gut microbiota composition compared with wild hosts. In the second option, wild hosts and those reared in different facilities could have similar gut microbial communities. Roeselers *et al.* (2011) observed variation between wild (recently caught) and domesticated zebrafish; however, the scale of these variations was no larger than that observed between or within different zebrafish lab facilities. In addition, the bacterial taxa identified as dominant in the gut of wild zebrafish were largely the same as those dominating the domesticated zebrafish gut. Altogether, these results indicate that wild zebrafish in their natural habitat and domesticated zebrafish maintained in aquaculture facilities acquire a common gut bacterial community. This suggests that features of the intestinal habitat in these fish select for specific bacterial taxa, revealing that a set of bacterial genera (a core gut microbiota) is present in domesticated and wild zebrafish despite differences in their local environments. The influence of the microbiota on the host may not only be derived from the composition of the microbial community or from the activities expressed by these microbes, because other factors could be involved as specific adaptations of the host, and on the modulation of microbe and host effects by environmental factors (Rawls *et al.* 2006). Little information is available about the interaction between microbial communities, host factors, and the physical environment (Spor *et al.* 2011). Recently, a study by Navarrete *et al.* (2012) assessed the relative contributions of host genetics and diet in shaping the gut microbiota of rainbow trout. Fish from different full-sib non-related families were fed diets

containing vegetable proteins or vegetable oils for two months in comparison to a control diet consisting of only fish protein and fish oil. These authors focused on transcriptionally active bacterial populations, which were examined based on RNA analysis using RT-PCR, TTGE profiles and TTGE band sequencing. Results showed that some bacterial groups were significantly ($P < 0.05$) associated with specific trout families, indicating that the host may influence microbiota composition. In addition, the effect of diet on microbiota composition was dependent on the trout family. The host factors, such as the genetic background, that select specific bacterial groups are unknown and could be the subject of future analysis.

4.4.2 Diet

Numerous studies have investigated the impact of dietary changes on the GI microbiota of aquatic animals (Table 4.2). Generally speaking this research has focused on: dietary form, the effect of replacing fishmeal with alternative proteins, dietary lipids and feed additives (e.g. phytobiotics, immunostimulants, probiotics and prebiotics). As some of these topics have been comprehensively reviewed elsewhere recently (Merrifield *et al.* 2010; 2011b; Ringø *et al.* 2010; 2012; Dimitroglou *et al.* 2011) the effects of dietary components on the gut microbiota will not be discussed in detail within this chapter.

Antimicrobial treatments are effective at reducing or preventing mortalities caused by the primary pathogen but, as many antibiotics are broad spectrum, they may impact upon the gut microbiota. This topic is often overlooked but several studies have reported that the indigenous gut microbiota may be altered in terms of total viable numbers and/or diversity of populations after antibiotic exposure (Austin and Al-Zahrani 1988; Lesel *et al.* 1989; Moffitt and Mobin 2006; Bakke-McKellep *et al.* 2007; Navarrete *et al.* 2008; Romero *et al.* 2012). Any reduction of microbial levels or diversity could lead to a reduction of the effective barrier provided by the commensal microbiota. Antibiotic treatment can eradicate susceptible microorganisms from the microbiota and facilitate the proliferation of resistant opportunistic pathogens by minimizing competition and promote opportunists that may occupy previously unavailable ecological niches. Additionally, it has been suggested that genetic material conferring antimicrobial resistance may be transferred from the remaining indigenous populations to opportunistic or potentially pathogenic visitors to the GI tract (Navarrete *et al.* 2008). In fish, this is a more dangerous situation than is the case for terrestrial animals as the rearing water readily supports and spreads bacterial pathogens. Future studies should further address this topic. In addition the use of feed additives to promote a healthy microbial balance after antibiotic treatments should be further explored.

4.4.3 Environmental factors

The early view (1970s–1980s) was that the existence of a stable microbiota in aquatic animals was controversial (Cahill 1990; Spanggaard *et al.* 2000), but over the past few decades significant numbers of studies have been carried out to characterize the microbiota in a wide range of fish species (Nayak 2010). One important attribute of GI microbiota is that bacterial components must be present in the majority of healthy individuals and represent populations that are readily stable over time (Berg 1996; Ringø and Birkbeck 1999). There are limited studies available that address the microbiota stability issue. Some of them are focused on the changes in a short time scale (weeks, months), but others compare microbiota composition between different seasons (or years).

Table 4.2 Overview of investigations of the gut microbiota of fish in response to changes in dietary form or composition.

Dietary factor	Experimental details	Observations	References
Dietary form	Goldfish fed on either pelleted diets and tubifex worms or pelleted diets and dried daphnia	The authors concluded that the gut microbiota was not influenced by the diets	Sugita <i>et al.</i> (1988)
	Arctic charr fed either a capelin roe diet or a commercial feed	Some changes in microbial composition	Ringø and Strøm (1994)
	Yellow grouper fed an extruded sinking diet or natural diet	Some observed changes in the autochthonous gut microbiota	Feng <i>et al.</i> (2010)
Dietary lipid	Rainbow trout fed high (16%) and low (5%) dietary lipid levels	Changes in microbial composition observed	Lesel <i>et al.</i> (1989)
	Arctic charr fed high (27%) and low (13%) dietary lipid levels	Changes in carnobacteria populations	Ringø and Olsen (1999)
	Arctic charr fed diets containing soybean, linseed and marine oils	Changes in carnobacteria populations	Ringø <i>et al.</i> (2002)
	The effect of dietary linoleic acid (18:2 <i>n</i> -6) supplementation in Arctic charr diets	Linoleic supplementation elevated the levels of <i>Lactobacillus</i> spp.	Ringø (1993)
	Arctic charr fed casein based diets supplemented with different fatty acids	Increase in lab in fish fed 7% linoleic acid and HUFA mix has not been elucidated	Ringø <i>et al.</i> (1998)
	Incorporation of dietary SBM in diets for Atlantic cod	Refer to review of Merrifield <i>et al.</i> (2011b)	Ringø <i>et al.</i> (2006), Refstie <i>et al.</i> (2006)
Protein sources	Incorporation of dietary SBM in diets for Atlantic salmon	Refer to review of Merrifield <i>et al.</i> (2011b)	Bakke-McKellep <i>et al.</i> (2007), Ringø <i>et al.</i> (2008)
	Incorporation of dietary SBM in diets for rainbow trout	Refer to review of Merrifield <i>et al.</i> (2011b)	Heikkinen <i>et al.</i> (2006), Merrifield <i>et al.</i> (2009)
	Incorporation of dietary SBM in diets for silver crucian carp (<i>Carassius auratus gibelio</i> ♀ × <i>Cyprinus carpio</i> ♂)	No significant differences in culturable bacterial populations enumerated	Cai <i>et al.</i> (2012)
	Incorporation of dietary SBM in diets for gilthead sea bream	Fish fed SBM diets showed distinctly different microbial profiles to FM fed fish	Dimitroglou <i>et al.</i> (2010)
	Incorporation of dietary yeast protein concentrate in diets for common carp	No significant differences in microbial profiles	Omar <i>et al.</i> (2012)
	Effects of krill and chitin meals on various fish species	Numerous species	Refer to review of Ringø <i>et al.</i> (2012)

Table 4.2 (continued)

Dietary factor	Experimental details	Observations	References
Feed additives (probiotics, prebiotics, immunostimulants, antibiotics and phytobiotics)	Probiotics: numerous bacterial and yeast species	Numerous species	Refer to the reviews of Merrifield <i>et al.</i> (2010), Dimitroglou <i>et al.</i> (2011)
	Prebiotics: numerous oligosaccharides and polysaccharides	Numerous species	Refer to the reviews of Merrifield <i>et al.</i> (2010), Ringø <i>et al.</i> (2010), Dimitroglou <i>et al.</i> (2011)
	Inclusion of <i>Thymus vulgaris</i> essential oil (TVEO) in rainbow trout diets	No statistical differences were observed between the gut microbial profiles of TVEO-treated and untreated fish	Navarrete <i>et al.</i> (2008)
	Red tilapia fed diets with or without alginic acid incorporation	No significant differences in microbial profiles	Merrifield <i>et al.</i> (2011a)
	Red tilapia fed control diets or diets with increasing inclusion levels of a commercial isoquinoline alkaloid sanguinarine source (Sangrovit®)	Culturable loads not affected but LAB levels significantly reduced with increasing Sangrovit® inclusion	Rawling <i>et al.</i> (2009)
	Common carp fed diets with or without β -glucans	Autochthonous: reduced the abundance of LAB, number of OTUs, species richness and diversity Allochthonous: reduced the number of OTUs and species richness	Kühlwein <i>et al.</i> (2013)
	Antibiotics	Numerous species	Refer to the review of Romero <i>et al.</i> (2012)

Key: FM, fishmeal; LAB, lactic acid bacteria; SBM, soybean meal.

It has been reported that the stability of the gut microbiota may be influenced by seasonal changes (Al-Harbi and Uddin 2004; Hagi *et al.* 2004). In a year-long study, Hagi *et al.* (2004) described the changes of LAB composition in common carp intestine; the main finding was the predominance of *Lactococcus lactis* in summer (20 °C) and *Lactococcus raffinolactis* in winter (10 °C); the change in predominant LAB was revealed to be due to the difference in the growth profile of the two species. Also using a culture-dependent approach, Al-Harbi and Uddin (2004) described the seasonal variation in the intestinal microbiota of hybrid tilapia (*Oreochromis niloticus* $\times$ *Oreochromis aureus*). *Aeromonas*, *Shewanella* and *Corynebacterium* were the most abundant species with prevalence near to 20% in summer, descending to near to 10% in winter. Considerable numbers of *Pseudomonas* were observed only in winter. Other minor components such as *Photobacterium*, *Cellulomonas* and *Bacillus* were present in some

seasons of the year. Recently, Hovda *et al.* (2011) used a molecular approach to examine the gut microbiota of farmed Atlantic salmon during an annual cycle. These authors reported that LAB were among the predominant bacterial groups as *Lactococcus*, *Weissella* and *Lactobacillus* were observed in all molecular profiles derived from samples collected during a year-long study. In contrast, Gram-negative bacteria such as *Vibrio* and *Photobacterium* and also an uncultivable spirochete were observed only at some time points.

In another case, Romero and Navarrete (2006) followed the microbiota composition of individual juvenile coho salmon from the same cohort collected during a 3 month period. During this period the fish were reared under controlled conditions and fed the same diet. The collected specimens started at 2 g (body weight) and finished at 15 g. The examination was focused on individually collected samples from intestinal contents. Using a culture-independent method, all individuals tested showed almost identical molecular profiles with dominant bacteria corresponding to *Pseudomonas* and *Aeromonas*. These data suggest that these bacteria may be part of the GI microbiota of coho salmon and seem to be stable during the stages analysed (2–15 g). It is important to notice that in several investigations it has been consistently reported that there is a predominance of a limited number of bacterial groups in salmonid guts within Chilean farms (Romero and Navarrete 2006; Navarrete *et al.* 2009; 2010a), in accordance with the observation by Holben *et al.* (2002).

One study evaluated the short term (weeks) stability of the rainbow trout gut microbiota when investigating a feed additive (Navarrete *et al.* 2010b). The diet assessment was focused on determining the effect of the dietary inclusion of *Thymus vulgaris* essential oil (TVEO) on microbiota composition, compared with a control diet without TVEO. Rainbow trout were reared under similar conditions and the gut microbiota was investigated over 5 weeks, sampling intestinal contents by stripping. Comparison of the molecular microbiota profiles was performed by using the Dice index (Cs) calculated using TTGE/RISA profiles derived from samples collected at the same time. This analysis showed relatively high similarities (>71%) between the TVEO-treated and untreated trout. No statistical differences were observed between the TVEO-treated and untreated fish. Thus, for these concentrations, TVEO induced negligible changes in the gut microbiota profiles. When the molecular profiles within the same groups (treated or untreated) were compared throughout the collection period, common bacterial components were mostly observed. These microbes were persistent throughout the trial, producing constant molecular profiles, indicative of the stability of the microbiota composition in both TVEO-treated and untreated fish. The stability of the TTGE pattern over time was revealed by the Dice index (Cs), which exhibited average values >65% for both TVEO-treated and untreated trout (Figure 4.6). The molecular identification showed that the intestinal microbiota of trout was composed of three phyla: Proteobacteria, Firmicutes, and Actinobacteria. These taxa have been reported previously in salmonids, and they represent the abundant bacterial populations present in the gut of these fish (Huber *et al.* 2004; Holben *et al.* 2002; Navarrete *et al.* 2009).

In order to further understand the relationship of the gut microbiota of fish to biotic and abiotic factors, Sullam *et al.* (2012) performed a meta-analysis based on 25 bacterial 16S rRNA gene sequence libraries obtained from the intestines of different fish species, including from different trophic levels and habitats (such as salmon, trout and zebrafish). They observed an increased representation of operational taxonomic units (OTUs) from the bacterial order Aeromonadales in freshwater fishes and Vibrionales in saltwater fishes. However, additional research is needed to determine whether the differences could be attributed to the availability

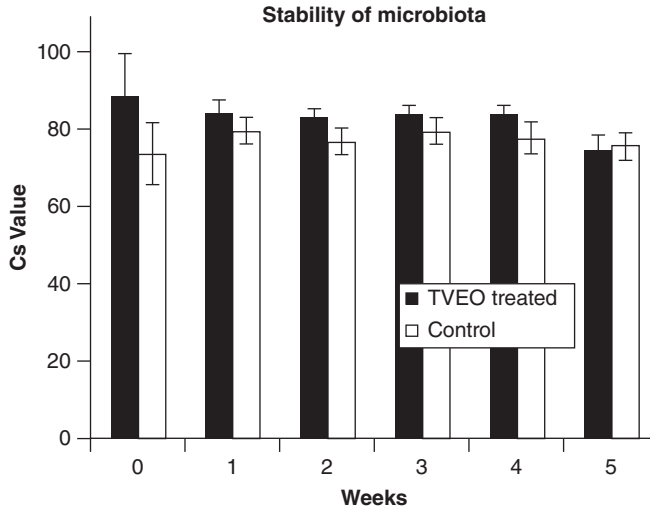


Fig. 4.6 Microbiota stability, taking the TTGE profile of week 0 as a reference. The Cs values did not significantly differ ($P > 0.05$). [Source: Navarrete *et al.* 2010b. Reproduced with permission of John Wiley & Sons.]

of microorganisms depending on environment or to host physiological differences associated with water salinity. To provide a broader context for their analysis, they compared fish microbiota data to data sets from diverse free-living and host-associated bacterial communities. This comparison revealed that fish gut microbiota compositions are often similar to those of other animals and contain relatively few free-living environmental bacteria. These results suggest that the gut microbiota composition of fishes may result from host-specific selective pressures within the gut and it could be more than a simple reflection of the microorganisms in their local habitat or diet. This host influence is coincident with observations made in the microbiota of rainbow trout genetic families (Navarrete *et al.* 2012).

4.5 CONCLUSION

In summary, present literature shows the presence of some phyla or bacterial groups in the microbiota of fish with some evidence that core microbiota may form, to some extent, irrespective of the rearing location or minor variations in environment and feeding regime. This new observation in zebrafish is worthy of future investigation as similar observations have been reported in humans, where it has been hypothesized that the human gut microbiota can broadly be grouped into three types, termed enterotypes. LAB have been the focus of numerous studies (*Chapter 6*) because this group could contain common, beneficial and potentially stable components of the microbial composition. However, microbial stability is still an important issue and the further development of strategies to manipulate and fortify the microbiota, such as probiotic and prebiotic applications, should help to stabilize beneficial microbial communities and improve fish health and aquaculture productivity. It is expected that massive sequencing methods could give more comprehensive information about the stability and factors that influence microbiota composition, as well as to help elucidate

the minor microbial components which are likely not detected when using the techniques commonly utilized in the literature to date. Further genomic and proteomic work will help to elucidate the importance of the microbe–host interactions at the mucosal interface, which will ultimately help to unravel the complexity of these microbial ecosystems.

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5 Methodological Approaches Used to Assess Fish Gastrointestinal Communities

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ABSTRACT

For historical reasons, much of the information available on the intestinal microbiota of fish is based on the use of conventional culture-dependent methods. This has consisted of sampling gut material and spreading gut homogenates on selective or general purpose agar, followed by incubation, colony counting and subsequent identification, typically by phenotypic/biochemical tests or during the last decade 16S rRNA sequencing. As is often the case with microbial communities from environmental samples, the gut microbiota of fish has been reported to be of low cultivability: cultivability using general purpose culture media has been reported to represent <0.1% of the total microbial community in the gastrointestinal (GI) tract of some fish species. A wide range of molecular ecology techniques are available based on the sequence variability of the 16S and 23S rRNA genes, and over the last 10–12 years such approaches have become more commonly used to investigate the gut microbiomes of fish species. The molecular based approaches used have depended on the aim of the studies: (1) clone libraries have been used to identify the microbiota composition; (2) fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE) and temporal temperature gradient electrophoresis (TTGE) have been used to analyse microbial community structure and diversity; (3) quantitative real-time PCR (qPCR) or fluorescent *in situ* hybridization (FISH) have been used to determine the abundance of particular taxa or total microbial levels; and (4) FISH and immunohistochemistry have been used to assess bacterial–host interactions

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at the mucosal brush border. In a limited number of recent studies, next-generation sequencing (NGS) approaches have been used to generate extensive libraries to extend our knowledge of the 'rare biosphere'. This review provides an overview of the techniques which have been used in evaluations of fish gut microbiota, the techniques with most relevance to future studies, and discusses the benefits and drawbacks of each approach with reference to the microbial populations present in the GI tract of fish.

5.1 CULTURE-DEPENDENT APPROACHES

Historically the general approach to study the microbiota of the gastrointestinal (GI) tract of fish has been the use of conventional culture based methods (Cahill 1990; Ringø and Gatesoupe 1998). This consists of sampling gut material and spreading gut homogenates on either selective or general purpose agar. The incubation temperature used should (although this has not always been the case) reflect the host's environmental conditions and thus the incubation duration should be longer when assessing the gut microbiota of cold water fish species where the growth rates of the indigenous gut microbes are likely to be slower. For example, some studies have utilized incubation periods of up to 4 weeks at 12 °C when assessing the gut microbiota of Atlantic cod (*Gadus morhua* L.) (Ringø *et al.* 2006a) and Atlantic salmon (*Salmo salar* L.) (Ringø *et al.* 2006b; 2008). Common incubation conditions for rainbow trout (*Oncorhynchus mykiss*) intestinal communities on general purpose media are 15–20 °C for 7–14 days (Spanggaard *et al.* 2000; Huber *et al.* 2004; Pond *et al.* 2006; Merrifield *et al.* 2009a; 2009b; Navarrete *et al.* 2010). In contrast, higher temperatures and shorter incubation periods are appropriate for warm water fish species; for example, 25 °C for 1–7 days in cyprinids (Al-Harbi and Uddin 2008; Sugita *et al.* 2009; Kühlwein *et al.* 2013) and 28–30 °C for 1–7 days in tilapia (*Oreochromis* spp.) (Al-Harbi and Uddin 2003; 2004; 2005; Ferguson *et al.* 2010; Standen *et al.* 2013). It should be noted of course that incubation periods, but not temperatures, should be adjusted when using selective media, as appropriate for the bacterial group(s) being investigated. This is commonly the case for the isolation of lactic acid bacteria (LAB) from cold water fish where it is often necessary to incubate for longer periods than required for general purpose media (e.g. Pond *et al.* 2006).

After an appropriate incubation period colonies are counted and a representative number of colonies are re-streaked for isolation. Traditionally, in older studies, isolates would be identified by biochemical assays and phenotypic observations, but identification using these techniques lacks sufficient discrimination power and in the last decade isolate identification has been undertaken using molecular methods such as 16S rRNA sequencing (Hagi *et al.* 2004; Ringø *et al.* 2006a; 2006b; 2008; Merrifield *et al.* 2009a). In addition, randomly amplified polymorphic DNA (RAPD) analysis has been used to identify selected groups such as probiotics or LAB (Balcázar *et al.* 2007a; 2007b; 2009; Vendrell *et al.* 2008; Avella *et al.* 2010).

Although there are numerous studies using a cultivation based approach, most of them are limited to using one or two types of media, typically general purpose; few studies have used several media within one investigation. Rare examples include Mudarris and Austin (1988) who used 14 media, Schulze *et al.* (2006) who used seven general and two selective media, and Yazawa *et al.* (1988) who used 11 different types of media. A number of early reviews have presented information based on the bacterial populations in the fish GI tract from culture-dependent observations (Cahill 1990; Ringø and Birckbeck 1999; *Chapter 4*).

However, as is often the case with microbial communities from environmental samples, the gut microbiota of fish has been reported to be of low cultivability (Table 5.1). Although

Table 5.1 Reported cultivability of the gut microbiota of fish species.

Fish species	Methodology	Notes	Cultivability	Reference
Rainbow trout <i>Oncorhynchus mykiss</i>	Cultivable bacteria: TSA, 7 days, 15 °C, aerobic	In general CFU g ⁻¹ were 1–2 log levels lower than total counts	Variable	Spanggaard <i>et al.</i> (2000)
	Total bacteria: DAPI			
	Cultivable bacteria: TSA, 7 days, 15 °C, aerobic	In general high cultivability (11–50%) reported. Low cultivability levels reported in some individuals (2%)	Variable (ca. 2–50%)	Huber <i>et al.</i> (2004)
	Total bacteria: DAPI			
	Cultivable bacteria: TSA, 7 days, 15 °C, aerobic	9–27% cultivability	Mean = 18%	Navarrete <i>et al.</i> (2010)
Coho salmon <i>Oncorhynchus kisutch</i>	Total bacteria: acridine orange			
	Cultivable bacteria: TSA, 10 days, 17 °C, aerobic	Average cultivable levels of 6×10^3 CFU g ⁻¹ GI tract and average total counts of 2×10^5 cells g ⁻¹ GI tract	ca. 1%	Romero and Navarrete (2006)
	Total bacteria: acridine orange			
Atlantic salmon <i>Salmo salar</i>	Cultivable bacteria: TSA, 10 days, 17 °C, aerobic	Average cultivable levels of 2.2×10^4 , 3.1×10^3 and 1.9×10^5 CFU g ⁻¹ for the stomach, pyloric caeca and intestine, respectively	<1%	Navarrete <i>et al.</i> (2009)
	Total bacteria: acridine orange			
Tilapia <i>Oreochromis niloticus</i>	*	Total viable counts: 5.4×10^9 g ⁻¹ intestinal contents. Direct counts: 1.1×10^{10} g ⁻¹ intestinal contents *	49%	Sugita <i>et al.</i> (1981)
Goldfish <i>Carassius auratus</i>	*	*	29–100%	Sugita <i>et al.</i> (1981)

(continued overleaf)

Table 5.1 (continued)

Fish species	Methodology	Notes	Cultivability	Reference
Carp <i>Cyprinus carpio</i>	*	*	55–100%	Sugita <i>et al.</i> (1981)
Bambooleaf wrasse <i>Pseudolabrus japonicus</i>	Cultivable bacteria: 1/20PYBG agar, 14 days, 25°C, aerobic Total bacteria: DAPI Cultivable bacteria: PYBG agar medium Total bacteria: DAPI	Cultivable levels of 1.0×10^6 to 2.0×10^9 CFU g ⁻¹ and total counts of 3.3×10^9 to 4.7×10^9 cells g ⁻¹ Cultivable levels of log 6.86 CFU g ⁻¹ and total counts of log 10.74 cells g ⁻¹ (note: $n = 1$)	0.022–48.6% ca. 0.1%	Sugita <i>et al.</i> (2005) Shiina <i>et al.</i> (2006)
Cupid wrasse <i>Thalassoma cupido</i>	Cultivable bacteria: 1/20PYBG agar, 14 days, 25°C, aerobic Total bacteria: DAPI Cultivable bacteria: PYBG agar medium Total bacteria: DAPI	Cultivable levels of 3.6×10^6 to 3.4×10^9 CFU g ⁻¹ and total counts of 3.2×10^9 to 4.2×10^9 cells g ⁻¹ Cultivable levels of log 5.73–6.81 CFU g ⁻¹ and total counts of log 10.82–10.93 cells g ⁻¹ (note: $n = 2$)	0.095–80.9% ca. 0.00001–0.0001%	Sugita <i>et al.</i> (2005) Shiina <i>et al.</i> (2006)
Scribbled toby <i>Canthigaster rivulata</i>	Cultivable bacteria: 1/20PYBG agar, 14 days, 25°C, aerobic Total bacteria: DAPI	Cultivable levels of 7.7×10^5 to 9.3×10^7 CFU g ⁻¹ and total counts of 2.9×10^9 to 4.3×10^9 cells g ⁻¹	0.018–2.7%	Sugita <i>et al.</i> (2005)
Grass puffer <i>Takifugu niphobles</i>	Cultivable bacteria: 1/20PYBG agar, 14 days, 25°C, aerobic Total bacteria: DAPI Cultivable bacteria: PYBG agar medium Total bacteria: DAPI	Cultivable levels of 2.3×10^7 to 2.5×10^9 CFU g ⁻¹ and total counts of 3.4×10^9 to 1.0×10^{10} cells g ⁻¹ Cultivable levels of log 6.13–8.68 CFU g ⁻¹ and total counts of log 10.67–11.18 cells g ⁻¹	0.3–50.4% ca. 0.1–1%	Sugita <i>et al.</i> (2005) Shiina <i>et al.</i> (2006)
Largescale blackfish <i>Girella punctata</i>	Cultivable bacteria: 1/20PYBG agar, 14 days, 25°C, aerobic Total bacteria: DAPI Cultivable bacteria: PYBG agar medium Total bacteria: DAPI	Cultivable levels of 6.2×10^4 to 4.6×10^7 CFU g ⁻¹ and total counts of 9.4×10^9 to 2.8×10^{10} cells g ⁻¹ Cultivable levels of log 4.00–6.45 CFU g ⁻¹ and total counts of log 10.81–11.27 cells g ⁻¹	0.0009–0.22% ca. 0.000001–0.1%	Sugita <i>et al.</i> (2005) Shiina <i>et al.</i> (2006)

Smallscale blackfish <i>Girella melanichthys</i>	Cultivable bacteria: 1/20PYBG agar, 14 days, 25°C, aerobic Total bacteria: DAPI	Cultivable levels of 9.5×10^3 to 2.7×10^8 CFU g ⁻¹ and total counts of 6.3×10^9 to 3.0×10^{10} cells g ⁻¹	0.0001–1.2%	Sugita <i>et al.</i> (2005)
Footballer <i>Microcanthus strigatus</i>	Cultivable bacteria: 1/20PYBG agar, 14 days, 25°C, aerobic Total bacteria: DAPI	Cultivable levels of 1.9×10^3 to 1.8×10^5 CFU g ⁻¹ and total counts of 4.2×10^9 to 1.0×10^{10} cells g ⁻¹	0.00003–0.0023%	Sugita <i>et al.</i> (2005)
Grey mullet <i>Mugil cephalus</i>	Cultivable bacteria: 1/20PYBG agar, 14 days, 25°C, aerobic Total bacteria: DAPI	Cultivable levels of 1.1×10^6 CFU g ⁻¹ and total counts of 5.1×10^9 cells g ⁻¹	0.021%	Sugita <i>et al.</i> (2005)
Surfperch <i>Ditrema temmincki</i>	Cultivable bacteria: PYBG agar medium Total bacteria: DAPI	Cultivable levels of log 6.09 CFU g ⁻¹ and total counts of log 10.84 cells g ⁻¹ (note: $n = 1$)	ca. 0.1%	Shiina <i>et al.</i> (2006)
Rockfish <i>Sebastes pachycephalus</i>	Cultivable bacteria: PYBG agar medium Total bacteria: DAPI	Cultivable levels of log 8.56–9.15 CFU g ⁻¹ and total counts of log 10.89–10.92 cells g ⁻¹ (note: $n = 2$)	ca. 1–10%	Shiina <i>et al.</i> (2006)

* Article in Japanese, information not presented in English.

estimates on the cultivability of gut microbes are restricted to a limited number of fish species, the cultivable bacterial levels in salmon (using tryptic soy agar (TSA) plates incubated for 10 days at 17 °C) represent ca. 1% of the total bacteria (coho salmon *Oncorhynchus kisutch*, Romero and Navarrete 2006; Atlantic salmon, Navarrete *et al.* 2009). Culture-dependent techniques therefore, even when using molecular methods for isolate identification, provide a rather skewed and restricted representation of the microbial communities in the GI tract of fish.

Despite the obvious limitations of culture-dependent approaches they are still useful if conducted appropriately, with a high number of isolates selected for molecular identification, and used in combination with culture-independent approaches. In future studies utilizing culture based approaches, it is important that microbiologists recognize the unique characteristics of fish and avoid applying protocols developed for terrestrial homeothermic animals. This is particularly true with respect to the selection of culture media and incubation conditions. Even now it is all too clear from both published literature and data presented at international symposia that the culture media used is not always appropriate. The most obvious cases include the use of selective media for the enumeration of *Bifidobacterium* spp., which are not common or abundant members of the gut microbiota of fish, and *Escherichia coli*, which is not an important enteropathogen of fish species. Although these bacteria have been identified in a limited number of studies in fish there are more relevant bacterial groups, which are more abundant and have known connotations for fish health status, which should take precedence. In addition, culture conditions will often be erroneously set at 37 °C for 24–48 h. These methodological approaches are appropriate for assessing the cultivable microorganisms in mammals but are not appropriate for poikilotherm animals such as fish.

5.2 MOLECULAR TECHNIQUES

A wide range of molecular ecology techniques are available based on the sequence variability of the 16S and 23S rRNA genes. These techniques allow for the identification and quantification of the intestinal microbiota, and in the past 5 years culture-independent molecular based techniques have become increasingly utilized for the assessment of the microbiota of the fish digestive tract (refer to Table 5.2). These techniques, however, are not currently utilized in the vast majority of probiotic and prebiotic studies.

Which molecular based approach to use depends on the aims of the study: clone libraries and metagenomics can be used to identify the microbiota composition; microbial community structure and diversity can be analysed by fingerprinting methods; while dot blot hybridization, quantitative real-time PCR (qPCR) or fluorescent *in situ* hybridization (FISH) can be used to measure the abundance of particular taxa (Zoetendal *et al.* 2004). This review will only discuss techniques that have been used in fish gut microbiota, prebiotic and probiotic studies or techniques with most relevance to future studies. For a broader discussion of molecular based techniques readers are referred to the reviews of Amann *et al.* (1995), McCartney (2002), Zoetendal *et al.* (2004), Ben Amor *et al.* (2009) and Roh *et al.* (2010).

5.2.1 PCR based methods

A key stage in the process of PCR based methods, often overlooked, is DNA extraction. The DNA extraction process is the foundation of any PCR based method. He *et al.* (2009)

Table 5.2 Overview, and examples, of the main methodologies utilized for the assessment of the gut microbiota of fish.

Method	Advantages	Disadvantages	Gut microbiota	References
Culture-dependent				
Culturing on general media followed by biochemical characteristics	Easy, inexpensive, quantitative	Culture-dependent, large number of isolates required to provide meaningful data, time consuming, metabolic plasticity of organisms may introduce error	Al-Harbi and Uddin (2003; 2004; 2005)	Bagheri <i>et al.</i> (2008)
Culturing on selective media	Easy, inexpensive, quantitative	Culture-dependent, selectivity of media is not guaranteed	Carnevali <i>et al.</i> (2006), Rollo <i>et al.</i> (2006), Silvi <i>et al.</i> (2008), Ghosh <i>et al.</i> (2010)	Zhou <i>et al.</i> (2007), Lv <i>et al.</i> (2007), Ghosh <i>et al.</i> (2008)
Culturing on general media followed by 16S rRNA sequence analysis of isolates	Confidence of species level identification of isolates, quantitative	Culture-dependent, large number of isolates required to provide meaningful data, time consuming	Ringø <i>et al.</i> (2006a; 2006b; 2008), Merrifield <i>et al.</i> (2009a)	Aubin <i>et al.</i> (2005)
Culturing on media followed by random amplified polymorphic DNA (RAPD) analysis of isolates	Easy, inexpensive, quantitative	Culture-dependent, large number of isolates required to provide meaningful data, time consuming, low reproducibility, phylogenetic information only available with comparison to RAPD profiles of known cultures	NR	Balcázar <i>et al.</i> (2007a; 2007b; 2009), Vendrell <i>et al.</i> (2008)

(continued overleaf)

Table 5.2 (continued)

Method	Advantages	Disadvantages	References	
			Gut microbiota	Probiotic study
Culture-independent				
16S rRNA gene clone libraries	Culture-independent, 16S rRNA sequence analysis, can extrapolate microbial diversity analysis	Some bias in the PCR process, loss of diversity	Shiina <i>et al.</i> (2006), Kim <i>et al.</i> (2007), Navarrete <i>et al.</i> (2009), Ward <i>et al.</i> (2009), Han <i>et al.</i> (2010)	Zhou <i>et al.</i> (2012)
Denaturing gradient gel electrophoresis (DGGE); temperature gradient gel electrophoresis (TTGE)	Culture-independent, 16S rRNA sequence analysis, can extrapolate microbial diversity analysis	Some bias in the PCR process, loss of diversity, semi-quantitative, possible migration of similar phylotypes to same gel point	Zhou <i>et al.</i> (2007; 2009a; 2009b), Liu <i>et al.</i> (2008), Merrifield <i>et al.</i> (2009b), Navarrete <i>et al.</i> (2009; 2010)	Kim and Austin (2006), Ferguson <i>et al.</i> (2010), He <i>et al.</i> (2011), Zhou <i>et al.</i> (2012)
rRNA intergenic spacer analysis (RISA)	Culture-independent, 16S rRNA sequence analysis, can extrapolate microbial diversity analysis, not as sensitive as DGGE/TTGE	Some bias in the PCR process, loss of diversity, semi-quantitative, possible migration of similar phylotypes to same point	Navarrete <i>et al.</i> (2009)	NR
LH-PCR	Culture-independent, 16S rRNA sequence analysis, can extrapolate microbial diversity analysis, not as sensitive as DGGE/TTGE	Some bias in the PCR process, loss of diversity, semi-quantitative, possible migration of similar phylotypes to same point	Heikkinen <i>et al.</i> (2006)	NR
Terminal restriction fragment length polymorphism (T-RFLP)	Culture-independent, can extrapolate microbial diversity analysis, not as sensitive as DGGE/TTGE	Some bias in the PCR process, loss of diversity, semi-quantitative, no phylogenetic information	Moran <i>et al.</i> (2005), Fjellheim <i>et al.</i> (2012)	NR
Fluorescence <i>in situ</i> hybridization (FISH)	Culture-independent, <i>in situ</i> , quantitative	Can probe only for known bacterial groups, time consuming for multiple species, difficulty detecting low metabolically active cells	Spanggaard <i>et al.</i> (2000), Asfie <i>et al.</i> (2003), Huber <i>et al.</i> (2004), Sugita <i>et al.</i> (2005)	Salinas <i>et al.</i> (2008), Del'Duca <i>et al.</i> (2013)

Direct fluorescence staining (e.g. nucleic acid/ protein stains such as DAPI, acridine orange, TRITC etc.)	Culture-independent, inexpensive, quantitative	Possible binding of cells/stain to extracellular material can make quantification difficult, careful optimization may be required for effective staining, detection of eukaryotic cells	Asfie <i>et al.</i> (2003), Sugita <i>et al.</i> (2005), Navarette <i>et al.</i> (2009; 2010)	Tovar <i>et al.</i> (2002), Salinas <i>et al.</i> (2008)
qPCR	Culture-independent, quantitative	Probes only for known bacterial groups	He <i>et al.</i> (2010), Kopečný <i>et al.</i> (2010), Li <i>et al.</i> (2013)	Avella <i>et al.</i> (2010; 2012)
Histochemistry	Culture-independent, <i>in situ</i> , quantitative	Probes only for known bacterial groups	NR	Gildberg and Mikkelsen (1998), Planas <i>et al.</i> (2006)
ELISA	Culture-independent, quantitative	Requirement of polyclonal antisera	Ringø <i>et al.</i> (1996)	Ringø <i>et al.</i> (1996), Ringø and Vadstein (1998), Ringø (1999)
Electron microscopy (SEM and TEM)	Culture-independent, visualization of colonization patterns and interactions with enterocytes	Time consuming, only applicable for epithelium associated cells, no phylogenetic information, semi-quantitative	Ringø <i>et al.</i> (2006b), Merrifield <i>et al.</i> (2009a; 2009b), Ghosh <i>et al.</i> (2010)	Andlid <i>et al.</i> (1995), Merrifield <i>et al.</i> (2010; 2011), Zhou <i>et al.</i> (2012)
Community libraries using NGS	Culture-independent, can extrapolate microbial abundance and diversity analysis, quantitative	Expensive, time consuming, some bias in the PCR process	Roeselers <i>et al.</i> (2011), van Kessel <i>et al.</i> (2011), Desai <i>et al.</i> (2012), Wu <i>et al.</i> (2012)	Peggs <i>et al.</i> (2014), Rodiles <i>et al.</i> (2014)

NR = to the authors' knowledge, not reported.

compared the effects of three different DNA extraction methods (lysozyme digestion, CTAB method and bead beating) on the analysis of different micro-ecological environments in a grass carp (*Ctenopharyngodon idella*) farm pond (pond sludge, feed) and the gut microbiota by 16S rRNA V3 DGGE. The results revealed differences in the DGGE fingerprints from the different DNA extraction methods. It is important therefore that the DNA extraction methods used should be robust and optimized before the analysis of GI microbial communities. Additional problems associated with PCR based techniques include heteroduplex detection, detection of chimeric rRNA operons, and low sensitivity to rare members present in mixed microbial communities (McCartney 2002).

5.2.2 Electrophoresis based methods

Although RISA (Navarrete *et al.* 2009), length heterogeneity PCR (Heikkinen *et al.* 2006), terminal restriction fragment length polymorphism (T-RFLP) (Moran *et al.* 2005; Fjellheim *et al.* 2012) and temporal temperature gradient electrophoresis (TTGE) (Navarrete *et al.* 2009; 2010; 2012) have been utilized to characterize the gut microbiota of fish, DGGE remains the most commonly utilized electrophoresis based method due to its relatively inexpensive and simple nature. DGGE, and TTGE, are based on the electrophoresis of targeted regions of the 16S RNA gene which display differing denaturing properties depending on the nucleotide composition; typically the 16S rRNA V3 region (after Muyzer *et al.* 1993) is used but the V6–V8 (Zhou *et al.* 2009a; Desai *et al.* 2012) and V4 (Bjornsdottir *et al.* 2009; Hermannsdottir *et al.* 2009) regions have also proven to be phylogenetically informative. RISA analysis targets the electrophoresis of the ribosomal intergenic spacer region between the small (16S) and large (23S) ribosomal subunits, which varies in length (from ca. 150 to 1500 nucleotides (nt)) in different bacterial strains. This approach may allow for a higher degree of identification in subsequent sequencing given the possibility of sequencing longer PCR amplicons.

These electrophoresis methods can provide a fast, inexpensive method for assessing the dominant gut microbiota of fish and the effects of prebiotics and probiotics on these populations. Fingerprinting profiles based on 16S rRNA are reported to represent 90–99% of the bacterial community (Zoetendal *et al.* 1998). A range of subsequent statistical methods (based on either binary or weighted analysis of banding patterns) have been developed to compare fingerprints. Such methods allow comparisons of profiles from samples to be made by using similarity matrices, dendrograms, and non-metric multidimensional scaling (nMDS) which show the relatedness of the microbial profiles, and principal component analysis (PCA) can be used to determine the influence of environment, host, or other factors on the phylogenetic patterns. Ecological statistics, such as species richness, species diversity and one-way analysis of similarity (ANOSIM) can also be applied. In order to gain information on specific bacteria, sequencing of excised bands is essential. However, given the low number of nucleotides used (e.g. ca. 150 nt for the V3 region of the 16S rRNA gene) a high level identification, and the ability to resolve between highly similar strains, are often restricted. As such, identification beyond genus level can be difficult. Another issue is the potential for PCR amplicons with similar or identical denaturing characteristics to migrate to the same location in the lane, thus providing difficulty in gel interpretation and subsequent sequencing of operational taxonomic units (OTUs). Particular care should be taken when comparing samples from different gels, as the normalization of bands across gels can introduce artifactual diversity. Even when using a set of markers in each gel, it is advisable to conduct within gel comparisons of key treatment groups. Experimental design should take this factor into account.

Another method used to investigate microbial community composition is terminal restriction fragment length polymorphism (T-RFLP). This method consists of the use of fluorescence-labelled primers in the PCR amplification (usually 16S rRNA) and a subsequent digestion of the amplicons using restriction enzymes; the fragments obtained are then separated and detected using an automated sequence analyser (Fraher 2012). The output of this method corresponds to different profiles (fingerprints) dependent on the composition of the communities of the samples. T-RFLP is a fast and semi-quantitative method; however, it does not provide phylogenetic identification and could also be affected by PCR bias.

5.2.3 Quantitative real-time PCR (qPCR)

qPCR can be used to estimate the numbers of specific bacteria in environmental samples using specific primer sets by comparing threshold cycle (C_T) values with a standard curve produced from known bacterial cell numbers plotted against C_T values. Such an approach is obviously useful when the objective is to determine the levels of a particular bacterial species/strain. It is useful therefore in probiotic studies and recently Avella and coworkers (2010) quantified *Lactobacillus rhamnosus* recovery in the gut of clownfish (*Amphiprion ocellaris*) using this method. A standard curve was generated based on C_T values of 16S rRNA genes based on known *Lb. rhamnosus* concentrations; C_T values obtained from bacterial numbers in the range $10^5 - 10^9$ CFU produced a linear relationship ($R^2 = 0.9914$). The study demonstrated that exposure of larvae and juveniles to *Lb. rhamnosus* via diet and/or rearing water led to high retention of the probiotic in the GI tract, and this was later verified using the same approach in zebrafish *Danio rerio* (Avella *et al.* 2012).

Such an approach is relatively straightforward when quantifying a known culturable strain because accurate C_T /CFU standard curves can be produced. It is however more difficult to quantify indigenous strains of interest or total bacterial numbers in this way. He *et al.* (2010) developed a qPCR method, based on the *rpoB* gene, to quantify the total autochthonous bacterial levels in the GI tract of tilapia (*Oreochromis niloticus* ♀ × *O. aureus* ♂). The *rpoB* gene was chosen because each cell contains only a single copy whereas the 16S rRNA gene potentially has multiple copies per genome (e.g. seven copies in the genome of *Escherichia coli*) and dominant indigenous bacteria were used to determine a standard curve between cell number and C_T values. A limitation of this method is the incomplete coverage of the *universal* primers and thus this approach does not truly reflect the total bacterial levels; however, this primer set was inclusive of more than 85% bacteria in the autochthonous GI communities of tilapia. Recently, Li *et al.* (2013) used qPCR to determine the relative abundance of Bacteroides and Firmicutes in the intestinal tract of common carp (*Cyprinus carpio*) using standards constructed with known amounts of plasmid DNA. The method can allow for the comparison of the relative abundance (copies) of these phyla between samples, and, when used in conjunction with plasmid standards derived from *universal* primer sets, can express the proportion (%) of the phyla with respect to total bacterial copies.

5.2.4 Clone libraries

16S rDNA (Kim *et al.* 2007; Navarrete *et al.* 2009; Ward *et al.* 2009; Han *et al.* 2010) and *cpn60* (Mansfield *et al.* 2010) clone libraries have been used to evaluate the intestinal microbial composition in fish. PCR products derived from *universal* primer sets are cloned to generate bacterial 16S rRNA/*cpn60* clone libraries from each fish using commercially available cloning kits

(e.g. TOPO TA cloning kit, Invitrogen; pGEM-T, Promega). The benefit of such an approach is the culture-independent nature, and libraries of near full-length 16S rDNA sequences improve the accuracy of species identification. Ward *et al.* (2009) demonstrated with the use of clone libraries that the gut microbiota of the omnivorous yellowbelly rockcod/bullhead notothen (*Notothenia coriiceps*) exhibited greater diversity than that of the carnivorous blackfin/Scotia Arc icefish (*Chaenocephalus aceratus*). However, many researchers have suggested that methods based on 16S rDNA gene sequences using *universal* primers may not accurately reflect the true underlying diversity of a given environment (Suzuki and Giovannoni 1996; Marchesi *et al.* 1998) and a large number of clones are required to provide reliable quantitative information. In addition, technical challenges such as PCR bias, varying ribosomal DNA copy numbers, and the efficiency of DNA extraction procedures all have the potential to significantly skew abundance estimates; therefore, assumption of a direct relationship between the number of sequences of a particular type in a clone library and the number of organisms in the environment may be inaccurate (Marchesi *et al.* 1998; Suzuki and Giovannoni 1996). In addition, the number of clones required (e.g. hundreds to thousands per sample) to obtain good coverage of gut microbial communities makes clone library preparations impractical.

5.2.5 Next-generation sequencing (NGS)

As described within this chapter, there are significant methodological challenges in surveying the tremendous and dynamic diversity of bacterial taxa within the fish intestine. By one estimate of human intestinal bacterial community richness, the number of unique bacterial phylotypes exceeds 1200 (Rajilic-Stojanovic *et al.* 2007). Bar-coding methods (e.g. DGGE, TTGE and RISA) have utility in simultaneous comparisons of multiple samples, yet the number of unique bands typically observed from a DGGE gel is orders of magnitude less than the extant intestinal diversity. While culture-independent methods avoid the most significant bias of laboratory cultivation, the biases intrinsic to PCR and bar-coding methods neglect less abundant bacterial taxa that may play critical roles in gut nutrition and disease.

Advances in DNA sequencing technology now allow massively parallel sequencing of PCR amplicons or genomic samples, providing sufficient coverage to include less abundant gut microorganisms. Each sequencing technology has its own set of biases and limitations. For amplicon sequencing the PCR biases (e.g. preferential amplification of abundant templates) are also present. In addition, some critical parameters to consider for NGS applications are: (1) the length of the sequencing read, (2) the numbers of sequencing reads, (3) sequencing errors, (4) the ability to include multiple samples, and (5) the cost per sample. The specific sequencing technology adopted will influence each of these parameters. For example, sequence reads with an average length of ~450 base pairs (bp) can be generated with a GS FLX system (454 Life Sciences, Branford, CT), whereas sequence reads with an average length of ~98 bp can be generated with a Genome Analyzer IIx (Illumina, Inc., San Diego, CA). As NGS technology is evolving rapidly and more companies are in this field (e.g. ABI SOLiD, Pacific Biosciences, Ion Torrent), clearly the number of reads, read lengths, and read accuracy will change over time. Sequence read length impacts phylogenetic resolution depending on the targeted domain of the 16S rRNA molecule (Youssef *et al.* 2009) and gene annotation accuracy (Wommack *et al.* 2008). Clearly longer sequence read lengths are preferable for 16S rRNA gene surveys, although by targeting specific phylogenetically informative domains (i.e. the V3 region of 16S rRNA) a shorter read length can still be used to achieve comparable results to nearly full-length sequences (Kim *et al.* 2011). The number of sequence reads is

also a critical parameter, and given the need to encompass the bacterial diversity within a fish gut many thousands of sequences should be generated per sample. While a GS FLX system will generate up to 1 million reads per full plate, a Genome Analyzer IIX can generate 40 million single reads per lane, with a total of 320 million in total for all eight lanes. The use of paired-end Illumina sequencing for 16S rRNA surveys retains the advantages of the larger number of reads that can be obtained with this technology, while including a quality control assembly step that minimizes sequencing errors (Bartram *et al.* 2011).

Depending on the number of samples and the desired sequencing coverage per sample, different sequencing technologies may therefore be appropriate. Since the error rate for NGS is higher than that for Sanger sequencing (Zagordi *et al.* 2010), trimming sequences for sufficient quality will reduce the number of useful sequences for phylogenetic analysis and this should be taken into account when planning the extent of sequencing coverage. Lastly, the ability to include multiple samples per run is important in the estimation of sequencing coverage per sample. In the case of 16S rRNA gene amplicon sequencing, unique 6 to 10 bp ‘bar-codes’ can be incorporated at the 5′ end of a primer, permitting post-sequencing segregation of sequences into specific sample sets (Hamady *et al.* 2008). This approach does require separate ordering of multiple unique primers, although this one-time purchase will be mitigated by the longer term utility of these primer sets for multiple experiments.

From a practical consideration of NGS adoption, the higher costs of NGS as compared to DGGE or other analyses should be considered. The per sample cost of NGS can be reduced by analysing multiple samples simultaneously, by reducing the number of sequences generated per sample, and/or by using a smaller region of a sequencing plate (i.e. one-quarter of a 454 pyrosequencing plate or one lane of an Illumina sequencer). The cost of sequencing per base pair has dropped precipitously and this trend is expected to continue, so that by combining cost-saving measures with massively parallel sequencing it is expected that bacterial community analyses will be increasingly conducted using NGS approaches. With cost-saving measures care must be taken to avoid reducing sequencing coverage to an extent that results in an inadequate survey. If possible, a preliminary survey may be useful in assessing the sequencing coverage needed, combined with generation of a rarefaction curve to establish the degree of sequencing redundancy achieved.

Revealing the more complete picture of intestinal microbial diversity via high-throughput sequencing has the consequence of producing a massive amount of data that poses a significant bioinformatics challenge. Fortunately, tools are available to help process and interpret this wealth of sequencing data. The first bioinformatics step is to trim the raw sequence data to remove errors. As mentioned previously, a paired-end strategy may be adopted wherein each sequence is assembled with its mate pair and any ambiguous sequences are discarded, resulting in higher quality and longer length 16S rRNA gene sequences (Bartram *et al.* 2011). Another strategy for removing errors associated with 454 pyrosequencing data is to use the algorithm PyroNoise, which results in a more accurate reflection of OTUs (Quince *et al.* 2009). No matter the sequencing technology, very rigorous trimming should be conducted to achieve a conservative estimate of bacterial diversity to avoid artifactually high estimates of bacterial richness (Huse *et al.* 2007; Reeder and Knight 2010). Sequences trimmed for quality may be rapidly compared to the database of 16S rRNA gene sequences using bioinformatics tools available at the Ribosomal Database Project (Cole *et al.* 2008). Each 16S rRNA gene sequence may be classified according to its closest phylogenetic affiliation using the RDP classifier (Wang *et al.* 2007), allowing the calculation of the relative abundance of bacterial

taxa at different phylogenetic ranks. The utilization of NGS to investigate the diversity of the bacterial populations in the GI tract of fish is becoming common practice; examples include studies in zebrafish (Roeselers *et al.* 2011; Rodiles *et al.* 2014), common carp (van Kessel *et al.* 2011; Li *et al.* 2013), grass carp (Wu *et al.* 2012), European sea bass *Dicentrarchus labrax* (Carda-Diéguez *et al.* 2013; Peggs *et al.* 2014) and rainbow trout (Desai *et al.* 2012). While the study of common carp GI microbiota by van Kessel *et al.* (2011) used multiple primer sets and classified 17,641 sequence reads, there was no mention of coverage or rarefaction curves. In the study of grass carp, the 16S rRNA V1–V3 region was targeted, and 259 to 2773 OTUs were identified from 6990 to 18,993 sequence reads (Wu *et al.* 2012). This sequence coverage resulted in rarefaction curves that approached the saturation plateau, and Good's coverage estimations revealed that 85% to 98% of the phylotypes were obtained. The study of Roeselers *et al.* (2011) revealed that rarefaction curves generated from 16S V1–V2 sequences in this study did not reach a plateau even after more than 9000 sequence reads. These studies reveal a far greater level of diversity of the gut microbiota of omnivorous cyprinids than previous studies that lacked an NGS approach have indicated. However, in the study on the carnivorous rainbow trout by Desai *et al.* (2012), which targeted the *cpn60* gene, estimates of Good's coverage ranged from 94.5% to 97.8% when using an average of only 1660 reads per sample. Although limited studies are available, this comparison between cyprinids and trout is suggestive of potentially large differences in the level of diversity. Thus it is important to assess the degree of sampling redundancy by generating rarefaction curves that plot the number of unique sequences at some percentage identity (e.g. 97%) versus the total number of sequences generated, which may be readily accomplished using the open-source software programs such as DOTUR as part of the MOTHUR project (Schloss *et al.* 2009).

In addition to surveys of bacterial communities, NGS permits the more facile *de novo* sequencing of bacterial genomes and community genomes. In particular for probiotic bacterial strains, *de novo* sequencing enables the determination of whether strains contain any potential virulence factors or pathways for secondary metabolite synthesis, and enables design of unique sequences for primer or probe design that are specific to the probiotic strain (Altermann *et al.* 2005; Callanan 2005; O'Flaherty and Klaenhammer 2011). The gene sequences from probiotic strains can be used to determine gene expression *in vivo* and investigate the impact of host microenvironments or different diet formulations on the activity of probiotic strains within the fish intestine. Entire microbial communities may also be subjected to community genomic (a.k.a. 'metagenomic') analysis, by direct sequencing of bacterial genomes or gene transcripts, or by cloning of the DNA or RNA extracted from an environmental sample (Rondon *et al.* 2000). Metagenomic analysis of human gut samples has revealed important contributions of gut microorganisms to obesity and identified bacterial phylotypes common among the gut microbiota of different individuals (Turnbaugh *et al.* 2009; Qin *et al.* 2010). Future work in understanding the contributions of probiotics to fish intestinal health and disease may investigate the functional genomics of probiotic strains and how these specific strains interact with their host and its diverse intestinal microbiota. There are many other bioinformatics analyses that may be undertaken to investigate (meta)genome sequences, which are beyond the realm of this chapter.

Clearly the field of probiotics as it relates to fish growth performance, immune competence, and disease will be better informed in the future through application of these culture-independent technologies.

5.3 FLUORESCENCE BASED METHODS

Quantitative means of culture-independent analysis with FISH (Spanggaard *et al.* 2000; Asfie *et al.* 2003; Huber *et al.* 2004) and direct nucleic acid stains such as DAPI (Spanggaard *et al.* 2000; Huber *et al.* 2004; Sugita *et al.* 2005; Shiina *et al.* 2006) and acridine orange (Navarrete *et al.* 2008; 2009; Abid *et al.* 2013) have been applied to quantitatively study the gut microbiota of fish.

Due to its speed, sensitivity and inexpensive nature the FISH technique is an effective way to derive cellular abundance information and is often utilized in ecological and environmental studies (Bottari *et al.* 2006). FISH analysis with rRNA-specific oligonucleotide probes designed to target bacterial taxa at different phylogenetic levels has proven to be a powerful tool to assess microbial diversity of the fish GI tract (Spanggaard *et al.* 2000; Huber *et al.* 2004). However, due to the viscous and dense nature of partially digested feed (i.e. digesta) which constitutes the gut contents inhabited by allochthonous microbes there are often difficulties yielding images that can be utilized effectively to quantify microbial populations (refer to Figure 5.1). Cells may often be too densely packed into digesta material and become clustered, leading to difficulties in reliably enumerating cells. Additionally, Huber *et al.* (2004) reported that FISH analysis with the bacteria-specific probe EUB338-mix only accounted for 80% of the total cell count (as determined by comparison with DAPI) in the GI tract of rainbow trout. In goldfish this value was reported to be lower, in the region of 50–70% (Asfie *et al.* 2003). The non-hybridized cells in such cases may be non-bacterial or it is possible that the cells were not permeabilized by the applied FISH conditions. In addition, viable but less metabolically active bacterial cells with lower ribosomal content may not be visualized with a ribosome-targeted approach. Despite this, with the application of extensive sets of probes, Huber *et al.* (2004) revealed high abundances of Gammaproteobacteria, *Aeromonas* spp. and Enterobacteriaceae in the intestinal tract of rainbow trout from a number of fish farms. Further issues regarding the efficacy and limitations of the FISH technique are discussed in detail by Amann *et al.* (1995) and Zoetendal *et al.* (2004). Direct nucleic acid stains may avoid the potential problems associated with hybridisation, but the issues relating to reliable enumeration of cells in viscous digestive material remains. Further, direct staining does not allow for the discrimination of different microbial species (thus providing total cell counts only), or indeed differentiation between microbial cells and eukaryotic cells.

5.4 ELECTRON MICROSCOPY

Electron microscopy can be an important tool for investigating intestinal mucosal microbial populations and in recent times the gut microbiotas of several fish species have been investigated with electron microscopy (for reviews refer to Ringø *et al.* 2003; 2007). Scanning electron microscopy (SEM) is useful for screening large sections of mucosal surfaces (see Figures 4.1 and 5.2) and transmission electron microscopy (TEM) allows for the observation of potentially endocytosed and paracellular bacterial cells, thus revealing translocation. These methods also allow the assessment of bacterial–host mucosal cellular interactions, probiotic and indigenous bacterial interactions and the resultant effects on enterocyte morphology. Despite providing indications of gut colonization by bacteria-, yeast- or Archaea-like cells,

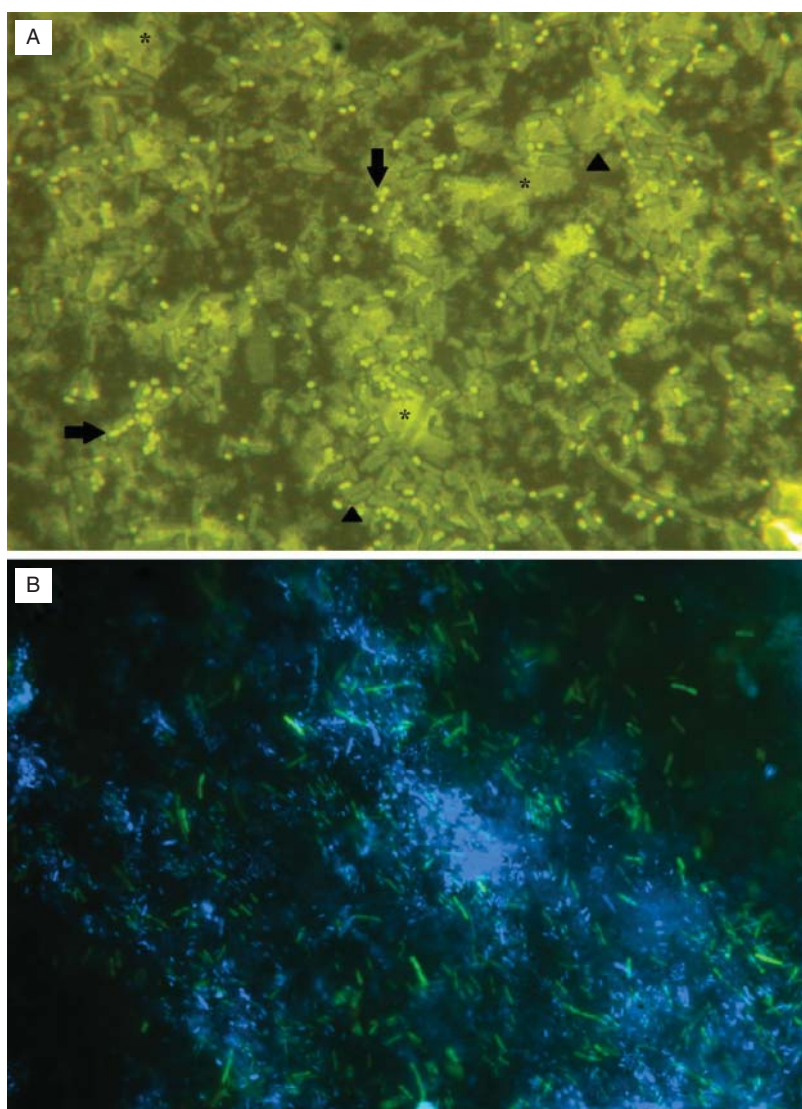


Fig. 5.1 Epifluorescence micrographs of the indigenous gut microbiota of fish. (A) *In situ* hybridization of rainbow trout gut microbiota with green fluorescein labelled probe EUB338-mix (detecting all bacteria) (Waines and Merrifield, unpublished data). Despite the clearly identifiable cocci (arrows) and rod-shaped bacteria (arrowheads) present within the sample, reliable enumeration is difficult due to the dense nature of the gut contents which leads to poor differentiation between individual cells in some cases. In addition, non-specific hybridization and fluorescence of background material (digesta and/or mucus) is present (five-pointed stars). (B) DAPI staining (blue) and *in situ* hybridization of common carp gut microbiota with green fluorescein (FITC) labelled probe VA (detecting *Aeromonas* and *Vibrio* spp.). (Source: Mark Rawling.) For colour detail see Plate 10.

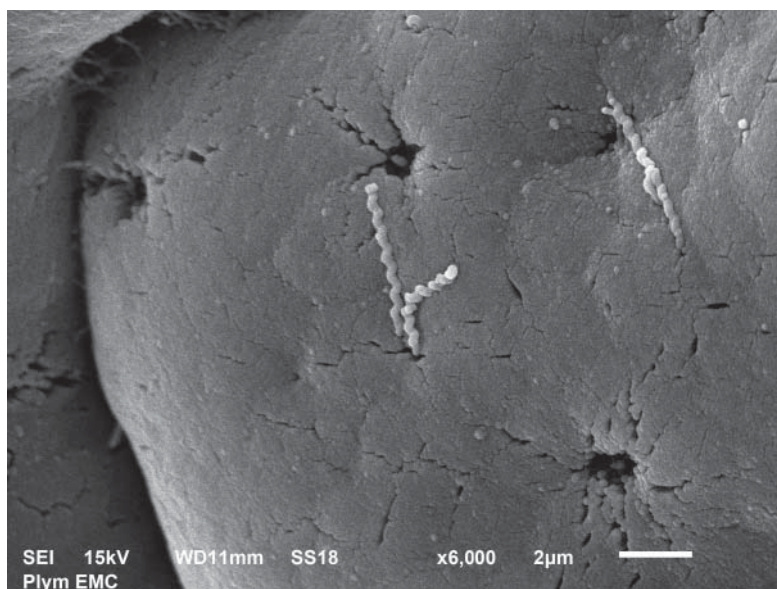


Fig. 5.2 Scanning electron micrograph of the intestine of Nile tilapia showing the presence of bacterial cells likely from the Spirochaete phylum. Scale bar = 2 µm. (Source: Ben Eynon.)

these techniques are somewhat limited without the employment of staining techniques for identification. Such techniques include immunogold labelling which has been used in conjunction with TEM to identify specific lymphoid cells in the fish intestinal epithelium (Picchietti *et al.* 2009) but to the authors' knowledge such techniques have yet to be utilized to study the gut microbiota of fish or to assess potential probiotic colonization.

5.5 MICROBIAL ACTIVITY AND FUNCTIONALITY

All of the approaches described in this chapter thus far have focused on microbial abundance. This is of course an important aspect to consider but it does not identify which members of the community are most active. One of the common approaches to assess the microbial activity and potential contribution to the host fish digestive processes has been the assessment of microbial community digestive enzyme production. More specifically, the abundance of bacteria displaying digestive enzyme activity are enumerated and isolated on specific media; for example, for amylase-, cellulase- and protease-producing bacteria, gut homogenates have been spread onto starch agar, carboxymethylcellulose (CMC) agar and peptone-gelatin agar plates, respectively (Ray *et al.* 2010; Pedrotti *et al.* 2013). Such an approach can provide important information on the potential contribution of cultivable microbes on the digestive activities of fish. A limited number of studies have quantified the levels of short-chain fatty acids (SCFAs) in the GI tract of fish. SCFAs, such as butyric acid, acetic acid and propanoic acid, are metabolic end products of microbial fermentation of fibre. Therefore the types of SCFAs present, and their levels, can potentially provide information on the types of bacterial groups present as well the activity of the community in the GI tract. Although this approach is widely used in the study of

mammalian GI microbiota, few studies are available in fish (Clements *et al.* 1994; Mountfort *et al.* 2002; Moran *et al.* 2005; Fidopiastis *et al.* 2006). Readers with an interest in the digestive enzyme activities and SCFA production of the GI microbiota of fish are referred to the reviews of Clements *et al.* (2009) and Ray *et al.* (2012).

The gut microbiotas of fish have also been extensively studied for their ability to antagonize bacterial pathogens, either to assess their intrinsic potential benefit to the host *in situ* or as a means to identify potential probiotic candidates. Typically, this involves the isolation of bacterial candidates on appropriate media, often LAB or *Bacillus* spp., and subsequently assessing potential antagonism using streak plates, spot overlays, agar wells, mucus adhesion and displacement assays or co-incubation in media or *ex vivo* sacs. Depending on the method used, the extent of antagonism can be determined qualitatively, semi-quantitatively or quantitatively. A plethora of studies are available on this topic in fish and they provide evidence of gut microbial activity and the potential role in limiting the proliferation and colonization of potential pathogens in the GI tract (Olsson *et al.* 1992; Ringø *et al.* 2000; Balcázar *et al.* 2008; Kim and Austin 2008; Askarian *et al.* 2012). For a review on this topic the reader is referred to previously published reviews (Ringø and Gatesoupe 1998; Gatesoupe 2008; Ringø *et al.* 2010).

Other methods of providing useful quantitative data on microbial activity, without the need for culturing, include the use of commercially available kits and fluorescent stains for the rapid determination of active microbial biomass and bacterial viability in environmental samples. The ability to accurately measure such parameters provides a level of understanding beyond that gained by merely enumerating cells which may or may not be playing an active role within the environment. To date the application of such methods to the investigation of fish gut microbiota has been limited. This is surprising given the widespread application of these methods in the field of environmental microbiology, and may be due in part to the fact that the cultivability of probiotic bacteria is generally high, thus enabling detection by more conventional means.

The LIVE/DEAD *BacLight* Bacterial Viability kit (Molecular Probes Inc., USA) is a method for microscopical enumeration of bacterial cells and has been developed to distinguish between live and dead bacteria. It is more sensitive than the conventional culture based approach, and is also simpler to perform than some of the molecular approaches described earlier in this chapter. Based on the differing permeability of the plasma membrane in each case, the kit consists of two fluorescent nucleic acid stains, Syto 9 and propidium iodide (PI). Syto 9 is membrane permeable and exhibits green fluorescence, whilst PI is not membrane permeable and exhibits red fluorescence. The use of these stains in combination therefore facilitates the quantification of live versus dead cells. This method has been applied in a wide variety of environmental microbiology studies, most notably in a large number of studies concerning the use of probiotic bacteria in humans that assessed survival rates of human-derived probiotics during heat treatment (Gardiner *et al.* 2000), bacteria in probiotic dairy products (Auty *et al.* 2001), and GI transit tolerance of probiotic strains of *Bifidobacterium* (Masco *et al.* 2007). Consequently, there would appear to be significant scope for its application to similar fish based investigations. However, it is important to bear in mind the need to optimize staining conditions beforehand, as staining efficiency may be influenced by factors such as pH and the physiological state of the bacteria. This may lead to variation in the level of fluorescence between individual cells (Boulos *et al.* 1999). In certain cases, non-specific binding to extra-cellular DNA may also be problematic if staining conditions are not correct. Another method of assessing bacterial viability is the use of fluorescent redox stains such as 5-cyano-2,3-ditolyl tetrazolium chloride (CTC). Whilst widely used in a broad range of

environmental microbiology studies, to the authors' knowledge its use in studies relating to the gut microbiota and probiotic colonization of the fish gut has not been documented. In metabolically active bacterial cells the CTC is reduced, resulting in the formation of insoluble formazan crystals after incubation. The enumeration of viable cells as a proportion of the total can then be facilitated by counterstaining with a general nucleic acid stain such as Syto 9 or DAPI. However, it is worth noting that whilst it represents a simple method of counting active bacteria, there are some disadvantages. For example, bacteria exhibiting a low level of metabolic activity (with a corresponding low level of intracellular formazan) may be below the limit of detection by some methods, and a lack of standardized methods makes comparison between studies difficult (Creach *et al.* 2003). As previously stated in this chapter, the association of cells with extracellular material (extracellular polymeric substances, digesta, sediment etc.) can also lead to difficulties in the reliable enumeration of cells using fluorescent probing of cells by different methods.

A commonly used non-microscopical method for the determination of active biomass is the analysis of nucleotide adenosine triphosphate (ATP) levels through bioluminescence assays. First developed in the 1960s, and based upon the measurement of light emitted due to the hydrolysis of ATP in the presence of the enzyme luciferase and its co-factor, D-luciferin, such assays are rapid and extremely sensitive. Another advantage of this method is that while microscopical analyses may sometimes be hampered by the close association of cells with extracellular material, direct extraction of microbial-derived ATP is achievable using less biased methods. Indeed, many commercially available kits are designed for the specific measurement of microbial ATP from environmental samples. Examples include HS (High Sensitivity) kits (manufactured by Biothema Ltd., Sweden), the ATP luminescence assay kit (Molecular Probes Inc.) and the ATP Bioluminescent Assay kit (Sigma-Aldrich, USA). ATP bioluminescence assays are sensitive and rapid, with the amount of emitted light directly proportional to the amount of intracellular microbial ATP. However, there is a requirement for specific detection equipment and a high risk of contamination from extracellular sources. While analysis of ATP levels can provide an accurate indication of the level of active biomass within a sample, it is not species specific and therefore should be used in conjunction with other more suitable methods where appropriate.

NGS technology can also be used to address questions of microbial activity within the fish intestine. RNA may be extracted from the intestinal contents and used to prepare a cDNA library. A cDNA library may be queried for specific transcripts via qPCR, or alternatively used for a metatranscriptome approach in which the collective transcripts from the intestinal microbiota are sequenced (e.g. by Roche 454 or Illumina) at sufficient depth of coverage to provide transcripts even from the rare microbiome. To our knowledge this approach has not yet been applied to examine the expression of fish intestinal microbiota, but it has been successfully applied in other animals (Poroyko *et al.* 2010). The cDNA sequences generated by this approach may be annotated using existing bioinformatics pipelines such as MG-RAST (<http://metagenomics.anl.gov/>) that can indicate specific functions associated with transcripts that have a significant BLAST hit. It should be noted that this approach is inherently limited to those expressed genes that have been previously described in the literature with an annotated function, and that a very large percentage of total cDNA sequences will not have a significant BLAST hit in GenBank or other databases. This reveals our collective ignorance as to the expressed functions of the intestinal microbiota and indicates the need for continued research to understand the contributions of the intestinal microbiota to fish health and disease.

5.6 SUMMARY

With the increasing cost effectiveness and accessibility of NGS it is anticipated that its application in studies of the gut microbiota of fish will increase rapidly in the coming years; as a result, our understanding of the true extent of microbial diversity will benefit greatly. It is likely also that more attention will be devoted by researchers to the gut microbiota of fish as the importance of this microbial community becomes better understood, in part through the information derived from studies in mammals, and as dietary formulations in aquafeeds evolve. The increased attention should lead to a combination of methodological approaches, including those that more accurately reflect microbial activity, which will provide a more robust picture of the communities, and their interactions with the host. This is particularly relevant given the recent findings from the Human Microbiome Project which show that although large variations in the abundance of the phyla composition of the human intestinal microbiome were observed, the metabolic pathways remained relatively stable (The Human Microbiome Project Consortium 2012). This therefore infers that the total metabolic capacity of the microbial community might be relatively stable, and thus its contribution to the host stable, even though the composition may differ between individual hosts. Further research is warranted to validate this hypothesis and to ascertain if this also applies to the gut microbiota of fish.

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6 Indigenous Lactic Acid Bacteria in Fish and Crustaceans

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ABSTRACT

The gut microbiota of fish plays an important role in mediating and stimulating host gastrointestinal (GI) development, aiding digestive function, maintaining mucosal tolerance, stimulating the host immune response and providing a level of protection against gastric infections. Lactic acid bacteria (LAB), which are generally considered as favourable bacteria due to their abilities to antagonize bacterial pathogens, are often identified as components of the gut microbiota of fish. Members of the *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus* and *Streptococcus* genera have been isolated by culture-dependent methods as minor components of the indigenous GI populations in salmonids. *Carnobacterium* species, such as *C. (piscicola) maltaromaticum*, *C. mobile*, *C. divergens*, *C. alterfuntum*-like, *C. inhibens* and *Carnobacterium* spp., have been thought to be the dominant/core LAB genus in salmonids and can account for up to 15% of the viable culturable populations. Recent culture-independent molecular analyses have led us to question whether *Carnobacterium* truly are the most prevalent LAB genus in salmonids. Techniques such as denaturing gradient gel electrophoresis (DGGE), temporal temperature gradient electrophoresis (TTGE), and 16S rRNA clone libraries confirm the presence of *Carnobacterium* spp. but have also identified *Lactobacillus* spp., *Streptococcus* spp., *Lactococcus lactis*, *Weissella cibaria* and

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Pediococcus sp. as indigenous species. In warm water fish species (e.g. sturgeon, zebrafish, carp, tilapia, grouper and cobia etc.), members of the genera *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Pediococcus*, *Leuconostoc* and *Weissella* have commonly been identified as the indigenous GI populations, while *Carnobacterium* species have rarely been identified. The dominant LAB in shellfish belong to the *Pediococcus* and *Lactobacillus* genera, including the species *Lb. plantarum*, *Lb. acidophilus*, *Lb. bulgaricus*, *Lb. sporogenes*, *Lb. casei*, *Lb. brevis*, *Lb. helveticus* and *P. acidilactici*. Although *Bifidobacterium* are commonly reported in the GI tract of mammals and terrestrial organisms, they are seldom reported in fish. Future experiments should attempt to quantify these bacterial groups using qPCR, FISH, metagenetic and metagenomic approaches. The importance of these bacteria in mediating host benefits, how they respond to dietary and environmental changes, and their potential to antagonize enteric pathogens, should be topics of future investigations.

6.1 INTRODUCTION

At the time of hatching, the digestive tract of fishes is a simple tube, histologically and functionally undifferentiated (Govoni *et al.* 1986). Early differentiation of the intestinal tube includes a proliferation of intestinal cells and the gut lengthens and twists as is essential for the intestine-specific functions; the anatomy and functions may eventually stabilize during larval development but then change again significantly at metamorphosis, especially if there is a major change in the diet.

In fish the initial intestine microbiota establishes at the yolk sac stage (Hansen and Olafsen 1999) and is initially composed of only few taxa within the digestive tract of sac fry (Yoshimizu *et al.* 1976; 1980). At this stage bacteria become ingested, leading to the establishment of an initial intestinal microbiota. Data on bacterial genera in the different regions of the GI tract show a progressive increase in the number of aerobic heterotrophic bacteria in adult yellow-tail (*Seriola quinqueradiata*) (Sakata *et al.* 1978) and in the juvenile and adult Dover sole (*Solea solea* L.) (MacDonald *et al.* 1986). Contrasting results on the changes of the intestinal microbiota have been reported comparing these data with cold water species such as the farmed Arctic charr (*Salvelinus alpinus* L.) (Ringø 1993a) and rainbow trout (*Oncorhynchus mykiss* Walbaum) (Austin and Al-Zahrani 1988). In turbot (*Scophthalmus maximus* L.) larvae, bacterial colonization of the GI tract coincides with the start of feeding and at this stage the microbiota is reportedly dominated by *Aeromonas* and *Vibrio* (Munro *et al.* 1994; Ringø *et al.* 1996). Readers with a specific interest on the overall composition of the gut microbiota of fish and the factors that modulate these communities are referred to Ringø *et al.* (2012a; 2012b) and *Chapter 4*, and readers with special interest in the importance of host–microbiota interactions within the fish GI tract are referred to the review of Pérez *et al.* (2010).

The aim of the present chapter is to present information on LAB reported in the GI tract of different fish and crustaceans. The results cited in the present review include works published in peer-reviewed international scientific journals, national journals, and abstracts presented in books from international conferences. The information presented is organized by fish families according to the current recognized taxonomical structure (FishBase 2012).

Readers with special interest on LAB in fish and crustaceans beyond what is presented in this chapter are referred to previous published reviews discussing this topic (Ringø *et al.* 1995; Ringø and Gatesoupe 1998; Ringø 2004; Ringø *et al.* 2005; Gatesoupe 2008; Merrifield *et al.* 2010a; Ringø *et al.* 2010; Lauzon and Ringø 2012).

6.2 LACTIC ACID BACTERIA

LAB are Gram-positive, often non-motile, catalase- and oxidase-negative, non-sporing bacteria that produce lactic acid as a major product of fermentative metabolism and are widespread in nature from soil, water, plants, milk products and animals (Stanier *et al.* 1975). LAB are able to tolerate bile and pancreatic enzymes (Cai *et al.* 1999; Ouwehand *et al.* 2002) and, therefore, they are often reported to be associated with the moist, nutrient-rich intestinal contents of endothermic animals.

There are also numerous studies that have demonstrated the presence of LAB in fish (e.g. Ringø and Gatesoupe 1998; Ringø 2004; Ringø *et al.* 2005; Balcázar *et al.* 2007a; Michel *et al.* 2007; Lauzon and Ringø 2012) and it is reported that dietary factors such as chromic oxide (Ringø 1993a; 1993b; 1994), dietary form (Ringø and Strøm 1994), feed additives (Giannenas *et al.* 2012), lipid levels (Ringø and Olsen 1999), lipid sources (Ringø *et al.* 2002a), polyunsaturated fatty acids (Ringø 1993c; Ringø *et al.* 1998) and vegetable products (Heikkinen *et al.* 2006; Ringø *et al.* 2006a; 2008; Mansfield *et al.* 2010; Merrifield *et al.* 2011a; Desai *et al.* 2012) may affect the level, composition and/or diversity of LAB populations. Furthermore, factors such as stress (Olsen *et al.* 2008; Ringø *et al.* 2014), hierarchy formation (Ringø *et al.* 1997), migration from freshwater and migration from seawater back to freshwater (Ringø 2000), seasonality (Hagi *et al.* 2004) and salinity (Ringø and Strøm 1994) also modulate LAB populations in the GI tract of fish.

The key problem in estimating the proportion of LAB in the GI tract of fish is the lack of quantitative analysis. Historically assessments of fish microbiota are dominated by culture-dependent studies; these studies have used a general purpose medium, and as a result LAB have not been routinely isolated in large numbers. This does not give an accurate estimation of LAB levels as most LAB require complex nutrients (and in many cases microaerobic conditions) and thus quantitative knowledge of LAB populations is somewhat limited. *Carnobacterium* spp. do not have such specific nutrient requirements and thus are routinely isolated from cold water fish on general purpose media when culture conditions are correct (i.e. low temperature and long term incubation) (Table 6.1). Many LAB such as *Lactobacillus* and *Enterococcus* have fastidious nutrient requirements and thus it is no coincidence that these species in earlier studies have been less frequently isolated from the gut of fish. However, when selective media such as de Man, Rogosa and Sharpe (MRS) are incorporated into quantitative investigations, high LAB recovery (i.e. $\geq 10^5$ CFU g⁻¹) from the GI tract of fish has been reported (Cai *et al.* 1999; Hagi *et al.* 2004; Bucio *et al.* 2006; Rawling *et al.* 2009; Kühlwein *et al.* 2013).

6.3 SALMONIDAE

While early pioneering studies suggested that the intestinal microbiota of salmonids were mostly composed of *Aeromonas* species, members of the Enterobacteriaceae family, and did not report LAB (e.g. Yoshimizu and Kimura 1976; Sakata 1990), subsequent studies provide information that suggests that this may not be correct. Much of our understanding of the presence of LAB in the GI tract of salmonids is derived from culture-dependent studies, and studies conducted over the last two decades reveal that the dominant viable LAB in the digestive tract of salmonids belongs to the *Carnobacterium* genus (Table 6.1). The information available is

Table 6.1 LAB identified from the GI tract of salmonids.

Species	LAB species isolated	Methodology	Reference
Rainbow trout (<i>Oncorhynchus mykiss</i>)	<i>C. maltaromaticum</i> , ^a <i>C. alterfuntum</i> -like and <i>C. divergens</i>	Culture based	Spanggaard <i>et al.</i> (2001)
	<i>Carnobacterium</i> spp. and <i>Streptococcus</i> spp.	Culture based	Spanggaard <i>et al.</i> (2000)
	<i>Carnobacterium</i> spp., <i>C. divergens</i> and <i>C. maltaromaticum</i>	Culture based, clone libraries and DGGE	Kim <i>et al.</i> (2007)
	<i>Lactobacillus</i> spp. and <i>Lactococcus</i> spp.	Culture based	Heikkinen <i>et al.</i> (2006)
	<i>Carnobacterium</i> spp. and <i>C. maltaromaticum</i> ^a	Culture based, DGGE and FISH	Huber <i>et al.</i> (2004)
	<i>C. maltaromaticum</i> ^a	Culture based	Pond <i>et al.</i> (2006)
	<i>Enterococcus</i> spp.	Culture based	Dimitroglou <i>et al.</i> (2009)
	<i>C. maltaromaticum</i> , <i>E. faecalis</i> , <i>Leu. citreum</i> and <i>B. adolescentis</i>	Clone libraries and qPCR	Mansfield <i>et al.</i> (2010)
	<i>Lc. lactis</i>	TTGE	Navarrete <i>et al.</i> (2010)
	<i>Lb. aviaries</i> , <i>Lactobacillus</i> sp., uncultured <i>Streptococcus</i> sp. and <i>Str. iniae</i>	TTGE	Navarrete <i>et al.</i> (2012)
	<i>E. faecium</i> , <i>E. faecalis</i> , <i>E. hirae</i> , <i>E. mundtii</i> , <i>Leu. mesenteroides</i> subsp. <i>mesenteroides</i> , <i>C. maltaromaticum</i> , <i>Lc. lactis</i> , <i>Lb. sakei</i> , <i>Lb. crispatus</i> , <i>Lb. delbrueckii</i> subsp. <i>lactis</i> , <i>Lb. johnsonii</i> , <i>Lb. fermentum</i> , <i>Bifidobacterium</i> sp., <i>P. dextrinicus</i> , <i>Str. infantarius</i> subsp. <i>infantarius</i> , <i>Str. mitis</i> , <i>Str. lutetiensis</i> and <i>Weissella</i> sp.	DGGE and pyrosequencing	Desai <i>et al.</i> (2012)
	<i>Bifidobacterium longum</i> and <i>Lactobacillus</i> spp.	Culture based	Vlková <i>et al.</i> (2012)
River trout (<i>Salmo trutta fario</i> L.)	<i>C. maltaromaticum</i>	Culture based	Skrodenyte-Arbaciauskiene <i>et al.</i> (2006)
Sea trout (<i>Salmo trutta trutta</i>)	<i>Carnobacterium</i> spp.	Culture based	Skrodenyte-Arbaciauskiene <i>et al.</i> (2008)
	<i>C. maltaromaticum</i>		
Brown trout (<i>Salmo trutta</i>)	<i>C. maltaromaticum</i> , <i>Str. macedonicus</i> , <i>Str. constellatus</i> and <i>W. cibaria</i>	Culture based and DGGE	Abid <i>et al.</i> (2014)
	<i>Bifidobacterium</i> -like	DGGE and qPCR	Kopečný <i>et al.</i> (2010)
Atlantic salmon (<i>Salmo salar</i>)	<i>C. divergens</i> ^b	Culture based	Strøm (1988)
	<i>C. maltaromaticum</i> ^a	Culture based	Ringø <i>et al.</i> (2006a)

Table 6.1 (continued)

Species	LAB species isolated	Methodology	Reference
	<i>C. maltaromaticum</i> , ^a <i>Streptococcus</i> spp., <i>Enterococcus</i> spp., <i>E. faecalis</i> and <i>Marinilactibacillus psychrotolerans</i>	Culture based	Bakke-McKellep <i>et al.</i> (2007)
	<i>C. inhibens</i> ^c	Culture based	Jöborn <i>et al.</i> (1999)
	<i>Carnobacterium</i> spp.	Culture based	Ringø <i>et al.</i> (2008)
	<i>Carnobacterium</i> spp., <i>Streptococcus</i> spp. and <i>Lactobacillus</i> spp.	Culture based	Ringø <i>et al.</i> (2000)
	<i>C. maltaromaticum</i> , <i>Enterococcus</i> spp. and <i>Enterococcus</i> spp./ <i>Lactococcus</i> spp.	Culture based	Skrodenyte-Arbaciauskiene <i>et al.</i> (2008)
	<i>Lactobacillus</i> spp., <i>Lb. fermentum</i> and <i>Lactococcus</i> sp.	DGGE	Hovda <i>et al.</i> (2007)
	<i>Lb. letivazi</i> , <i>Lb. satsumensis</i> , <i>Lb. aviarius</i> and <i>Pediococcus</i> sp.	DGGE	Liu <i>et al.</i> (2008)
	<i>Carnobacterium</i> spp., <i>Lb. sakei</i> and <i>Leu. citreum</i>	Culture based	Askarian <i>et al.</i> (2012)
	<i>Lb. fermentum</i> , <i>Lc. lactis</i> and <i>W. cibaria</i>	DGGE	Hovda <i>et al.</i> (2012)
Chinook salmon (<i>Oncorhynchus tshawytscha</i>)	<i>Enterococcus</i> spp. and <i>Lactobacillus</i> spp.	Culture based	Moffit and Mobin (2006)
Arctic charr (<i>Salvelinus alpinus</i> L.)	<i>Lactobacillus</i> spp. and <i>Streptococcus</i> spp.	Culture based	Ringø (1993a)
	<i>Lactobacillus</i> spp. and <i>Streptococcus</i> spp.	Culture based	Ringø (1993b)
	<i>Lactobacillus</i> sp., <i>Leuconostoc</i> sp. and <i>Streptococcus</i> sp.	Culture based	Ringø and Strøm (1994)
	<i>C. maltaromaticum</i> , ^a <i>C. mobile</i> , <i>C. divergens</i> and <i>Streptococcus</i> spp.	Culture based	Ringø and Olsen (1999)
	<i>Carnobacterium</i> spp., <i>C. divergens</i> and <i>C. maltaromaticum</i>	Culture based	Ringø <i>et al.</i> (2006b)
	<i>Carnobacterium</i> spp. and <i>Streptococcus</i> spp.	Culture based	Ringø <i>et al.</i> (2002)
Salmonids ^d	<i>C. maltaromaticum</i> , <i>Lb. curvatus</i> , <i>Lb. sakei</i> , <i>Lb. plantarum</i> , <i>Lc. lactis</i> subsp. <i>cremoris</i> , <i>Lc. lactis</i> subsp. <i>lactis</i> , and <i>Leu. mesenteroides</i>	Culture based	Balcázar <i>et al.</i> (2007a)

^aPreviously *Carnobacterium piscicola*.^bOriginally identified as *Lb. plantarum*, reclassified as *Carnobacterium divergens* (Ringø *et al.* 2001).^cPreviously *Carnobacterium* strain K1.^dNot specified: either brown trout, rainbow trout or Atlantic salmon.Genera abbreviations: *C.* = *Carnobacterium*, *E.* = *Enterococcus*, *Leu.* = *Leuconostoc*, *Lb.* = *Lactobacillus*, *Lc.* = *Lactococcus*, *Str.* = *Streptococcus*, *W.* = *Weissella*.

heavily focused on rainbow trout and Atlantic salmon (*Salmo salar* L.) and most studies have used only a general purpose medium. Recently, however, molecular based investigations of the GI microbiota of salmonids reveal a more diverse LAB population composed of members from the *Carnobacterium*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus* and *Weissella* genera (Table 6.1).

6.3.1 Carnobacteria

Carnobacteria are ubiquitous LAB and have been isolated from both cold and temperate environments, and they are frequently isolated from foods, meat, some dairy products and fish. A range of *Carnobacterium* species have been identified as components of the microbiota of the GI tract of salmonids (Table 6.1). To our knowledge the first study to isolate carnobacteria from fish was carried out by Strøm (1988). Strøm isolated a strain (Lab01), initially identified as *Lactobacillus plantarum* but later reclassified as *Carnobacterium divergens* (Ringø *et al.* 2001), from the GI tract of wild Atlantic salmon. This bacterium has subsequently been shown to have antagonistic activity against several fish pathogens (Ringø 2008) and has been used in *ex vivo* studies evaluating its ability to adhere to the intestine and to study the interactions between *C. divergens* and fish pathogens (Ringø *et al.* 2007; Kristiansen *et al.* 2011). Figure 6.1 shows the typical morphology of *C. divergens* Lab01 (Ringø *et al.* 2001). *C. divergens*, *Carnobacterium maltaromaticum* (previously characterized as *Carnobacterium piscicola*), *Carnobacterium alterfuntum*-like, and *Carnobacterium* spp. have all been identified from the GI tract of rainbow trout (Spanggaard *et al.* 2000; 2001; Huber *et al.* 2004; Pond *et al.* 2006; Kim and Austin 2006a; Balcázar *et al.* 2007a; Kim *et al.* 2007; Mansfield *et al.* 2010; Desai *et al.* 2012; Abid *et al.* 2014). *C. maltaromaticum*, *C. mobile*, *C. divergens*, *Carnobacterium inhibens* and *Carnobacterium* spp. have been identified from Atlantic

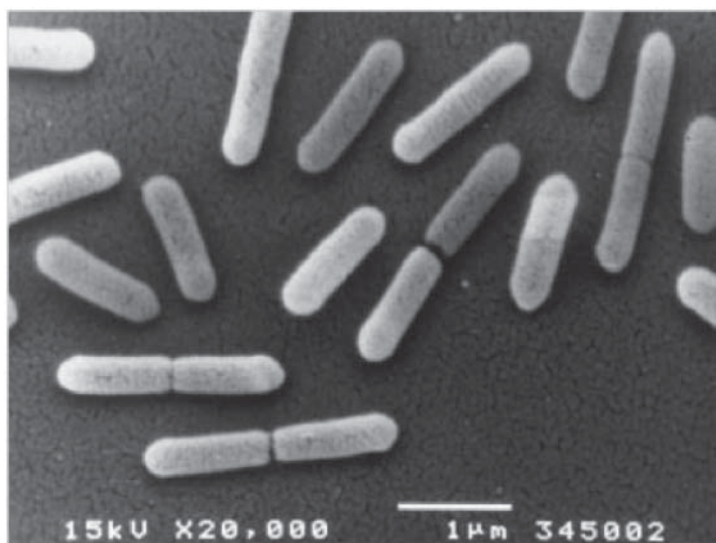


Fig. 6.1 Scanning electron microscopy of *Carnobacterium divergens* Lab01 isolated from the gastrointestinal tract of Atlantic salmon (*Salmo salar* L.). (Source: After Ringø *et al.* 2001.)

salmon (Strøm 1988; Jöborn *et al.* 1997; 1999; Ringø 2000; Ringø *et al.* 2000; 2006a; 2008; Balcázar *et al.* 2007a; Bakke-McKellep *et al.* 2007; Skrodenyte-Arbaciauskiene *et al.* 2008; Cantas *et al.* 2011). In addition, carnobacteria have been identified from Arctic charr (*Salvelinus alpinus* L.) (Ringø and Olsen 1999; Ringø 2000; Ringø *et al.* 1998; 2002b; 2006b; 2006c), river trout (*Salmo trutta fario*) (Skrodenyte-Arbaciauskiene *et al.* 2006) and sea trout (*Salmo trutta trutta*) (Skrodenyte-Arbaciauskiene *et al.* 2008) and brown trout (*Salmo trutta*) (Abid *et al.* 2014).

Furthermore, some quantitative information regarding endogenous *Carnobacterium* species is available. Skrodenyte-Arbaciauskiene *et al.* (2006) sampled the whole gut contents (allochthonous bacteria) of river trout (*Salmo trutta fario*) and cultured isolates on tryptic soy agar (TSA) for 3 days at 20 °C. *C. maltaromaticum* represented 3.8% and 4.2% of the 100 isolates from trout sampled from the Skroblus river and the Zeimena river, respectively. In laboratory reared brown trout (*Salmo trutta*) the culturable autochthonous LAB were dominated by *C. maltaromaticum*; all isolates from MRS agar from the pyloric caeca, anterior intestine and posterior intestine were identified as *C. maltaromaticum* (Abid *et al.* 2014). Skrodenyte-Arbaciauskiene *et al.* (2008) sampled material from the anterior digesta of freshwater Atlantic salmon and sea trout (*Salmo trutta trutta*) and cultured isolates on TSA for 5 days at 17 °C. The isolates from Atlantic salmon ($n = 52$) and sea trout ($n = 50$) were subsequently identified by partial 16S rDNA sequencing. The results demonstrated that *Carnobacterium* spp. represented 15.4% and 6.0% of the culturable heterotrophic populations of Atlantic salmon and sea trout, respectively. These levels comprised *C. maltaromaticum* and an unidentified *Carnobacterium* spp. in sea trout, but only *C. maltaromaticum* was identified in Atlantic salmon. Ringø *et al.* (2008) reported *Carnobacterium* levels in the distal intestine of Atlantic salmon to be of log 3.8 and 5.2 CFU g⁻¹ for the autochthonous and allochthonous communities, respectively. In a recent study of juvenile Atlantic salmon, Cantas *et al.* (2011) investigated the culturable allochthonous intestinal microbiota of triploid and diploid fish. Significantly higher total bacterial counts within each gut section (proximal intestine (PI), midgut (MG) and distal intestine (DI)) were observed in triploids but a significant decrease in *Carnobacterium* spp. was observed in triploids compared to diploids. Whether these differences contribute to fish health or disease resistance has not been elucidated and merits further investigations.

In respect of both culturable and non-culturable populations, the levels of carnobacteria in the intestinal contents of rainbow trout are reported to be relatively low. Huber *et al.* (2004) determined the microbial composition of the intestines of rainbow trout from three fish farms by both classical culture-dependent methods (isolation on TSA after incubation at 15 °C for 7 days) and culture-independent molecular methods (DGGE and FISH). No *Carnobacterium* isolates were isolated using culture based methods or FISH from the intestine of trout at farm 1, at farm 2 or from the first visit to farm 3 (10/09/97). However, upon a second visit (09/12/97) to farm 3 the culture based results demonstrated that *Carnobacterium* spp. accounted for 33% of the culturable populations and FISH indicated that this represented 3% of total bacterial levels. DGGE and subsequent sequencing indicated that these were *C. maltaromaticum*. Kim *et al.* (2007) also investigated the microbial community of farm reared rainbow trout. Of the 75 culturable microorganisms isolated, the authors reported one strain of *Carnobacterium* sp. and one strain belonging to *C. divergens*. Clone libraries derived from the intestinal contents and intestinal mucus revealed that two out of 14 (14.3%) and one out of 31 (3.2%) clones from intestinal contents and mucus were *C. maltaromaticum*. In contrast to these low levels, Pond

et al. (2006) and Mansfield *et al.* (2010) reported that *C. maltaromaticum* was a dominant component of the gut contents of laboratory reared rainbow trout. According to clone library analyses, Mansfield *et al.* (2010) reported that *C. maltaromaticum* represented 55%–97.2% of the total bacterial community of the distal gut contents of rainbow trout.

In contrast to the aforementioned studies, a number of studies have assessed the gut microbiota of salmonids and have not retrieved carnobacteria as components of the indigenous gut microbiota (e.g. Heikkinen *et al.* 2006; Moffit and Mobin 2006; Dimitroglou *et al.* 2009; Merrifield *et al.* 2009a; 2009b; Navarrete *et al.* 2010; 2012). It is difficult to draw conclusions as to what levels of *Carnobacterium* spp. are typically present in the gut of salmonids from the somewhat contradictory findings of the present literature – and the different and not always appropriate (to isolate carnobacteria) methodology used can compromise our conclusions. Studies are required to determine carnobacteria levels in the gut of salmonids in order to better understand their importance as components of the normal salmonid microbiota.

Despite this, it is clear that carnobacterial populations are of potential importance to the host fish as *Carnobacterium* isolates have been extensively tested both *in vitro*, to ascertain their impacts on transient pathogenic bacteria, and *ex vivo* and *in vivo* as probiotics. Kim and Austin (2006a) extensively characterized two probiotic carnobacteria isolates derived from the intestine of rainbow trout: *C. maltaromaticum* (B26) and *C. divergens* (B33). Both carnobacteria grew between 0 and 37 °C in 0–10% NaCl at pH 5–10, were plasmid free, were resistant to a broad spectrum of antibiotics, and inhibited *in vitro* growth of *Aeromonas salmonicida*, *Aeromonas hydrophila*, *Streptococcus iniae* and *Vibrio anguillarum*. These characteristics suggest that such carnobacteria species in the intestines of fish may play an important defensive role against pathogenic organisms. Indeed, Kim and Austin (2006a) used these strains as probiotics and demonstrated that transplanting high levels of *C. maltaromaticum* and *C. divergens* to the intestines of trout via dietary applications stimulated non-specific immunity. However, as the application led to carnobacterial levels of >90% of culturable levels, which is generally far higher than natural carnobacterial levels reported, it is difficult to ascertain their degree of importance in mediating normal immune function and mucosal tolerance. However, it has been reported from *in vitro* studies that incubation of *C. maltaromaticum* and *C. divergens* with (undefined) gut cells did not induce any effects on IL-1 β , IL-8, TGF- β and TNF- α mRNA levels (Kim and Austin 2006b). However, future *ex vivo* and *in vivo* studies will yield more precise information on the importance of carnobacterial–epithelial interactions in fish.

Several studies suggest that the carnobacteria populations within the gut of fish are affected by dietary factors. For example, Ringø *et al.* (2006a) observed that the presence of dietary krill meal reduced culturable epithelial associated *C. maltaromaticum* populations in the hindgut of Atlantic salmon to non-detectable levels, down from log 3.23 CFU g⁻¹ in the control fish. Mansfield *et al.* (2010) reported that *C. maltaromaticum* represented 55% of the total bacterial community of the distal gut contents of rainbow trout fed a fishmeal based diet, and when the dietary protein source was changed to casein or soybean meal the abundance increased to 87.8% and 97.2%, respectively. Indigenous *C. maltaromaticum* populations in the gut of Atlantic salmon have also been reported to be sensitive to oxytetracycline (Bakke-McKellep *et al.* 2007). Carnobacteria composition also appears to be affected by dietary carbohydrates (Ringø and Olsen 1999; Ringø *et al.* 2006b). Arctic charr fed high levels of dietary carbohydrates displayed different adherent culturable carnobacteria profiles in the intestine, such as *C. mobile* in the small intestine and *C. maltaromaticum* in the distal intestine; however, fish fed low levels of dietary carbohydrates displayed only *C. divergens* in both intestinal regions.

It is important to note also that the higher dietary carbohydrate level reduced the frequency of carnobacteria isolation within replicate fish (Ringø and Olsen 1999). In a later study, it was observed that the presence of dietary inulin tended to lower culturable autochthonous carnobacteria levels (by ca. 90%) in the hindgut of Arctic charr, which seemed to benefit *C. maltaromaticum* at the expense of *C. divergens* (Ringø *et al.* 2006b).

6.3.2 *Lactobacillus*

Although a great deal of effort has focused on the assessment of *Lactobacillus* spp. as probiotics for salmonids, fewer studies have identified *Lactobacillus* as indigenous components of the intestinal microbiota. Additionally, it has been suggested that the identification of intestinal *Lactobacillus* spp. in older studies based on culture-dependent identifications probably erroneously describe some strains of '*Lactobacillus* spp.' which may belong to the *Carnobacterium* genus (Ringø and Gatesoupe 1998). The first study showing that fish contained lactobacilli in the GI tract was conducted by Kraus (1961) who isolated lactobacilli from herring (*Clupea harengus* L.). In their study of several freshwater fishes (Cyprinidae, Escoidae and Percidae), Kvasnikov *et al.* (1977) reported several cultivable lactobacilli in the intestinal contents of the fish. More recently, several studies have identified members of the *Lactobacillus* genus in the GI tract of trout (Heikkinen *et al.* 2006; Skrodenyte-Arbaciauskiene *et al.* 2008; Balcázar *et al.* 2008; Azizpour 2009; Navarrete *et al.* 2010; Pérez-Sánchez *et al.* 2011a; Navarrete *et al.* 2012; Desai *et al.* 2012; Vlková *et al.* 2012), Atlantic salmon (Ringø *et al.* 2000; Moffit and Mobin 2006; Hovda *et al.* 2007; 2012; Askarian *et al.* 2012; Ringø *et al.* 2014) and Arctic charr (Ringø *et al.* 1998; 2000).

A clear limitation, in most cases, is the lack of identification of such isolates to species level. Confirming the need to use either selective media or preferably culture-independent methods, Hovda *et al.* (2007) failed to isolate *Lactobacillus* spp. in the gut of rainbow trout using standard culture based protocols, but using DGGE and subsequent sequence analyses *Lactobacillus* spp. were identified from the PI, MG and DI of Atlantic salmon. These were identified as *Lactobacillus* spp., *Lb. fermentum* and *Lb. fermentum*-like. Recently, *Lb. curvatus*, *Lb. sakei*, *Lb. plantarum* and *Lb. fermentum* have been isolated from the gut contents of salmonids (Balcázar *et al.* 2007a; 2008). Balcázar *et al.* (2008) further characterized the *Lb. plantarum* and *Lb. fermentum* isolates and assessed their properties with regards to pathogen antagonism and epithelial colonization. Even though *Lb. plantarum* and *Lb. fermentum* did not show antibacterial activities against three fish pathogens tested in a well assay, *Lb. plantarum* reduced the mucus adhesive capacity of *A. hydrophila* and *A. salmonicida*, whereas *Lb. fermentum* reduced the adhesion of *A. hydrophila*, *A. salmonicida* and *Yersinia ruckeri*. These findings suggest that indigenous *Lactobacillus* populations have the potential to retard pathogenic colonization of the epithelial mucus layer within the intestine of fish, which is important because adhesion to epithelial mucus is thought to be an important step in intestinal infection, providing a foundation for mucosal interaction and translocation (Ringø *et al.* 2010; Sica *et al.* 2012; Chapter 3). How effective these strains are *in vivo*, under different environmental conditions, is yet to be ascertained. However, the efficacy of probiotic applications of *Lactobacillus* spp. for salmonids is clear, particularly in regards to immunity and disease resistance (Merrifield *et al.* 2010a), and thus is indicative that natural *Lactobacillus* spp. are probably of some importance to the host. In fact *Lb. plantarum*, isolated from the intestinal mucosa of rainbow trout, was administered to the same fish species to investigate the effects on the immune response, and it was observed that this strain was able to up-regulate pro-inflammatory cytokine gene

expression in the head kidney and intestine (Pérez-Sánchez *et al.* 2011b). Upon lactococcosis challenge, elevated intestinal mucosal antibody (IgT) mRNA levels and survivability were observed compared to the control group (Pérez-Sánchez *et al.* 2011b).

A large stumbling block in extrapolating inferences from probiotic studies is the paucity of information pertaining to natural *Lactobacillus* levels in salmonid fish. Some studies provide a brief insight into the quantity of these *Lactobacillus* spp. within the GI tract of salmonid species. Heikkinen *et al.* (2006) observed that *Lactobacillus* spp. accounted for 4.7–13.7% of the culturable bacterial community isolated from the intestinal contents of rainbow trout. Azizpour (2009) reported isolation of *Lb. plantarum* from the whole intestine of rainbow trout, identified by morphology and biochemical tests, and indicated that the bacterium accounted for 9% of the isolates tested. Although extensive information pertaining to the proportion of *Lactobacillus* spp. from culture-independent analysis is lacking, Huber *et al.* (2004) enumerated the bacterial community structure of the GI tract of the same fish species using FISH and demonstrated that 10–12% of the total microbiota was identified as belonging to the Gram-positive bacteria with low GC content. However, as the low GC Gram-positive group includes a number of genera, this does not give a reliable estimation of the level of *Lactobacillus* spp. Indeed, according to a recent pyrosequencing based study the abundance of lactobacilli, identified as *Lb. sakei*, *Lb. crispatus*, *Lb. delbrueckii* subsp. *lactis*, *Lb. johnsonii*, *Lb. fermentum*, in the distal gut of rainbow trout constituted <1% of the sequences obtained and were absent in many replicates (Desai *et al.* 2012). Further such studies are required to determine *Lactobacillus* levels in the GI tract of salmonids.

Little is known of the factors affecting the indigenous *Lactobacillus* levels of salmonids, but Moffit and Mobin (2006) observed that dietary erythromycin treatment elevated *Lactobacillus* levels in the intestine of Chinook salmon (*Oncorhynchus tshawytscha*) which were otherwise absent or at non-detectable levels before and after treatment. Dietary soybean meal has been reported to reduce the prevalence of culturable *Lactobacillus* spp. in the intestinal contents of rainbow trout from ca. 14% of the cultivable population to 5% (Heikkinen *et al.* 2006).

6.3.3 Lactococcus

The genus *Lactococcus* was proposed by Schleifer and colleagues in 1985 to reclassify some species of the genera *Lactobacillus* and *Streptococcus*. Members of this genus have been isolated from different sources, such as dairy products, plants and animals. Although *Lactococcus* species are generally considered non-pathogenic, some species have been associated with fish diseases. Among them, *Lactococcus garvieae* is the aetiological agent of lactococcosis, an emergent disease, which affects cultured freshwater and marine fish with special incidence in rainbow trout (Eldar and Ghittino 1999; Vendrell *et al.* 2006).

Some studies have demonstrated that *Lactococcus* spp. are members of the normal fish microbiota. Heikkinen *et al.* (2006) observed that lactococci accounted for 5.9–14.0% of the culturable bacterial community isolated from the intestinal contents of rainbow trout, but Desai *et al.* (2012) reported that lactococci, such as *Lc. lactis*, were not present in the distal gut of the majority of rainbow trout sampled and when present their abundance was low (i.e. <0.1% of the total sequence reads). Hovda *et al.* (2007) detected *Lactococcus* species in the PI, MG and DI of Atlantic salmon by DGGE analysis, but *Lactococcus* spp. were not detected by culture-dependent methods. Skrodenyte-Arbaciauskiene *et al.* (2008) sampled the intestinal contents of two salmonid species, freshwater Atlantic salmon and sea trout, and observed that a bacterial strain which could not be differentiated between *Lc. garvieae* and *Enterococcus*

sp. U5-1 represented 11.5% of the culturable heterotrophic bacteria of Atlantic salmon. Pérez *et al.* (2011) proposed a new subspecies, *Lc. lactis* subsp. *tractae*, which was isolated from the intestinal mucus of brown trout (*Salmo trutta*) and rainbow trout.

In 2008, Itoi and colleagues put forward the controversial hypothesis that since *Lc. lactis* has rarely been isolated from marine environments, the cells may enter into a viable but non-culturable state. This hypothesis merits further evaluation. In their study on *Lc. lactis* subsp. *lactis* isolated from the GI tract of marine and freshwater fish, Itoi *et al.* (2009) revealed extensive diversity in phenotypic variation and suggested that the strain(s) isolated from the intestine of fish may be useful as probiotics in aquaculture as the bacterium seems to adapt to various environments. Indeed, some information is also available on the probiotic properties of selected *Lactococcus* strains. Balcázar *et al.* (2007b) characterized two *Lc. lactis* strains, *Lc. lactis* subsp. *lactis* (CLFP 100) and *Lc. lactis* subsp. *cremoris* (CLFP 102), isolated from the intestinal microbiota of rainbow trout. Both strains showed high adhesion to fish intestinal mucus and reduced the mucus adhesion success of fish pathogens, such as *A. salmonicida*, *C. maltaromaticum*, *Lc. garvieae* and *Y. ruckeri*. The ability to adhere to mucosal surfaces is an important factor in the competitive exclusion of pathogens, and adhesion to the intestinal mucosa has been suggested to enhance the ability to stimulate the immune system. In fact, Balcázar *et al.* (2007c) observed a correlation between colonization with *Lc. lactis* and non-specific humoral responses such as alternative complement pathway activity and lysozyme activity in brown trout. Besides inducing enhanced immune responses, *Lc. lactis* exhibited protection against furunculosis in rainbow trout (Balcázar *et al.* 2007b) and brown trout (Balcázar *et al.* 2009).

6.3.4 *Leuconostoc*

Species of the genus *Leuconostoc* have been frequently isolated from several food products such as dairy and meat products, vegetables, and fermented food (Hemme and Foucaud-Scheunemann 2004). However, limited information is available on the presence of *Leuconostoc* species in salmonids (Ringø and Strøm 1994; Ringø *et al.* 1998; Balcázar *et al.* 2007b; 2009; Askarian *et al.* 2012). Ringø and Strøm (1994) showed that approximately 4.5% of the cultivable microbiota in the faeces of Arctic charr fed a capelin (*Mallotus villosus*) roe diet belonged to the genus *Leuconostoc*. In a later study, Ringø *et al.* (1998) isolated several *Leuconostoc* species associated with the epithelial mucosa of the stomach, PI and DI of Arctic charr fed different polyunsaturated fatty acids. Unfortunately, no attempt was made to identify the *Leuconostoc* to species level by 16S rRNA gene sequencing in the studies of Ringø and Strøm (1994) and Ringø *et al.* (1998). Mansfield *et al.* (2010) identified *Leuconostoc citreum* as a minor component of the total bacteria community in the distal intestine of rainbow trout fed a fishmeal based diet. However, this community, equivalent to 2.3% of the clones derived from the fishmeal fed fish, was not present in fish fed a diet whereby the protein was derived from casein or soybean meal.

Balcázar *et al.* (2007b) isolated a *Leuconostoc mesenteroides* strain (CLFP 196) from the intestinal microbiota of rainbow trout, which produced antibacterial compounds against several fish pathogens. Further studies demonstrated that administration of this strain enhanced the immune response and disease resistance of rainbow trout (Balcázar *et al.* 2007b) and brown trout (Balcázar *et al.* 2009). Perez-Sanchez *et al.* (2011a) isolated *Leu. mesenteroides* subsp. *mesenteroides* from the intestine of rainbow trout, and although this strain showed some positive *in vitro* characteristics it failed to improve rainbow trout disease resistance to lactococcosis

in a subsequent study (Perez-Sanchez *et al.* 2011b). More recently, Askarian *et al.* (2012) isolated autochthonous *Leu. citreum* from PI of Atlantic salmon fed a diet supplemented with 5% chitin. This bacterium was further tested for digestive enzyme production but it did not belong to the most promising enzyme-producing gut bacteria isolated.

6.3.5 Streptococcus

Species of the genus *Streptococcus* have been isolated from different sources, including fish. However, the taxonomy of this genus has been the subject of important changes, because several species have been reclassified into the *Lactococcus*, *Enterococcus* and *Vagococcus* genera, based on biochemical characteristics, as well as molecular analyses (Schleifer and Kilpper-Bälz 1984; Schleifer *et al.* 1985; Collins *et al.* 1989).

Streptococcus species were initially described as part of the intestinal microbiota in salmonids by Trust and Sparrow (1974). Since then, this genus has been isolated from the digestive tract of several fish species, including Arctic charr (Ringø 1993a; 1993b; Ringø and Strøm 1994; Ringø and Olsen 1999; Ringø *et al.* 2002b), Atlantic salmon (Ringø *et al.* 2000; Bakke-McKellep *et al.* 2007) and rainbow trout (Spanggaard *et al.* 2000; Desai *et al.* 2012; Navarrete *et al.* 2012) (Table 6.1). Ringø *et al.* (2000) observed that streptococci accounted for 0.6% of the culturable bacterial community isolated from the digestive tract of Atlantic salmon. As is the case with other LAB, *Streptococcus* species also appear to be affected by dietary treatment. In fact, Ringø *et al.* (2002b) observed that *Streptococcus* levels in the hindgut of Arctic charr were log 4.3 and 3.6 CFU g⁻¹ in fish fed diets supplemented with soybean or marine oils, respectively; however, no streptococci were isolated in the fish fed diets supplemented with linseed oil. Bakke-McKellep *et al.* (2007) observed that diets containing soybean meal reduced culturable adherent *Streptococcus* spp. populations in the MG and DI of Atlantic salmon to non-detectable levels, whereas the levels in the gut of fishmeal fed fish were log 2.3 and 2.8 CFU g⁻¹ in the MG and DI, respectively. Dietary supplementation of inulin also promoted the establishment of *Streptococcus* spp. populations in both intestinal regions.

More recently, Navarrete *et al.* (2012) identified *Streptococcus* sp. and *Str. iniae* in the DI of rainbow trout using TTGE, and the study revealed that the streptococci populations could be influenced by diet and the host fish genetics. *Streptococcus infantarius* subsp. *infantarius*, *Streptococcus mitis* and *Streptococcus lutetiensis* were recently observed as minor components (i.e. <0.01%) of the distal intestinal microbiome of rainbow trout by pyrosequencing (Desai *et al.* 2012).

6.3.6 Enterococcus

Members of the genus *Enterococcus*, classified in the past as group D *Streptococcus*, have been isolated from different sources, including salmonids (González *et al.* 2000; Bakke-McKellep *et al.* 2007; Dimitroglou *et al.* 2009) (Table 6.1). González *et al.* (2000) sampled material from the skin, gills and intestines of freshwater fish (mostly brown trout) and cultured isolates on BM agar, which is a modified MRS medium with sodium acetate and ammonium citrate omitted, for 36 h at 28 °C. The results demonstrated that *Enterococcus* species, particularly *Enterococcus durans*, represented 3.2% of the culturable LAB. In contrast with *Streptococcus* levels, Bakke-McKellep *et al.* (2007) observed that diets containing fishmeal or inulin reduced adherent *Enterococcus* populations in the MG and DI of Atlantic salmon to non-detectable levels,

whereas the presence of soybean meal led to enterococci (mostly *Enterococcus faecalis*) levels of log 2.8 and 3.1 CFU g⁻¹ in MG and DI, respectively. Dimitroglou *et al.* (2009) investigated the effect of mannan oligosaccharide (MOS) on the gut microbiota of juvenile rainbow trout and observed that *Enterococcus* spp. increased (from 3% to 19% of the total culturable bacterial population) in MOS-supplemented groups when compared with control groups. However, when compared to the total bacterial community, clone libraries revealed that *E. faecalis* was a minor component of the total bacteria community (1.8%) in the distal intestine of rainbow trout fed a fishmeal based diet and was absent in trout fed a casein or soybean meal based diet (Mansfield *et al.* 2010). More recently, pyrosequencing revealed that the total enterococci abundance, comprising *E. faecium*, *E. faecalis*, *Enterococcus hirae* and *Enterococcus mundtii*, accounted for <0.1% of the sequences generated from the distal gut contents of rainbow trout (Desai *et al.* 2012).

Moffit and Mobin (2006) isolated erythromycin-resistant *Enterococcus* spp. from Chinook salmon reared in raceways, and Skrodenyte-Arbaciauskiene *et al.* (2008) observed that the intestinal contents of Atlantic salmon contained isolates representing 11.5% of the culturable heterotrophic bacteria which could not be differentiated between *Enterococcus* sp. U5-1 and *Lc. garvieae*.

6.3.7 *Vagococcus*

The genus *Vagococcus* was proposed by Collins *et al.* (1989) to accommodate some motile Lancefield group N cocci, which were phylogenetically distinct from lactococci. Species of this genus have been frequently isolated from diseased fish. In fact, *Vagococcus salmoninarum* was originally isolated from diseased rainbow trout in North America (Wallbanks *et al.* 1990) and, since then, has been recovered from Atlantic salmon, rainbow trout and brown trout with peritonitis (Schmidtke and Carson 1994; Ruiz-Zarzuela *et al.* 2005). Although members of the genus *Vagococcus* have been isolated from different sources (Jaffrès *et al.* 2010; Wang *et al.* 2011), little is known about them as members of the normal intestinal microbiota in salmonids. González *et al.* (2000) isolated LAB from the skin, gills and intestines of freshwater fish (mostly brown trout) and reported two isolates, which were identified as *Vagococcus fluvialis*, but no specific information was provided regarding the precise isolation source of these strains.

6.3.8 *Weissella*

To our knowledge, no culture based studies have reported *Weissella* in the digestive tract of salmonids; however, three recent culture-independent studies have reported indigenous *Weissella* or *Weissella*-like populations in the GI tract of salmonids (Desai *et al.* 2012; Hovda *et al.* 2012; Abid *et al.* 2014). In a study of triploid female rainbow trout fed different plant based diets, Desai *et al.* (2012) reported Firmicutes with ca. 82–90% sequence similarity to *Weissella paramesenteroides* (i.e. the nearest neighbour) in the distal gut contents. *Weissella cibaria* has been identified from the GI tract of Atlantic salmon (Hovda *et al.* 2012) and brown trout (Abid *et al.* 2014). There is no information on the levels of these species or their significance to the host but recent studies have revealed that dietary application of *W. cibaria* reduced intestinal *Vibrio* levels and elevated serum Ig levels of hybrid sorubims (*Pseudoplattystoma* sp.) (Mouriño *et al.* 2012). *Weissella* spp. have also been reported as opportunistic pathogens for rainbow trout in China (Liu *et al.* 2009).

6.3.9 Bifidobacterium

Bifidobacterium constitute a significant portion of the human and mammalian intestinal microbiota and have beneficial effects on their host (Vaughan *et al.* 2000). These obligate anaerobes compete with other species of intestinal flora and transient organisms for nutrients and attachment sites in the gut. Recent studies have also reported the presence of bifidobacteria in the GI tract of honey bees, bumblebees and cockroaches (Vlková *et al.* 2012).

Using a nested PCR approach, based on initial PCR using *Bifidobacterium* 16S rRNA specific primers, and secondary PCR targeting the 16S rRNA V3 region, Kopečný *et al.* (2010) reported the presence of bifidobacteria in the GI tract of brown trout. Unfortunately no sequence analyses were presented for the presumed bifidobacterial DGGE bands obtained from trout samples, but sequence alignments for bands obtained from other host organisms (i.e. wasps and cockroaches) were low, with the highest reported similarity of bands being 91–94% similar to *Bifidobacterium adolescentis*. Given that the highest sequences obtained from this study were below 95% similarity, the general level for acceptance of genus classification, we suggest that these bacteria are *Bifidobacterium*-like strains. The authors used qPCR to provide quantitative data which indicated that the *Bifidobacterium*-like populations accounted for 1.1% of the bacterial community relative to the 'total' bacterial community. However, the total community was estimated based on qPCR of *Bacteroides uniformis* and, given that *Bacteroides* are often a minor component of the gut microbiota of fish, including trout, this value could be an overestimation and an accurate quantification cannot be concluded from this study. This study is however the first study, to the authors' knowledge, to report the presence of bifidobacteria-like species from the GI tract of fish. A second study published in 2010 also provided evidence of a minor presence of bifidobacteria in the gut of rainbow trout (Mansfield *et al.* 2010). The construction of clone libraries of bacterial communities from the distal intestine of rainbow trout revealed one bifidobacterial clone (*Bifidobacterium adolescentis*) present in one group of fish, accounting for 0.1% of the clones generated (Mansfield *et al.* 2010). More recently, Vlková *et al.* (2012) identified presumptive bifidobacteria in the distal intestine of one of 15 rainbow trout examined. The levels recovered were $\log 3.5 \text{ CFU g}^{-1}$ compared to total bacterial levels of $\log 8.85 \text{ bacteria g}^{-1}$ (determined from FISH) and thus bifidobacterial levels represented $<0.0005\%$ of the total bacterial community. A representative isolate of this population was identified by 16S rRNA sequence analysis as *B. longum* (98% similarity). Using pyrosequencing of the distal gut contents of rainbow trout, Desai *et al.* (2012) recovered a very small number of sequences identified as *Bifidobacterium* sp. 12_1_47BFAA from one sample.

6.4 GADIDAE

Several papers have reported LAB in the GI tract of Atlantic cod (Daczowska-Kozon 1988; Strøm 1988; Strøm and Olafsen 1990; Strøm and Ringø 1993; Ringø *et al.* 2006c; Seppola *et al.* 2006; Lauzon *et al.* 2010; Løvmo Martinsen *et al.* 2011; Askarian *et al.* 2013; Zhou *et al.* 2013).

Daczowska-Kozon (1988) reported low population levels of LAB in the gut of cod from the Baltic Sea; the level varied between <200 and 380 LAB g^{-1} intestine. Strøm (1988) isolated *C. divergens*-like strains and *C. maltaromaticum*-like strains from the intestine of wild juveniles and adults and farmed Atlantic cod and reported that their population level was

approximately 10^4 CFU g⁻¹. Strøm and Olafsen (1990) investigated the gut microbiota of four Atlantic cod groups: wild caught fry (group 1), fry held in a fish farm for one month (group 2), fish fed an artificial diet for one year (group 3) and fish sampled from a farm after an outbreak of cold water vibriosis (group 4). The results of this study showed that the proportion of mucosal *Lactobacillus* was affected by feeding regimes. The highest proportion of *Lactobacillus* (constituting 30% of the culturable microbiota) was detected in the gut of group 3 and 10% was reported in group 1 and in group 4. Surprisingly, *Lactobacillus* accounted for <1% of the gut microbiota in group 2. In a later study, Strøm and Ringø (1993) reported that lactobacilli constituted <1% of the gut microbiota in 14-day-old cod larvae. However, it should be noted that the larvae were not fed during the 14 day experiment.

The study of Ringø *et al.* (2006c) investigated the allochthonous and autochthonous microbiota in the PI, MG and hindgut chamber (HC; fermentation chamber) of 1-year-old Atlantic cod fed fishmeal, standard soybean meal and a bioprocessed soybean meal. The results demonstrated that the gut microbiota was modulated by dietary manipulation. The only LAB isolated in this study belonged to *Carnobacterium* sp., identified by 16S rRNA gene sequencing, and was generally detected as both allochthonous and autochthonous in all three segments of the GI tract of fish fed fishmeal and soybean meal. However, the *Carnobacterium* sp. was less abundant in the GI tract of bioprocessed soybean meal fed fish.

Seppola *et al.* (2006) isolated allochthonous and autochthonous LAB from the DI and HC of fed and starved (3 days) cod. All strains isolated from the HC belonged to carnobacteria, in contrast to the DI where only 10.2% of the isolates were carnobacteria. Lauzon *et al.* (2010) reported *Lc. lactis* ssp. *lactis*, *Va. fluvialis* and *Enterococcus pseudoavium* in the gut of cod larvae. Løvmo Martinsen *et al.* (2011) observed that *C. maltaromaticum* constituted a minor part of the bacterial community (5×10^3 CFU g⁻¹) in the MG of cod. Askarian *et al.* (2013) isolated a *Carnobacterium* sp. from the GI tract of cod which displayed protease and cellulase activities and some phytase activity and inhibited *in vitro* growth of *A. salmonicida*, *V. anguillarum*, *Moritella viscosa* (the causative agent of winter ulcer) and a pathogenic *C. maltaromaticum*.

Zhou *et al.* (2012) investigated the autochthonous gut microbiota of cod fed diets with or without 5% chitin supplementation using DGGE. From each of the two dietary groups, samples were taken from the PI and DI of three individual fish. The results revealed *Lactococcus* sp. in both segments of both dietary groups, independent of the dietary treatment, whereas *Lc. lactis* was only detected in the DI of the chitin fed group.

Information on the LAB in the GI tract of saithe (*Pollachius virens* L.) is reported by Schröder *et al.* (1980), Strøm (1988) and Strøm and Ringø (unpublished results). In the early study of Schröder and co-authors a *Lb. plantarum*-like strain was isolated from the GI tract. However, the strain was only identified by physiological and biochemical characterization, but as it grew at pH below 5.5 it possible that it belonged to genus *Lactobacillus*. The strain grew well at low temperature and inhibited the growth of *Vibrio* sp. In the studies of Strøm (1988) and Strøm and Ringø (unpublished results), autochthonous LAB, present at approximately 10^4 CFU g⁻¹, were isolated from the intestine of saithe. These isolates were identified as *C. divergens* and *C. maltaromaticum* based on 16S rRNA gene sequencing (Ringø and Strøm unpublished results).

In a study carried out on 12 fish species, wild and farmed, from various fishing grounds in Tunisia, Boulares *et al.* (2011) isolated *Lb. brevis* from whiting (*Merlangius merlangus*) and reported that the bacterium was dominant among the culturable LAB in the intestine.

6.5 CLUPEIDAE

A number of studies have reported LAB from members of the Clupeidae family but less information is available on the level, or activity, of these populations. Early reports on herring have suggested that the lactobacilli isolated from the whole intestine of herring belonged to *Lb. brevis* or *Lb. plantarum* (Kraus 1961). Although not identified to species level, LAB levels have been reported to be 190 bacteria g⁻¹ in the gut of spawning herring and 9.3×10^3 bacteria g⁻¹ in intensively fed fish (Daczowska-Kozon 1988). More recent studies on the gut microbiota of Clupeidae have isolated *E. hirae*, *Lb. paracasei* subsp. *tolerans*, *Weissella viridescens* and *Leu. citreum* in Jenyns's sprat (*Ramnogaster arcuata*) (Sica *et al.* 2010) and *W. viridescens* from Brazilian menhaden (*Brevoortia aurea*) (Sica *et al.* 2010). Sahnouni *et al.* (2012) isolated different LAB from the contents of the intestinal tracts of sardine (*Sardina pilchardus*) and bug (*Boops boops*). The strains were identified based on morphology and biochemical tests as *E. faecium*, *Lb. plantarum*, *Lc. lactis* subsp. *diacetylactis*, *Lc. lactis* subsp. *lactis* and *Leuconostoc* sp. These bacteria were further tested for their ability to inhibit 10 pathogenic bacteria and for the production of proteolytic, amylolytic and lipolytic activities. These studies provide some information on LAB identity and *in vitro* characteristics but clearly more comprehensive studies on the LAB of Clupeidae are warranted.

6.6 ANARHICHADIDAE

To the authors' knowledge the only information with regards to LAB in the GI tract of anarhichadidae is reported by Ringø *et al.* (2001). In this study, the authors reported an adherent *C. divergens* isolate from the whole intestine of wolffish (*Anarhichas lupus* L.) fry, and the isolate has been reported to inhibit *in vitro* growth of *A. salmonicida* subsp. *salmonicida* and *V. anguillarum* (Ringø 2008).

6.7 ACIPENSERIDAE

Despite extensive research on the isolation and characterization of LAB in finfish, limited reports are available on sturgeons (Mahious *et al.* 2006b; Askarian *et al.* 2008; 2009; Ghanbari *et al.* 2009; Askarian *et al.* 2011; Soltani *et al.* 2013). However, the effects of dietary supplements including inactive yeast and prebiotics (inulin and oligofructose) on the indigenous LAB have been studied using culture based techniques in sturgeons (Mahious *et al.* 2006a; Reza *et al.* 2009; Hoseinifar *et al.* 2011a; 2011b). Preliminary information on the presence of LAB in intestinal microbiota of Siberian sturgeon (*Acipenser baerii*) has been presented by Mahious *et al.* (2006b). Although molecular and culture based techniques revealed that the majority of isolates belonged to the *Bacillus subtilis* group (19.8%) and members of the Enterobacteriaceae family (29.1%), isolation of *Lc. lactis* was also reported. In contrast, Rurangwa *et al.* (2009) reported that *Lb. delbrueckii* subsp. *lactis* was one of the most prevalent species isolated from the gut of Siberian sturgeon fed fermentable carbohydrates. Isolation of LAB from the GI tract of beluga (*Huso huso*) and Persian sturgeon (*Acipenser persicus*) has also been reported (Askarian *et al.* 2009; Ghanbari *et al.* 2009; Askarian *et al.* 2011; Soltani *et al.* 2013). Askarian *et al.* (2009) isolated and characterized indigenous LAB in different parts of the digestive tract

(oesophagus, stomach, PI and DI) in beluga and Persian sturgeon. Mean LAB levels in beluga and Persian sturgeons were 7.9×10^6 CFU g⁻¹ and 5.4×10^6 CFU g⁻¹, respectively. Maximum LAB levels were reported in the DI of beluga (3.8×10^6 CFU g⁻¹) and Persian sturgeon (4.6×10^6 CFU g⁻¹) while minimum levels were present in the PI (3.2×10^5 CFU g⁻¹ in beluga and 1.1×10^5 CFU g⁻¹ in Persian sturgeon). 16S rRNA partial sequencing of LAB isolates ($n = 70$) from beluga and Persian sturgeon revealed that 25 isolates were identified (98–99% sequence alignment) as *Lb. curvatus*, eight isolates as *Lactococcus raffinolactis*, five isolates as *Lc. lactis* spp. *cremoris*, and two isolates as *Streptococcus* spp. Furthermore, in Persian sturgeon 22 isolates were identified as *Leu. mesenteroides* and eight isolates as *Enterococcus seriolicida*. Based on these results, the authors concluded that the DI is the best region for isolating LAB in the GI tract of beluga and Persian sturgeon and that the LAB community composition differed between the species (Askarian *et al.* 2009). It was interesting to note therefore that, in a later study, Askarian *et al.* (2011) observed that dietary administration of *Lb. curvatus* (isolated by Askarian *et al.* 2009) improved the growth performance and digestive enzyme activities of beluga whereas the application of *Leu. mesenteroides* (isolated by Askarian *et al.* 2009) did not. In contrast, in Siberian sturgeon *Leu. mesenteroides* application improved the growth performance and digestive enzyme activities but *Lb. curvatus* did not. These studies would infer that the dominant LAB communities in these sturgeon species display host specificity and that they might have important influences on the host.

Ghanbari *et al.* (2009) isolated and characterized LAB from intestine of beluga and Persian sturgeons inhabiting the Caspian Sea using culture based techniques followed by biochemical tests. Their results showed relatively high levels of LAB in the intestine of beluga (log 5.3 CFU g⁻¹) and Persian sturgeon (log 6.4 CFU g⁻¹). Biochemical tests of the gut isolates indicated prevalence of *Lb. sakei*, *Lb. plantarum*, *Lb. alimentarius*, *Lb. casei* in beluga and *Lb. sakei* and *Lb. brevis* in Persian sturgeon. In a more recent study, Soltani *et al.* (2013) reported that culturable autochthonous LAB ranged from log 2.93 to 5.61 CFU g⁻¹ intestine in Persian sturgeon. 16S rRNA gene sequence analysis revealed that the LAB community was dominated by *Lc. garvieae* and *Lc. lactis*. In addition the authors reported that *Pediococcus pentosaceus*, *W. cibaria* and *E. faecalis* were minor components of the LAB community.

Characterization of indigenous microbiota, especially LAB, using culture-independent molecular approaches like PCR-DGGE, next-generation sequencing and FISH, which provide a more comprehensive assessment of bacterial communities, has so far been neglected in sturgeon studies. Thus, determination of indigenous LAB and their levels in different life stages of sturgeon species, for the development of optimum prebiotic and probiotic applications, is an obvious topic for future research.

6.8 PERCIDAE AND SCIAENIDAE

It has been demonstrated that LAB species are members of the *normal* microbiota of some Percidae (Bucio *et al.* 2006) and Sciaenidae (Sica *et al.* 2010). *Lb. coryneformis* subsp. *torquens*, *Lb. sakei*, *Bifidobacterium dentium* and lactobacilli have been isolated from perch (*Perca fluviatilis*) (Bucio *et al.* 2006; Vlková *et al.* 2012), *Lactobacillus* spp. from ruffe (*Gymnocephalus cernuus*) (Bucio *et al.* 2006), *W. viridescens* and *Leu. mesenteroides* subsp. *mesenteroides* from striped weakfish (*Cynoscion guatucupa*) (Sica *et al.* 2010) and *Leu. mesenteroides* subsp. *mesenteroides* from *Micropogonias furnieri* (Sica *et al.* 2010).

6.9 MORONIDAE

Significant changes in the gut microbiota have been reported during larval growth of European sea bass (*Dicentrarchus labrax*). Analysis performed at 11 days post hatching (dph) revealed low levels of culturable anaerobic and aerobic bacteria, enterobacteria (10^5 CFU g⁻¹) and LAB (10^4 CFU g⁻¹) (Silvi *et al.* 2008). The total culturable microbiota levels increased significantly at 22 days dph: higher numbers of anaerobic (10^9 CFU g⁻¹), aerobic (10^9 CFU g⁻¹) and enterobacteria (10^8 CFU g⁻¹) were observed, and concomitantly LAB levels (10^3 CFU g⁻¹) were reduced. Among the LAB the following species were identified: *Lb. curvatus* (17.4% and 16.7% of the total LAB community at 11 and 22 dph, respectively), *Lb. delbrueckii* subsp. *delbrueckii* (8.5% and 7.4% at 11 and 22 dph, respectively), *Lb. brevis* (29.3% and 31.3%, at 11 and 22 dph, respectively), *Lb. fructivorans* (7.1% and 8.3% at 11 and 22 dph, respectively), *Lactobacillus* spp. (19.0% and 18.5% at 11 and 22 dph, respectively) and *Lc. lactis* subsp. *lactis* (18.7% and 17.8% at 11 and 22 dph, respectively) (Silvi *et al.* 2008). Counts at 30, 50 and 70 dph evidenced a decrease in the levels of anaerobes and aerobes as well as a decrease in total LAB, particularly at 30 dph, where the LAB were identified as *Lb. fructivorans*, *Lb. brevis*, *Lb. curvatus*, *Lb. acidophilus*, *Lb. viridescens*, *Lb. delbrueckii* subsp. *delbrueckii*, *Lb. lindneri*, *Lactobacillus* spp., *Aerococcus viridans*, *Lc. lactis* subsp. *lactis* and *Leu. mesenteroides* subsp. *mesenteroides*. The diversity of LAB species decreased marginally at 50 dph and at 70 dph, where only five different LAB species remained: *Lb. acidophilus* (27.0%), *Lb. delbrueckii* subsp. *delbrueckii* (10%), *Lb. fructivorans* (14.0%), *Lb. lindneri* (46%) and *Lactobacillus* spp. (4.0%) (Silvi *et al.* 2008).

Carnevali *et al.* (2006) isolated an indigenous *Lb. delbrueckii* subsp. *delbrueckii* (AS13B) strain from the intestine of adult sea bass. The strain showed good capability to colonize the gut of sea bass larvae, thereby modifying the gut microbiota, and exerting positive effects on the survival of the treated sea bass (Silvi *et al.* 2008). In addition, the probiotic strain elevated the levels of intestinal T cells, in keeping with increased total body TcR- β transcripts, and increased acidophilic granulocytes concomitantly with lower transcription of pro-inflammatory genes (IL-1- β , TGF- β , IL-10, Cox-2) (Picchiatti *et al.* 2009; Abelli *et al.* 2009), indicating that it might be an important component of the indigenous microbiota.

Recently, Bourouni *et al.* (2012) isolated 47 LAB strains from the intestinal tract of farmed sea bass and examined the strains phenotypically and phylogenetically. Most of the strains belonged to *Enterococcus* and were identified as *E. faecium* (59% of the isolates), *E. faecalis* (21%), *E. sanguinicola* (8%), *E. mundtii* (2%) and *E. pseudoavium* (2%). In addition *Lc. lactis* (2%) and *Ae. viridans* (2%) were isolated. The LAB strains were further tested for antimicrobial activities *in vitro* against pathogenic and spoilage bacteria and 32 strains inhibited the growth of the test bacteria.

6.10 SPARIDAE

The microbial community reported in the early developmental stages (35 dph) of gilthead sea bream (*Sparus aurata*), from the same farm as the sea bass described by Silvi *et al.* (2008), was quite different – indicating therefore a host selective pressure rather than reflecting the fish farm of origin (Carnevali *et al.* 2004). The anaerobe/aerobe ratio was 0.43 and *Lactobacillus*

were detected at 35 dph. At 66 dph, the levels of anaerobes and aerobes decreased significantly compared with those at 35 dph and concomitantly *Lactobacillus* levels increased to log 3.96 CFU g⁻¹ larvae. The composition remained similar at 90 dph (Carnevali *et al.* 2004). In a recent study, Sorroza *et al.* (2012) isolated and identified by 16S rRNA gene sequencing *Va. fluvialis* from the gut contents of gilthead sea bream. The bacterium was subsequently tested for its ability to inhibit *in vitro* growth of several fish pathogens, resistance to bile and pH, adhesion to mucus and efficacy in a challenge study. Based on their results the authors concluded that *Va. fluvialis* may be important for the control of vibriosis.

6.11 PLEURONECTIFORMES

Little information is available on LAB in the intestine of flatfish. Boulares *et al.* (2011) isolated *Leu. mesenteroides* and *Lb. brevis*, and Sorroza *et al.* (2012) isolated *Va. fluvialis* from the sole gut. In an unpublished study by Ringø, adherent *C. maltaromaticum* strains were isolated from juvenile turbot intestine which inhibited the growth of *A. salmonicida* subsp. *salmonicida* and *V. anguillarum* *in vitro*. Concerted efforts are required to focus on the identification and functionality of indigenous LAB populations in flatfish.

6.12 CYPRINIDAE

A number of studies have focused on the indigenous gut LAB from cyprinids (Table 6.2).

6.12.1 Common carp (*Cyprinus carpio* L.)

Cai *et al.* (1999) isolated 54 LAB strains from the intestines of common carp in the Nakorn-Pathom Province, Thailand. These LAB belonged to the genera *Leuconostoc*, *Enterococcus*, *Lactobacillus*, *Pediococcus* and *Lactococcus*. A later study by Jankauskiene (2002) investigated the autochthonous and allochthonous LAB in the digestive tract of 65 carps. Bacteria belonging to genus *Lactobacillus* were detected in the digesta of 42 fish (64.6% of the fish) and associated to the wall of the digestive tract of 22 fish (33.8% of the fish). Sugita and Mizuki (2012) evaluated the allochthonous gut microbiota in the PI and DI of common carp reared in an indoor tank by constructing clone libraries. Of the 93 clones constructed, five clones belonged to *Lc. raffinolactis*, and the results showed that there were some differences between the two parts of the intestine investigated as *Lc. raffinolactis* was only detected in the DI. Hagi *et al.* (2004) investigated the composition of intestinal LAB in common carp in Kasumigaura during different seasons. During the summer, LAB levels in the GI tract were 3.3×10^6 CFU g⁻¹ (ca. 0.17% of the total viable count) and comprised *Lc. lactis* (80% of the LAB isolates identified) and *E. seriolicida* (20%). During the winter however LAB levels were reduced to 8.0×10^5 CFU g⁻¹ (ca. 1.6% of the total viable count) and were comprised only of *Lb. raffinolactis*. The study thus indicates a clear shift in culturable LAB communities with seasonality; this was confirmed in a later study on common carp which revealed that the predominant LAB was *Lc. lactis* in the summer and *Lc. raffinolactis* in the winter (Hagi and Hoshino 2009). In addition, Hagi and Hoshino (2009) also isolated *Enterococcus pseudoavium*, *Lactobacillus fuchuensis*, *Lb. sakei* and *Leuconostoc gasicomitatum*. Five summer and five winter LAB isolates were tested with respect to growth, level of cholic acid

Table 6.2 LAB identified in the GI tract of cyprinids.

Species	LAB identified	Reference
Common carp (<i>Cyprinus carpio</i> L.)	<i>Leuconostoc</i> , <i>Enterococcus</i> , <i>Lactobacillus</i> , <i>Pediococcus</i> and <i>Lactococcus</i>	Cai <i>et al.</i> (1999)
	<i>Lc. lactis</i> , <i>Lc. raffinolactis</i> and <i>E.</i> <i>seriolicida</i>	Hagi <i>et al.</i> (2004)
	<i>Lc. lactis</i> , <i>Lc. raffinolactis</i> , <i>E.</i> <i>pseudoavium</i> , <i>Lb. fuchuensis</i> , <i>Lb. sakei</i> and <i>Leu. gasicomitatum</i>	Hagi and Hoshino (2009)
	<i>Lb. fermentum</i>	Azizpour <i>et al.</i> (2009)
	<i>E. faecium</i>	Gopalakannan and Arul (2011)
	<i>Streptococcus</i> sp.	Al-Harbi and Uddin (2008)
	<i>Lb. casei</i> , <i>Lb. paracasei</i> , <i>Lb. plantarum</i> and <i>Lb. sakei</i>	Andani <i>et al.</i> (2012)
	<i>Lc. raffinolactis</i>	Sugita and Mizuki (2012)
	<i>Lactobacillus</i> spp.	Jankauskiene (2002)
	<i>Bifidobacterium asteroides</i> and lactobacilli	Vlková <i>et al.</i> (2012)
	<i>Lc. garvieae</i> , <i>Lactococcus</i> sp., <i>Lactobacillus</i> sp., <i>Vagococcus</i> sp., <i>W.</i> <i>cibaria</i>	Kühlwein <i>et al.</i> (2013)
Koi carp (<i>Cyprinus carpio haematopterus</i>)	<i>Lc. raffinolactis</i>	Michel <i>et al.</i> (2007)
Gibel carp (<i>Carassius gibelio</i>)	<i>Lc. lactis</i> subsp. <i>lactis</i>	Zhang <i>et al.</i> (2013)
	<i>Lc. lactis</i> subsp. <i>lactis</i>	He <i>et al.</i> (2011a)
	<i>Lb. plantarum</i> , <i>Lc. raffinolactis</i> and <i>Lc.</i> <i>lactis</i>	Ghiasi (2011)
Deep bodied crucian carp (<i>Carassius cuvieri</i>)	<i>Lc. lactis</i> and <i>Lc. raffinolactis</i>	Hagi <i>et al.</i> (2004)
Goldfish (<i>Carassius auratus</i> L.)	<i>Str. parauberis</i> and <i>Lactococcus</i> spp.	de Paula Silva <i>et al.</i> (2011)
	<i>Bifidobacterium longum</i> and lactobacilli	Vlková <i>et al.</i> (2012)
Silver carp (<i>Hypophthalmichthys molitrix</i>)	<i>Lc. lactis</i> and <i>Lc. raffinolactis</i>	Hagi <i>et al.</i> (2004)
Grass carp (<i>Ctenopharyngodon idella</i>)	<i>Leuconostoc</i> , <i>Lactobacillus</i> and <i>Streptococcus</i>	Wu <i>et al.</i> (2012a)
Rohu (<i>Labeo rohita</i>)	<i>Pediococcus</i> spp. and <i>P. acidilactici</i>	Halami <i>et al.</i> (1999)
Zebrafish (<i>Danio rerio</i>)	<i>Lb. fermentum</i> , <i>Lc. lactis</i> subsp. <i>cremoris</i> , <i>Lc. lactis</i> subsp. <i>lactis</i> , <i>Lc. lactis</i> , <i>Lc.</i> <i>piscum</i> , <i>Lc. plantarum</i> , <i>Lc. raffinolactis</i> and <i>Leu. citreum</i>	Rawls <i>et al.</i> (2004)
	<i>Str. thermophilus</i> and <i>Lc. plantarum</i>	Gioacchini <i>et al.</i> (2012)

Genera abbreviations: *E.* = *Enterococcus*, *Lb.* = *Lactobacillus*, *Lc.* = *Lactococcus*, *Leu* = *Leuconostoc*, *P.* = *Pediococcus*, *Str.* = *Streptococcus*, *W.* = *Weissella*.

resistance and antibacterial activity against eight indicator strains (*A. salmonicida*, atypic *A. salmonicida*, *A. hydrophila*, *A. caviae*, *A. sobria*, *A. jandaei*, *A. enteropelogenes* and *E. faecalis*). The results revealed that all the potential probiotics grew over a wide temperature range even though they were originally isolated in different seasons, and their levels of cholic acid resistance and antibacterial activity were better than, or at least equal to, those of their corresponding type strains. In the study of Azizpour *et al.* (2009), the authors characterized LAB isolated from the intestines of various samples of common carp from commercial farms in West Azerbaijan, Iran. The authors reported that *Lb. fermentum* was the dominant LAB isolated from the intestines, and accounted for 10% of the isolates. Gopalakannan and Arul (2011) isolated LAB from the intestinal mucus of common carp and the most promising isolate, *E. faecium*, based on ability to inhibit *in vitro* growth of several pathogens, was further tested as a probiotic in an *in vivo* *A. hydrophila* challenge.

Recently, Vlková *et al.* (2012) reported *Bifidobacterium*, as well as lactobacilli, in the intestine of common carp. Presumptive *Bifidobacterium* counts of log 2.6 and 3.1 CFU g⁻¹ were observed in two of the 19 common carp tested. When compared with direct counts using FISH this population comprised <0.0012% of the total bacterial community. A representative isolate was identified as *Bifidobacterium asteroides* (99% similarity) by 16S rRNA sequence analysis.

6.12.2 *Carassius* spp.

Hagi *et al.* (2004) investigated the composition of intestinal LAB in deep-bodied crucian carp (*Carassius cuvieri*) in Lake Kasumigaura during different seasons. In deep-bodied crucian carp, as well as the common carp, LAB levels in the GI tract were higher during the summer (27°C) than in the winter (9.8°C). The proportion of LAB with respect to the total culturable populations represented ca. 10% in the summer and 33% during the winter. The authors reported that the predominant intestinal LAB of both carp species was *Lc. lactis* in the summer and *Lc. raffinolactis* in the winter.

De Paula Silva *et al.* (2011) reported that the gut bacteria of goldfish (*Carassius auratus* L.) contained *Streptococcus parauberis* and *Lactococcus* spp. and Vlková *et al.* (2012) reported *Bifidobacterium*, as well as lactobacilli, in the intestine of goldfish. Presumptive *Bifidobacterium* counts of log 2.18 CFU g⁻¹ were observed in one of the six goldfish tested. When compared with direct counts using FISH this population comprised <0.00015% of the total bacterial community. Representative isolates were identified as *Bifidobacterium longum* (99% similarity) by 16S rRNA sequence analyses.

Lc. lactis subsp. *lactis* was reported to be a component of the gut of gibel carp (*Carassius gibelio*) by He *et al.* (2011a). The study revealed that the bacterium was suppressed when the fish were fed Saccharoculture (a yeast based feed additive) in combination with flavomycin. Recently, Zhang *et al.* (2013) confirmed the presence of *Lc. lactis* subsp. *lactis* in the GI tract of gibel carp and observed that relative abundance (as a proportion of the whole bacterial community) was higher with respect to the allochthonous microbiota than to the autochthonous microbiota.

6.12.3 Other cyprinids

Although greater attention has been paid to assessing the gut microbiota of common carp and *Carassius* spp., some information is also present with respect to other species from the order

Cypriniformes (Table 6.2). Hagi *et al.* (2004) investigated the composition of intestinal LAB in silver carp (*Hypophthalmichthys molitrix*) in Lake Kasumigaura during different seasons. As was observed for the common carp and deep-bodied crucian carp, the LAB levels in the GI tract were higher during the summer (27 °C) than in the winter (9.8 °C). However, this difference was more subtle in the silver carp (LAB levels were log 5 CFU g⁻¹ during the summer and winter) compared to the common carp and deep-bodied crucian carp (LAB levels were log 6 and log 7 CFU g⁻¹ in the summer, respectively, and log 5 CFU g⁻¹ in the winter). The proportion of LAB with respect to the total culturable populations was less than 1% in silver carp at both sampling points. The authors reported that the predominant intestinal LAB of all carp species was *Lc. lactis* in the summer and *Lc. raffinolactis* in the winter. Ghiasi (2011) also reported the presence of *Lc. raffinolactis* and *Lc. lactis* as allochthonous intestinal LAB in silver carp as well as *Lb. plantarum*.

In an early study Halami *et al.* (1999) isolated and characterized *Pediococcus* sp. and *P. acidilactici* from the gut of rohu (*Labeo rohita*). These bacteria were tested for antibacterial production against several different pathogenic LAB, but they did not belong to the most promising LAB tested, which were isolated from fowl and mushroom.

In a recent study Wu *et al.* (2012a) used a next-generation sequencing approach to investigate the intestinal microbial community of grass carp (*Ctenopharyngodon idellus*). A complex microbial community was revealed and the community was rich in cellulose-degrading bacteria such as *Anoxybacillus*, *Leuconostoc*, *Clostridium*, *Actinomyces* and *Citrobacter* typical of herbivorous species. In addition, 1.61%–7.65% of the communities were identified as *Lactobacillus* spp. and 1.59% as *Streptococcus* spp.

The zebrafish (*Danio rerio*) model has been introduced in a wide variety of research fields including studies to elucidate the importance of the gut microbiota in relation to health and disease of vertebrates. *Lb. fermentum*, *Lc. lactis* subsp. *cremoris*, *Lc. lactis* subsp. *lactis*, *Lc. lactis*, *Lc. piscum*, *Lc. plantarum*, *Lc. raffinolactis*, *Leu. citreum* and *Str. thermophilus* have been identified as constituents of the gut microbiome of zebrafish (Rawls *et al.* 2004; Gioacchini *et al.* 2012). Further studies have reported that a wide range of LAB species, particularly lactobacilli, display favourable characteristics for zebrafish intestinal colonization and mucosal adhesion (Gioacchini *et al.* 2012; Zhou *et al.* 2012).

6.13 CHANNIDAE

The snakeheads are members of the freshwater perciform fish family Channidae. Allameh (2012) reported that *Ae. viridans*, *Lb. delbrueckii* subsp. *delbrueckii* and *E. faecalis* were common in the stomach and intestine of snakehead juveniles (*Channa striatus* Bloch 1793) while strains belonging to *Lb. fermentum* and *Leu. mesenteroides* subsp. *mesenteroides* were detected in the stomach and intestine of adult fish. Antagonistic activities tested against *A. hydrophila*, *Pseudomonas aeruginosa* and *Shewanella putrefaciens* indicated that *E. faecalis*, *Lb. fermentum* and *Leu. mesenteroides* subsp. *mesenteroides* displayed highest *in vitro* inhibition against the pathogenic bacteria (Allameh 2012; Allameh *et al.* 2012). More recently, Allameh *et al.* (2013) isolated *Lb. fermentum* from the stomach of snakeheads.

6.14 SILURIFORMES

A number of studies have reported LAB to be present in the GI tract of catfishes but few investigations have focused on determining the levels or their importance to the host. Perhaps the most informative study is that of Hagi *et al.* (2004) which investigated the composition of intestinal LAB in channel catfish (*Ictalurus punctatus*) during the summer and winter. LAB levels accounted for ca. 10% of the culturable populations and were composed of *Lc. lactis* (100% of 20 isolates) in the summer and *Lc. raffinolactis* (100% of 20 isolates) in winter. In other studies *Lb. casei* subsp. *paracasei* and *Lb. brevis* have been identified from the gut of African catfish (*Clarias gariepinus*) (Bucio *et al.* 2006), *Lactobacillus* spp. from wels catfish (*Silurus glanis*) (Bucio *et al.* 2006) and *Lc. lactis* from the Amur catfish (*Silurus asotus* L.) (Sugita *et al.* 2007). In the study of Sugita *et al.* (2007), 49 strains of *Lc. lactis* were isolated from the intestinal content of the Amur catfish. These LAB were tested for their antibacterial abilities to inhibit *in vitro* growth of five species of *Aeromonas*. In a later study (Sugita *et al.* 2009) the authors subsequently reported that *Lc. lactis* strain C7 showed higher antibacterial potential than the strains tested in the 2007.

Le Nguyen *et al.* (2008) isolated *Lb. plantarum* from the intestinal contents of the Pangasius (*Pangasius hypophthalmus*). In a study evaluating the microbial diversity of mucus and intestinal contents of yellow catfish (*Pelteobagrus fulvidraco*), Wu *et al.* (2010) reported two LAB, *Enterococcus saccharominimus* and *Lc. garvieae*, from a clone library constructed from the intestinal contents, but no LAB were detected in the mucus clone library and no cultivable LAB were isolated from intestinal contents or mucus. In a more recent study, Wu *et al.* (2012b) characterized the bacterial community in the stomach of yellow catfish. Of the 45 phylotypes reported from 65 positive clones from the gastric contents, two clones showed 94.8% similarity to *Lactobacillus aviaries*. In contrast to this result, no lactobacilli were reported from the cultured bacteria isolated from the stomach contents or from the clone library from stomach mucus.

6.15 CICHLIDAE

A number of studies have identified LAB from the GI tract of tilapia. *Streptococcus* spp. have been identified as components of the culturable aerobic intestinal microbiota of tilapia in a number of studies (Al-Harbi and Uddin 2003; 2004; 2005). Al-Harbi and Uddin (2003) reported *Streptococcus* spp. levels of 0.58% of the total culturable community in the intestine of hybrid tilapia (*Oreochromis niloticus* × *Oreochromis aureus*) but in their later studies it was apparent that seasonality impacted these levels. For example, *Streptococcus* spp. were absent in the intestine of hybrid tilapia during the summer but accounted for ca. 5% of the culturable population in the autumn and 3% in the winter (Al-Harbi and Uddin 2004). In a study evaluating the effect of florfenicol and *Saccharomyces cerevisiae* on the gut bacterial community of hybrid tilapia, *Lc. lactis* was detected in the intestine of all groups investigated, but the abundance was highest in the group fed sequential florfenicol (8 weeks) and *S. cerevisiae* (8 weeks) and lowest when the fish were fed *S. cerevisiae* (He *et al.* 2011b).

In Nile tilapia (*Oreochromis niloticus*) reared in brackish water *Streptococcus* spp. accounted for 12% of the culturable intestinal community (Al-Harbi and Uddin 2005). In a later study of Nile tilapia, Jatoba *et al.* (2008) isolated *Lb. plantarum* and *Lb. brevis* from

the intestine and these LAB were able to inhibit the growth of several pathogens *in vitro* and improved the immune status *in vivo* (Jatoba *et al.* 2011). Merrifield *et al.* (2010b) evaluated *Chlorogloeopsis* as a dietary supplement for red Nile tilapia (*Oreochromis niloticus*), and reported an uncultured *Lactobacillus* sp. in the posterior intestinal communities of the fish. *Weissella* spp. have also been identified by DGGE and sequence analyses as an indigenous member of the autochthonous and allochthonous gut microbiota of red Nile tilapia (Merrifield *et al.* 2011b; Ferguson and Merrifield unpublished data).

Vijayabaskar and Somasundaram (2008) isolated LAB from the digestive tract of Mozambique tilapia (*Oreochromis mossambicus*), but no further information was presented with regards to whether these bacteria were allochthonous, autochthonous or a mixture of both.

6.16 SERRANIDAE

It has been demonstrated that LAB species are members of the *normal* microbiota of grouper (Sun *et al.* 2009; Zhou *et al.* 2009; Yang *et al.* 2012). Sun *et al.* (2009) observed that three LAB species, *Lc. lactis* MM1, *Lb. casei* MM2 and *E. faecium* MM4, accounted for 0.1–0.7% of the total culturable bacterial community of the whole intestine of healthy juvenile orange-spotted grouper (*Epinephelus coioides*). The *Lc. lactis* MM1 and *E. faecium* MM4 strains exhibited antagonism against bacterial pathogens and showed good resistance to artificial gastric and intestinal fluids under *in vitro* conditions (Sun *et al.* 2010; Yang *et al.* 2010). A subsequent *in vivo* study showed that individual dietary administration of the two candidate probiotics at a dose of 10^8 CFU g⁻¹ for 60 days could significantly improve the feed utilization of juvenile orange-spotted grouper (Sun *et al.* 2012a). Interestingly, enhanced specific activities of digestive enzymes were observed in the probiotic groups, while the two probiotic strains did not secrete any digestive enzymes (protease, amylase and lipase) under *in vitro* conditions. Therefore, the authors speculated that the probiotics may induce the secretion of digestive enzymes and this might have led to improved digestive processes, enhancing the digestibility of feed and thereby the nutritive utilization of feed (Sun *et al.* 2012a). Also, dietary administration of *Lc. lactis* and *E. faecium* individually improved the general immune responses (Sun *et al.* 2012a), and elevated the autochthonous intestinal microbial diversity of juvenile *E. coioides* by stimulating some potentially beneficial and unidentified bacteria (Sun *et al.* 2012b; 2012c).

Using DGGE, a *Lc. lactis* subsp. *lactis* LMG-like bacterium was detected in the stomach and pyloric caeca of adult yellow grouper (*Epinephelus awoara*) (Zhou *et al.* 2009), while an *Enterococcus* sp.-like bacterium was detected in the MG and DI of orange-spotted grouper fed the probiotic *Bacillus clausii* for 60 days (Yang *et al.* 2012).

6.17 RACHYCENTRIDAE

Xing (2010) characterized a *P. pentosaceus* strain (#4012) isolated from the intestine of cobia (*Rachycentron canadum*), and the strain demonstrated *in vitro* inhibitory effects on pathogenic *Photobacterium damsela* subsp. *piscicida*, the causative agent of fish photobacteriosis (Liu *et al.* 2003). Dietary administration of *P. pentosaceus* 4012 for 2 weeks improved the growth rate of cobia, and significantly elevated the respiratory burst activity of peripheral blood

leukocytes. In addition, the strain significantly increased the survival rate of fish challenged with *P. damsela* subsp. *piscicida*.

6.18 MUGILIDAE

In a study evaluating culturable bacteria in the gut of flathead grey mullet (*Mugil cephalus*), Ghosh *et al.* (2011) tested 18 isolates for antibacterial activities against several fish, shrimp and human pathogens. Eleven LAB strains were identified by 16S rRNA gene sequencing: *Lb. brevis* (five isolates), *Lb. plantarum* (two isolates), *Lb. paralimentarius*, *Lb. mindensis*, *Lactobacillus* sp. and an uncultured lactobacilli. In a more recent study, Ghosh *et al.* (2014) revealed several lactobacilli – *Lb. brevis*, *Lb. casei*, *Lb. plantarum*, *Lactobacillus heilongensis* and *Lactobacillus kimchii* – in the GI tract of *M. cephalus*.

6.19 COASTAL FISH

Coastal fish inhabit the sea between the shoreline and the edge of the continental shelf. During the last decade a number of studies have investigated the intestinal microbial communities in Thai, Japanese and Argentine coastal fish (Shiina *et al.* 2006; Buntin *et al.* 2008; Itoi *et al.* 2008; Sequeiros *et al.* 2010; Sica *et al.* 2010; Tanaka *et al.* 2012). Readers with special interest in the family names of the fish species investigated are referred to the original studies.

Shiina *et al.* (2006) evaluated the allochthonous gut microbiota of *Takifugu niphobles* by creating clone libraries and reported that 16.3% and 5.8% of the isolates were related to *Lactococcus* and *Streptococcus*, respectively. Of the LAB identified, the authors reported *Lc. raffinolactis*, *Lc. lactis*, *Streptococcus australis* and *W. confusa*. Furthermore, one of the sequences deposited in the DDBJ/EMBL/GenBank databases, sequence AB194668, showed similarity to *Leuconostoc kimchii*/*Leu. citreum*/*Leuconostoc argentinum*. In a later study, Buntin *et al.* (2008) isolated 116 LAB strains from the gut of several fish species from Thailand. Three of these, two *P. pentosaceus* and one *E. faecium*, were further tested for antibacterial activities against several human pathogens. Itoi *et al.* (2008) isolated allochthonous gut microbiota from six coastal fish species. In total 668 bacteria were isolated, of which 227 isolates were presumptive LAB. Four of these were identified as *Lc. lactis* subsp. *lactis* but the remaining LAB were not identified. Sequeiros *et al.* (2010) isolated 53 LAB from the intestinal contents of a Patagonian fish, *Odontesthes platensis*. One of the LAB, strain TW34, was identified as belonging to the *Lc. lactis* clade and, based on its antimicrobial spectrum, antimicrobial activities against several fish pathogens and the stability of the bacteriocin activities, the authors suggested that *Lc. lactis* TW34 could provide an alternative for lactococcosis control and recommended challenge experiments with *O. platensis*. Sica *et al.* (2010) isolated 21 LAB from the gut contents of four coastal fish species from a mesotidal coastal plain estuary in Argentina. 16S rRNA gene sequencing of the isolates revealed that LAB isolated from *Ramnogaster arcuata* sampled in December were *E. hirae*, *Lb. paracasei* subsp. *tolerans* and *Weissella viridescens*, while fish sampled in May contained *Leu. citreum* and *W. viridescens*. Of the other fish species sampled in May, *Leu. mesenteroides* subsp. *mesenteroides* and *W. viridescens* were detected in the intestine of *Cynoscion guatucupa*, *W. viridescens* in the intestine of *Brevoortia aurea* and *Leu. mesenteroides* subsp. *mesenteroides* in the intestine of *Micropogonias furnieri*. In a

recent study, Tanaka *et al.* (2012) evaluated the bacterial diversity of the intestinal tracts of six Japanese coastal fish by constructing clone libraries, but the only LAB reported were isolates showing high similarity to *Streptococcus sanguinis* isolated from *Girella punctata*, and *S. sanguinis* was a major bacterium in the intestinal tract of the fish species (Haruo Sugita, personal communication 2012).

6.20 SHELLFISH

The use of indigenous bacterial species is paramount in probiotic applications from both legislative and ecological perspectives. It is therefore essential to consider the native bacterial species present in a given host species to aid decisions on suitable probiotic species. However, unlike finfish, there is a paucity of studies which have investigated the indigenous gut bacteria in shellfish species.

Despite this, LAB have been reported to be indigenous to shellfish species including shrimp and prawns (Cai *et al.* 1999; Lalitha and Surendran 2004; Kennedy *et al.* 2006; Vieira *et al.* 2007; 2008; Zhou *et al.* 2007; Kosin and Rakshit 2010; Vieira *et al.* 2010; Leyva-Madrigal *et al.* 2011; Liu *et al.* 2011; Kongnum and Hongpattarakere 2012), swimming crab (*Callinectes* and *Portunus* spp.) (Uaboi-Egbenni *et al.* 2010; Talpur *et al.* 2012), mud crab (*Scylla paramamosain*) (Li *et al.* 2012), lobster (Daniels *et al.* 2010; 2013; Meziti *et al.* 2010), scallop (Campa-Cordova *et al.* 2011) and abalone (Sarkono *et al.* 2010) (Table 6.3). In an early study by Cai *et al.* (1999) the presence of several LAB belonging to the *Lactococcus*, *Enterococcus* and *Pediococcus* genera were reported in the intestine of the giant freshwater shrimp (*Macrobrachium rosenbergii*). Three specific isolates were identified to species level as *Lc. garvieae*, *P. acidilactici* and *E. faecium* using 16S rRNA gene sequencing. Later, Lalitha and Surendran (2004) reported that *Enterococcus* spp. accounted for 8.3% of the identified gut bacteria in adult *M. rosenbergii*. Kennedy *et al.* (2006) investigated the microbiota associated with *M. rosenbergii* larvae, and among the bacteria isolated the authors identified a small proportion of lactobacilli, accounting for approximately 4.5% of the culturable microbiota. *Lactobacillus* were only present at the early life stages however, being absent in larval stages past day 4 of development, and this absence continued up to stage six of post larval development. More recently, Vieira *et al.* (2007) isolated two LAB from the digestive tract of juvenile *Litopenaeus vannamei* using culture-dependent agar plating techniques; one of these strains was later identified as *Lb. plantarum* by 16S rRNA gene sequencing (Vieira *et al.* 2008). It might be suggested from additional experiments that this LAB strain is transient in nature within the digestive tract of *L. vannamei*, as probiotic supplementation trials with *Lb. plantarum* showed that six days after changing from the probiotic enriched diet to a control diet, total LAB counts in the gut were low and not significantly different from control shrimps (Vieira *et al.* 2008). Moreover, this and a later study by Vieira *et al.* (2010) identified detectable levels of LAB, using culture-dependent methods, in the digestive tract of *L. vannamei*. Kosin and Rakshit (2010) identified autochthonous LAB from *L. vannamei* using selective media (for probiotic and thermo-tolerant bacteria). These LAB were identified as *Lb. plantarum* and *Leu. mesenteroides* subsp. *mesenteroides/dextranicum* by API 50 CHB and API 50 CHL. Leyva-Madrigal *et al.* (2011) isolated *P. pentosaceus* and *Streptococcus haemolyticus* from the intestine of *L. vannamei*. The LAB were tested for extracellular enzymatic activities and inhibitory activities and were used as probiotics to reduce WSSV and IHHNV in naturally infected whiteleg

Table 6.3 LAB identified from the GI tract of crustaceans and molluscs.

Species	Source	Isolation source	LAB identified	Reference
Giant freshwater prawn (<i>Macrobrachium rosenbergii</i>)	Natural	GI tract	<i>Lc. garvieae</i> , <i>P. acidilactici</i> and <i>E. faecium</i>	Cai <i>et al.</i> (1999)
	Aquaculture	Water, larva and <i>Artemia</i> (live feed)	<i>Lactobacillus</i> spp.	Kennedy <i>et al.</i> (2006)
Pacific white shrimp (<i>Litopenaeus vannamei</i>)	Aquaculture	Water, pond residues (soil and mud), inner shell surface and GI tract	18 isolates; two identified to spp. level <i>Lb. plantarum</i> and <i>Leu. mesenteroides</i>	Kosin and Rakshit (2010)
	Aquaculture	GI tract	14 LAB isolates	Viera <i>et al.</i> (2007)
	Aquaculture	GI tract	<i>Lactobacillus</i> spp. and <i>Str. faecalis</i>	Zhou <i>et al.</i> (2007)
	Aquaculture	GI tract	LAB	Viera <i>et al.</i> (2008)
	Aquaculture	GI tract	LAB	Viera <i>et al.</i> (2010)
	Aquaculture	GI tract	<i>E. faecalis</i>	Liu <i>et al.</i> (2011)
Chinese shrimp (<i>Fenneropenaeus chinensis</i>)	Natural	GI tract	Uncultured bacterium from phylum Firmicutes (not necessarily LAB)	Meziti <i>et al.</i> (2010)
Norway lobster (<i>Nephrops norvegicus</i>)	Aquaculture	GI tract	<i>W. confusa</i> and <i>W. cibaria</i>	Daniels <i>et al.</i> (2010)
European lobster (<i>Homarus gammarus</i>)				
Mud crab (<i>Scylla paramamosain</i>)	Aquaculture	GI tract	<i>W. confusa</i> and <i>W. cibaria</i>	Daniels <i>et al.</i> (2013)
	Aquaculture	GI tract	<i>Streptococcus mutans</i> (diseased), <i>Weissella fabaria</i> (farmed) and <i>Lactobacillales</i> bacterium 1247 (hatchery)	Li <i>et al.</i> (2012a)
Blue swimming crab (<i>Portunus pelagicus</i>)	Natural	GI tract	<i>Lb. plantarum</i> , <i>Lb. salivarius</i> , <i>Lb. rhamnosus</i> , <i>W. confusa</i> and <i>W. cibaria</i>	Talpur <i>et al.</i> (2012)
Swimming crab (<i>Callinectes</i> sp.)	Natural	GI tract	<i>Str. agalactiae</i>	Uaboi-Egbenni <i>et al.</i> (2010)
Abalone (<i>Haliotis asinina</i>)	Aquaculture	GI tract and environment	<i>Lactobacillus</i> spp. and <i>Lb. paracasei</i>	Sarkono <i>et al.</i> (2010)
Oyster (<i>Crassostrea corteziensis</i>)	Aquaculture	GI tract	LAB strain NS61	Campa-Córdova <i>et al.</i> (2011)
Giant lion's paw scallop (<i>Nodipecten subnodosus</i>)	Aquaculture	GI tract	LAB strain NS61	Nava-Hernández (2008)

Genera abbreviations: *E.* = *Enterococcus*, *Lb.* = *Lactobacillus*, *Lc.* = *Lactococcus*, *P.* = *Pediococcus*, *Str.* = *Streptococcus*, *W.* = *Weissella*.

shrimp. Based on their results, the authors concluded that the isolates of *P. pentosaceus* and *Str. haemolyticus* were good candidates to be used as feed additives in whiteleg shrimp culture. Liu *et al.* (2011) identified *E. faecalis* from the microbiota of the Chinese shrimp (*Fenneropenaeus chinensis*) by using DGGE. However, construction of a clone library (72 clones were sequenced) did not reveal members of the *Enterococcus* genera which indicates that it was not amongst the most abundant bacteria present. More recently a study of the GI tract of wild and cultured yellow shrimp (*Metapenaeus brevicornis*), banana shrimp (*Penaeus merguensis*) and white shrimp (*L. vannamei*) isolated 202 LAB strains, via culture-dependent techniques, of which 78–79% were coccoid LAB, potentially *Streptococcus*, *Lactococcus* and/or *Enterococcus* (Kongnum and Hongpattarakere 2012). In an earlier study analysing the GI tract of *L. vannamei*, low population levels of presumptive *Lactobacillus* spp. and *Str. faecalis* were present in the intestine of control and short-chain fructooligosaccharides (scFOS) fed shrimp (Zhou *et al.* 2007). Studies assessing the GI tract of post-larval European lobster *Homarus gammarus* identified the presence of *Weissella* spp. using PCR-DGGE (Daniels *et al.* 2010; 2013).

Recent studies have isolated LAB in two species of wild swimming crab: *Streptococcus agalactiae* has been identified in the GI tract of swimming crab *Callinectes* sp. (Uaboi-Egbenni *et al.* 2010) and *Lb. plantarum*, *Lb. salivarius*, *Lb. rhamnosus*, *W. confusa* and *W. cibaria* have been identified in the GI tract of blue swimming crab (*Portunus pelagicus*) (Talpur *et al.* 2012). A recent study of mud crabs also identified intestinal LAB, namely *Weissella fabaria* and *Streptococcus mutans* (Li *et al.* 2012). Talpur *et al.* (2012) tested the *Portunus pelagicus* derived isolates for antibacterial effects against *V. harveyi*, *Vibrio parahaemolyticus* and *Photobacterium piscicida* and the general finding was that the lactobacilli possessed the highest antibacterial activity. Nava-Hernández (2008) isolated LAB strain NS61 from the giant lion's paw scallop (*Nodipecten subnodosus*) which was later tested as a probiotic for the oyster *Crassostrea corteziensis* (Campa-Cordova *et al.* 2011). Sarkono *et al.* (2010) isolated four culturable presumptive LAB strains, identified as members of the genus *Lactobacillus*, from the fluid of the digestive tract of abalone (*Haliotis asinina*). However, identification was only based on cell shape, Gram reaction, catalase, motility and endospore formation. The LAB isolates were also tested for their ability to inhibit *in vitro* growth of *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus*, but as the inhibition zones were generally low no clear conclusion could be drawn.

It should be noted that certain species of LAB detected in shellfish could potentially be causative agents of disease. For example, Cheng and Chen (1998) identified an *Enterococcus*-like bacterium closely related to *E. seriolicida* which was detected in diseased *Macrobrachium rosenbergii* and caused 100% mortality when injected into healthy shrimp. Similar disease effects appear to be caused by another LAB, *Lc. lactis*, which in a separate study was isolated from diseased *M. rosenbergii* and identified as a disease causing bacteria which resulted in 100% mortality in 2 days (Wang *et al.* 2008). Shellfish may also harbour LAB which are known human pathogens. In a recent study Uaboi-Egbenni *et al.* (2010) isolated a well-known pathogen, *Str. agalactiae*, from natural populations. Thus any potential probiotics isolated from shellfish should be tested for their pathogenicity to humans. It is clear from the limited data available that more studies are required to assess the levels, composition and functionality of LAB in shellfish. Future studies should investigate the full microbial community and not merely seek to isolate a limited number of LAB isolates for potential use as probiotics.

6.21 SUMMARY

To our knowledge the first article dealing with LAB in fish was published by Kraus in 1961 and, until the end of the 1980s and the beginning of the 1990s, interest in LAB in fish and shellfish was generally low. However, during the last decade, our knowledge of the importance of gut microbes on host development and functions in terrestrial vertebrates and more recently in fish has grown. Concomitantly with the moratorium on the use of antibiotic growth promoters in feeds, interest in fish gut microbes and methods to fortify these communities has increased exponentially. Based on the information presented in this chapter a general conclusion can be drawn: intestinal LAB are not generally the dominant gut bacteria in fish and shellfish. However, their relevance and importance to the host likely outweigh their proportional abundance.

In this chapter we have presented an overview of LAB as members of the intestinal microbiota of fish and shellfish species. LAB isolated from the GI tract of fish and shellfish species belong to the following families and genera: *Aerococcaceae*, *Aerococcus*; *Carnobacteriaceae*, *Carnobacterium*; *Enterococcaceae*, *Enterococcus* and *Vagococcus*; *Lactobacillaceae*, *Lactobacillus* and *Pediococcus*; *Leuconostocaceae*, *Leuconostoc* and *Weissella*; and *Streptococcaceae*, *Lactococcus* and *Streptococcus*. Extremely low levels of bifidobacteria have been reported from a limited number of individuals of a small number of fish species. To our knowledge, *Tetrageonococcus* within the family *Enterococcaceae*, and *Oenococcus* within the family *Leuconostocaceae*, have not been reported.

Since conventional characterizations of microorganisms have depended on cultivation based techniques, our understanding of the intestinal microbiota composition has been restricted to those that can be cultured. It is acceptable and informative to continue to isolate and identify LAB in the GI tract of fish and shellfish in order to elucidate their drug resistance, enzyme production and ability to inhibit *in vitro* growth of potential fish pathogens. However, as the intestinal microbiota of fish and crustacea are complicated ecosystems modulated by internal and external factors, next-generation sequencing, metagenetic approaches and elucidation of LAB activity and functionality *in situ* are topics that merit extensive investigations.

These approaches will aid in the understanding of commensal and symbiotic LAB–host interactions in the GI tract, aid the identification of probiotic candidates, and extend our knowledge on the pathogenic LAB. Indeed indigenous pathogenic and opportunistic LAB such as *Streptococcus*, *Enterococcus*, *Lactobacillus*, *Carnobacterium* and *Lactococcus* have been reported in the GI tract of aquatic animals. In freshwater fish, Austin and Robertson (1993) reported that *Str. milleri* caused disease to carp. Later, Perera *et al.* (1994) suggested that *Str. iniae* was associated with mortality of hybrid tilapia, while Al-Harbi (1994) reported that *Streptococcus* sp. caused disease in hybrid tilapia in Saudi Arabia. Eldar *et al.* (1994) isolated and identified two new streptococcal species (*Streptococcus shiloi* and *Streptococcus difficile*) which caused meningoencephalitis in tilapia. Readers with special interest in pathogenic LAB are referred to Ringø and Gatesoupe (1998), Ringø (2004), Gatesoupe (2008), Loch *et al.* (2008; 2011; 2012), Austin and Austin (2012), Lauzon and Ringø (2012) and Leisner *et al.* (2012).

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7 Probiotics and Prebiotics: Concepts, Definitions and History

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ABSTRACT

Probiotics and prebiotics are considered as novel functional ingredients that can be applied to influence the host's microbiota, which in turn plays an important role in the nutrition, development, health and well-being of the host. Following the 2006 EU ban on antimicrobial growth promoters in agri-business production, the use of alternative prophylactic methods has been emphasized. This chapter presents the concepts, definitions and history of probiotics and prebiotics, summarizing the fields of their applications and corresponding benefits.

7.1 INTRODUCTION

Increasingly, technologies to enhance human and animal health through natural solutions are gaining in popularity. Recently, it has become clear that the intestinal microbiota plays an important role in the health and well-being of aquatic animals analogously to terrestrial animals. Microbial metabolic activities ensure the uptake of energy and the absorption of nutrients, promote gastric development, and stimulate epithelial cell proliferation and differentiation, while maintaining mucosal tolerance and providing protective functions against pathogens (Guarner and Malagelada 2003). As a result, there is great scientific interest in modulation of the intestinal microbiota in favour of beneficial bacteria through the use of novel functional food ingredients such as probiotics, prebiotics and in some cases the combination of probiotics with prebiotics (i.e. synbiotics).

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7.2 THE PROBIOTIC CONCEPT AND HISTORY

Probiotics, generally defined as live microorganisms with different beneficial characteristics, are increasingly becoming accepted as an alternative prophylactic measure for humans and animals, either to treat pathogen-related diseases or to be used in preventive treatments. The scientific definition of the term *probiotic* has been debated for several decades, and no legal definition exists which might reduce the credibility of probiotic products on the market. Probiotic research has mainly focused on the host's gastrointestinal (GI) tract, while applications to other organs or host surfaces have been investigated to some extent.

Microorganisms, especially lactic acid bacteria (LAB), have long been associated with food fermentation, one of the oldest methods of food preservation. Dating back to 3200 BC, the Egyptians produced fermented milk and dairy products during the Pharaonic period (Abou-Donia 2008). However, it was only a little over a century ago that understanding and interest in the possible health benefits related to these products came about.

At the turn of the twentieth century, Metchnikoff (1907) suggested that the longevity of Bulgarian peasants was linked to their heavy consumption of fermented milk. Following the research of Tissier (1900) on the importance of bifidobacteria in the gut of infants, Metchnikoff (1907) proposed the possibility of modulating the gut microbiota and replacing harmful microbes with beneficial microbes. He thought the beneficial effects of *feeding* fermented milk were due to implantation and proliferation of *Lactobacillus bulgaricus* in the intestine. At the end of the First World War, many children suffered from intestinal disorders. Encouraged by the work of Metchnikoff, Isaac Carasso created Danone in Barcelona in 1919 where yogurt production took place using ferments from the Pasteur Institute. The products were sold on prescription in pharmacies in Barcelona. Later, Rettger and Cheplin (1921) showed that the implantation of *Lactobacillus acidophilus* in the human intestine could help combat constipation, diarrhoea and other intestinal problems. This bacterium originated from the human intestine and such indigenous bacteria were believed to be more likely to produce the desired effect in the gut. Meanwhile, in 1917 Nissle (1918) isolated an *Escherichia coli* strain which successfully treated acute cases of infectious intestinal disease such as shigellosis and salmonellosis. This strain is an example of a non-LAB probiotic available today and sold as Mutaflor® (Ardeypharm GmbH, Germany).

Based on Metchnikoff's philosophy that modulation of the intestinal microbiota can contribute to health and longevity by hindering putrefactive bacteria, Dr Minoru Shirota aimed to isolate intestinal LAB that could help fight harmful bacteria in the body. In 1935, Shirota marketed the probiotic strain *Lactobacillus casei* Shirota in a milk drink called Yakult in Japan (Yakult Honsha Co., Ltd) which is now available worldwide. In the early 1950s a group of physicians, the Association for Microbial Therapy, led by Hans Kolb, Helmut Mommsen and Hans Peter Rusch, explored treatments with microbes to prevent the deleterious effects of antibiotics (Rusch 2002). In 1957, Gordon and colleagues indicated that a successful lactobacilli treatment required the strain to be non-pathogenic, of intestinal origin, and capable of establishing itself in the gut following its application as a large number of viable cells ($10^7 - 10^9$ g⁻¹). They investigated the bacteriological effects of a *Lb. acidophilus* product on the intestinal microbiota of patients receiving an oral antibiotic and observed a pronounced drop in staphylococcal numbers at a later stage of the probiotic-antibiotic treatment. In the 1960s, fermented milk products containing *Lb. acidophilus* were promoted by the dairy industry. In the following decades, other intestinal *Lactobacillus* species with beneficial properties were marketed:

Lactobacillus reuteri, *Lactobacillus helveticus* and *Lactobacillus rhamnosus*. Also, members of the genus *Bifidobacterium* (especially *Bifidobacterium lactis*) were added to probiotic products (Tannock 2003). *Lactobacillus* and *Bifidobacterium* became the main probiotic groups, but the probiotic potential of *Pediococcus*, *Lactococcus*, *Bacillus* and yeasts have also been demonstrated (Soccol *et al.* 2010). Today, a wide range of probiotic products is available for human consumption.

Interest in the probiotic concept also reached the field of animal husbandry in the 1960s. Dietary supplements became available for farm animals with the inclusion of LAB as health-promoting agents (Tannock 2003). The benefits claimed included improved weight gain, reduced mortality and feed costs, alleviation of stress and decreased incidence of infections (Parker 1974). The first study to apply the concept of competitive exclusion in poultry using caecal contents from adult chickens was conducted by Nurmi and Rantala (1973) who sought to protect chicks against *Salmonella* infection. In the 1980s, the most common probiotics for animal feeds belonged to three bacterial and one yeast genera: *Lactobacillus*, *Streptococcus*, *Bacillus* and *Saccharomyces* spp. At that time, at least 20 different probiotic preparations were available on the European market, with *Enterococcus faecium* (formerly *Streptococcus faecium*), *Lb. acidophilus* and *Bacillus cereus* var. *toyoi* being the most widely applied (Vila *et al.* 2010). In livestock, probiotics are mainly used during difficult physiological and technological periods to treat various digestive disorders. Fuller (1999) reviewed the use of probiotic products in animal husbandry and concluded that several claims were mentioned, but not always supported by good scientific data. Similarly, the outcome of basic studies on the effect of probiotic bacteria in health maintenance of farm animals, such as swine, poultry, cattle and sheep, is summarized by Corcionivoschi *et al.* (2010). They concluded that the lack of clinical trials impedes the creation of specific recommendations on the application of probiotics to treat various digestive disorders in farm animals. A review on the role of probiotics in the poultry industry conducted by Kabir (2009) indicates that probiotics could be applied as nutritional tools in poultry feeds for promotion of growth and meat quality, modulation of intestinal microbiota and inhibition of pathogens, as well as immunomodulation. Over the last decade, the use of probiotics in animal agriculture has increased, but their commercial success in poultry production has been limited (Flint and Garner 2009). Following the 2006 EU ban on antimicrobial growth promoters, to reduce the use of antibiotics in agri-business productions, new opportunities for the development of probiotic products have arisen.

Research on the application of probiotic microorganisms in aquaculture started over two decades ago (for review see Gatesoupe 1999). The main reason for this was the need for alternative prophylactic methods to remedy the survival problems encountered at different stages of intensive rearing. The success and availability of human and livestock probiotics have influenced the selection of microorganisms tested in aquatic rearing systems, particularly the early research in this field. These solutions may prove safe for human consumption, but their efficiency has been variable in aquatic animals (Azad and Al-Marzouk 2008). This has led to the search for tailor-made solutions from the autochthonous microbiota of aquatic animals. Given the differences in natural GI microbiota of aquatic and terrestrial animals, the main probiotics researched and studied for aquaculture practices include a wide range of microorganisms, such as *Aeromonas*, *Alteromonas*, *Enterobacter*, *Flavobacterium*, *Pseudomonas*, *Pseudoalteromonas*, *Phaeobacter*, *Roseobacter*, *Shewanella*, *Vibrio*, *Arthrobacter*, *Bacillus*, *Clostridium*, *Micrococcus*, LAB, *Debaryomyces* and *Saccharomyces* species

(Gatesoupe 1999; Gram and Ringø 2005; Lauzon 2010; Merrifield *et al.* 2010; Nayak 2010; Prado *et al.* 2010; Dimitroglou *et al.* 2011). The application of probiotics in aquaculture has been related to several beneficial effects, namely modulation of the intestinal microbiota and immune system, as well as enhanced survival, development, nutrition and disease resistance. However, further research is required to establish their exact modes of action (Tinh *et al.* 2008; Nayak 2010).

7.2.1 Etymology of ‘probiotic’ and its definitions

The term *probiotic* is composed of the preposition *pro*, meaning ‘for’ in Latin or ‘in front of/before’ in Greek, and *biotic*, a Greek adjective relating to the noun *bios* meaning ‘life’. While the overall meaning implies that it favours life, ‘for life’, it could also suggest that a probiotic is a compound that becomes activated *in situ* to support life and enhance vitality. This etymology of the term *probiotic* is reflected by the different definitions proposed through the years, which were based on the understanding of mechanisms and sites of action, delivery forms and methods as well as the hosts treated.

To our knowledge, the term *probiotic(s)* was first mentioned by Kollath (1953), defined as organic and inorganic supplements required to restore the health of malnourished patients. These supplements were considered common in vegetable food as vitamins, aromatic substances, enzymes or possibly other substances connected with vital metabolic processes. The latter included bacteria and their constituents delivered via vegetables (Rusch 2002). This is perhaps the first allusion made to the indirect use of microorganisms as probiotics. Vergin (1954) then proposed that the use of a probiotic diet, including fermentation products, could contribute to the microbial balance of the body otherwise disturbed following an antibiotic treatment. Kolb (1955) supported this suggestion which in some way approaches the definition accepted today. Later, Lilly and Stillwell (1965) defined probiotics as growth-promoting factors produced by microorganisms.

After the denotation of action towards vital processes, microbial balance and growth stimulation of the host (or its indigenous gut microorganisms), the potential of probiotics to modulate the host immune response was proposed by Fujii and Cook (1973), who defined probiotics as ‘compounds that build resistance to infection in the host but do not inhibit the growth of microorganisms *in vitro*’. So at that time, probiotics were considered as compounds and/or microorganisms with different functions contributing to health restoration, maintenance or enhancement through routes not related to microbial antagonism or competition. In 1974, Parker refined the definition of probiotics as acting towards the digestive tract: ‘organisms and substances which contribute to intestinal microbial balance’. This may be considered as the first direct mention of probiotics in the context of gut health, though Vergin (1954) may have suggested the action of the probiotic diet towards the intestinal microbiota in describing ‘the microbial balance of the body’. Fuller (1989) also focused on the intestinal microbial balance in his definition, but he further emphasized the notion of the viability of probiotics and their health enhancing effect in animals with the definition of ‘live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance’. The imprecision of Parker’s (1974) definition would otherwise have included antibiotics.

Later, the definition was broadened by Havenaar and Huis in’t Veld (1992) with respect to host and habitat of the microbial flora: ‘viable mono- or mixed culture of microorganisms, which applied to animal or man, beneficially affects the host by improving the properties of the indigenous microbiota’. This allowed for the effects of probiotics on other organs or

compartments of the host. It also opened the way for different administration methods. Indeed, the fact that there are other compartments in man and animals colonized by symbiotic microorganisms, such as the oral cavity, skin, vagina or fish gills, had been so far neglected. This is in line with Gatesoupe (1999) who emphasized that in aquaculture, a probiotic treatment through rearing water could also be of importance since the overall microbiota of fish and shellfish depends on the external environment. Verschuere *et al.* (2000) presented a similar but extended definition as follows: 'live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment'.

The idea that probiotics may be applied via the rearing water further complicates the definition process because it impacts upon closely related terms such as bioaugmentation, bioremediation and biocontrol. With respect to animals reared in aquatic environments, it has been suggested that microbes that are antagonistic to pathogens, but are not found to populate the host's GI tract, should be termed biocontrol agents (Maeda *et al.* 1997; Moriarty 1998). Aquatic microbial applications which improve water quality by the breakdown of waste or pollutants have been termed bioaugmentation or bioremediation (Moriarty 1997; 1998; Gatesoupe 1999). Given the large variations in defining criteria for aquatic probiotic applications, and the overlap with related concepts, Merrifield *et al.* (2010) proposed a more inclusive and broader definition for a probiotic intended for use with aquatic animals: 'any microbial cell provided via the diet or rearing water that benefits the host fish, fish farmer or fish consumer, which is achieved, in part at least, by improving the microbial balance of the fish'. In this context direct benefits to the host were considered as immunomodulation, improved disease resistance, reduced stress response and improved GI morphology, among others, and benefits to the fish farmer or fish consumer as improved fish appetite, growth performance, feed utilization, improvements in carcass and flesh quality, and reduced malformations.

In 1996, both Salminen and Schaafsma generalized the definition by not considering the influence of the indigenous microbiota on the health effects observed. Rather, they attributed the beneficial effects to the probiotics themselves: 'a live microbial culture or cultured dairy product which beneficially influences the health and nutrition of the host' (Salminen 1996). Schaafsma (1996) was also the first to mention the importance of the probiotic dose required to achieve enhanced effects: 'living microorganisms which upon ingestion in certain numbers exert health effects beyond inherent basic nutrition'. Salminen *et al.* (1999) introduced the idea that viability of probiotics was not a prerequisite for functionality, that is, 'microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host'. However, this implies that bacterial metabolites and bacteria-derived immunostimulants may then be considered as probiotics. This was based on recent findings where the use of inactivated cells led to positive health effects (Ouweland and Salminen 1998). In fact, this may be considered advantageous with respect to regulatory issues since non-viable probiotics and microbial cell wall components are less likely to cause safety concerns than their live counterparts. Despite these findings, a group of experts adopted the following definition in an FAO/WHO report from 2002: 'Live microorganisms which when administered in adequate amounts confer a health benefit on the host'. In 1988, the US Food and Drug Administration (FDA) defined live microbes used in animal feeds as 'direct-fed microbials'. Those intended for therapeutic effect in humans, that is, used as drugs, have been classified as 'live biotherapeutics' by the FDA's Center for Biologics Evaluation and Research, and the

Office of Vaccines Research and Review (Vaillancourt 2005). They may include bacteria or yeasts to be used in disease prevention or treatment. In parallel, the International Study Group on New Antimicrobial Strategies (ISGNAS) has proposed the distinction between medical, pharmaceutical and alimentary probiotics (Rusch 2002).

As many people now refer to all the previously discussed microbial applications as probiotic treatments (*sensu lato*), it is important to be aware that the term may be used quite liberally.

7.2.2 Probiotic selection criteria

Previous researchers have published varied opinions on what are considered to be important characteristics for the selection of probionts for applications in aquaculture (e.g. Spanggaard *et al.* 2001; Gram and Ringø 2005; Balcázar *et al.* 2006; Vine *et al.* 2006; Farzanfar 2006; Gómez and Balcázar 2008). Merrifield *et al.* (2010) collated such characteristics and extended them to produce the following comprehensive list of criteria (where E indicates an essential criterion and F indicates a favourable criterion). The probiont:

- must not be pathogenic, not only with regards to the host species but also with regards to aquatic animals in general and human consumers (E)
- must be free of plasmid-encoded antibiotic resistance genes (E)
- must be resistant to bile salts and low pH (E)
- should be able to adhere to and/or grow well within intestinal mucus (F)
- should be able to colonize the intestinal epithelial surface (F)
- should be recognized as safe for use as a feed additive (F)
- should display advantageous growth characteristics (e.g. a short lag period, a short doubling time and/or growth at host rearing temperatures) (F)
- should exhibit antagonistic properties towards one or more key pathogens (F)
- should produce relevant extracellular digestive enzymes (e.g. chitinase if chitin-rich ingredients are to be incorporated into the diet, or cellulase if the diet is rich in plant ingredients) and/or vitamins (F)
- should be indigenous to the host or the rearing environment (F)
- should remain viable under normal storage conditions and be robust enough to survive industrial processes (F).

From a practical and commercial perspective, it is clearly recognized that bacterial species from the US FDA's Generally Recognized as Safe (GRAS) list and/or the EFSA's Qualified Presumption of Safety (QPS) list will be favourable with respect to commercialization of probiotic products, as further discussed in *Chapter 12*. It is clear that finding a specific microbe that has all, or even most, of these characteristics is highly unlikely, and therefore a number of researchers, and commercial products, have embraced the simultaneous application of a number of microbial species/strains in 'multi-species' preparations in order to extend the number of beneficial characteristics that can be achieved with the application.

7.3 THE PREBIOTIC CONCEPT AND DEFINITION

The use of probiotics in many cases is difficult in the feed production industry because of the low viability of the bacteria after pelleting, during storage, as well as problems with

feed handling and preparation. Additionally, there is a possibility of environmental issues as probiotics enter into the environment. As an alternative, prebiotics have been assessed in an attempt to overcome issues associated with probiotic applications. Instead of introducing probiotic bacteria, the aim of prebiotics is to stimulate selected beneficial indigenous microbiota populations. A prebiotic was first defined as 'a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or the activity of limited number of bacteria in the colon, and thus improves host health' (Gibson and Roberfroid 1995). In order for a food ingredient to be classified as a prebiotic, the authors suggested that it should (1) be neither hydrolysed nor absorbed in the upper part of the GI tract, (2) be a selective substrate for one or a limited number of beneficial bacteria commensal to the colon, which are stimulated to grow and/or are metabolically activated, (3) consequently, be able to alter the colonic flora in favour of a healthier composition, and (4) induce luminal or systemic effects that are beneficial to host health.

The initial developmental research in prebiotics dates back to the end of the 1970s in Japan where several carbohydrates, especially fructooligosaccharides (FOS), were selectively fermented by bifidobacteria and had the capacity, upon feeding, to stimulate their growth in mammals (Yazawa *et al.* 1978). Prebiotics mainly consist of oligosaccharides which have proved to be able to promote beneficial bacterial growth within the GI tract (Gibson *et al.* 2003). Prebiotic oligosaccharides are able to provide the necessary energy to selective species of bacteria which are responsible for the production of lactic, acetic and other short-chain organic acids. Such oligosaccharides are mainly mannan oligosaccharides (MOS), FOS (including short-chain fructooligosaccharides, scFOS) and *trans*-galactooligosaccharides (TOS, including galactooligosaccharides, GOS). Inulin is a fructan polysaccharide which also has documented probiotic qualities. Not all of these glycans have been tested in aquaculture at this time.

In a more recent review for human intestinal microbiota, the prebiotic definition was updated. Gibson *et al.* (2004) suggested that a prebiotic has to (1) resist gastric acidity, hydrolysis by (mammalian) enzymes and GI absorption, (2) be fermented by the intestinal microbiota, and (3) stimulate selectively the growth and/or activity of intestinal bacteria associated with health and well-being. According to Gibson *et al.* (2004), each one of these criteria is important but the third criterion, concerning the selective stimulation of growth and/or activity of beneficial bacteria, is the most important and the most difficult to achieve. This is especially true because this step requires anaerobic sampling, and reliable quantitative microbial analysis needs to be undertaken for a wide range of bacterial species (i.e. total aerobic and anaerobic bacteria as well as total counts of Bifidobacteria, Enterobacteria, *Lactobacillus* etc.). For this purpose it is suggested that advanced molecular techniques, such as metagenomics and qRT-PCR, should be used. Additionally, Gibson *et al.* (2004) evaluated eight possible prebiotic candidates: inulin, TOS, lactulose, isomaltooligosaccharides, lactosucrose, xylooligosaccharides, soybean oligosaccharides and GOS. According to their criteria only three of these glycans could be classified as prebiotics: inulin, TOS and lactulose. However, in a more recent review, Roberfroid (2007) stated that only two fulfil the criteria for prebiotic classification: inulin and TOS. Therefore the term *prebiotic*, in this chapter as is the case more broadly across the scientific community, is generally used to describe glycans that potentially meet the suggested criteria, rather than those that have been definitively proven to meet the criteria.

In endothermic animals, prebiotics have a long history, but in aquaculture the first prebiotic study was reported by Hanley *et al.* (1995). Since then numerous papers have been published and the most common 'prebiotics' used in fish include inulin, FOS,

scFOS, MOS, GOS, xylooligosaccharides (XOS), arabinoxylooligosaccharides (AXOS), isomaltoligosaccharides (IMO), GroBiotic®-A, Previda™ (containing galacto-glucmannans from hemicellulose extract and a combination of oligosaccharides) and Chinese medicines (for review, see Burr *et al.* 2005; Merrifield *et al.* 2010; Ringø *et al.* 2010; Dimitroglou *et al.* 2011). However, according to the criteria for prebiotic classification given by Gibson *et al.* (2004), Gibson and Roberfroid (1995) and Roberfroid (2007), the evidence for the prebiotic status of these products for fish is not sufficient and strictly speaking they should not be classified as prebiotics. These criteria and subsequent prebiotic classification were based on human nutrition and applications but the use, definition and criteria of prebiotics for aquatic animals may be more complicated. For example, the anatomic structure of the GI tract in fish displays large variation (refer to *Chapter 1*). There are fish species with and without a discernible stomach, the number of pyloric caeca can vary from nil to 1000, the intestine can be short or up to 20 times the body length, and some species have a hindgut (fermentation) chamber. Furthermore, aquatic animals do not have a well-defined colon.

Based on the prebiotic studies carried out using aquatic animals we suggest the following criteria for prebiotic classification: (1) resistance to hydrolysis by the host's enzymes and GI absorption, (2) improvement in intestinal microbial balance, and (3) conferring host benefits (such as improved disease resistance, non-specific immunity, gut morphology, growth, survival and/or nutrient digestibility). However, one should bear in mind that not all 'prebiotics' have fulfilled these criteria.

7.3.1 Prebiotics: biochemistry and host benefits

Most non-digestible oligosaccharides (NDOs) are composed of 3–10 sugar moieties with glycosidic bonds in the beta-configuration, thus rendering them resistant to hydrolysis of the endogenous enzymes of animals including fish which only recognize the alpha glycosidic linkage typical in starch.

Prebiotic oligosaccharides are able to provide the necessary energy to selective species of bacteria which are responsible for the production of short-chain organic acids. Indeed, metabolic cross-feeding is a process whereby metabolites from one bacterial species are the energy source for other bacteria with a resulting production of short-chain fatty acids (SCFAs), primarily lactic, acetate, propionate and butyrate. The functional benefits of prebiotics may induce SCFA production leading to modulation of blood lipid, GI/systemic immunomodulation, energy sources resulting in intestinal cell proliferation, and improved intestinal barrier function (Tungland and Meyer 2006). Reduction in pH aids mineral absorption and general nutritional support. Their synergistic promotion of commensal and symbiotic bacteria in turn provides competitive exclusion of pathogens, indirectly enhancing pathogen resistance, reducing toxic microbial metabolites and suppressing intestinal inflammation (Pedron and Sansonetti 2008). These SCFAs are very important in the growth and physiology of intestinal tissue and systemic metabolism in humans and animals, accounting for a large proportion of the energy requirements of colonocytes. Little information is available for fish, but it has been shown that fish enterocytes can absorb SCFAs (Mountfort *et al.* 2002). Acetate is a primary fuel for skeletal muscle, heart and brain. In humans, the crypt cells of the absorptive epithelium are stimulated by propionate. It has also been reported that it can reduce hepatic glucose output and modulate cholesterol biosynthesis. Butyrate is the most common short-chain fatty acid produced by intestinal microbiota and accounts for most of the oxygen requirements for the gut microorganisms in fish. The fatty acid may up-regulate glutathione S-transferase and

catalase expression in the distal gut. The latter are important enzymes of the primary antioxidant enzyme system of the animal and help prevent oxidative stress and free radical generation. Butyrate is also closely linked with the osmoregulation processes of the gut and the absorption of sodium (Hamer *et al.* 2008).

7.3.2 *Trans*-galactooligosaccharides (TOS) and galactooligosaccharides (GOS)

TOS are produced from lactose by enzymatic transgalactosylation. The oligomers are linear and consist of lactose and several galactose molecules with β -1,6 or β -1,4 linkages; they are not hydrolysed by human small-intestinal β -galactosidase and will pass undigested into the colon (Burvall *et al.* 1980). The effect of TOS on gut microbiota is conflicting. Leforestier *et al.* (2007) reported that TOS modified the gut microbiota in mice, while Alles *et al.* (1999) reported that TOS had no effect on human gut microbiota even though TOS were completely fermented in the colon. GOS consist of 2–20 molecules of galactose and glucose (Figure 7.1) and can be produced through enzymatic treatments of lactose by enzymatic reactions using β -galactosidases from *Lb. reuteri*, *Aspergillus oryzae* or *Kluyveromyces lactis* (Yang and Silva 1995; Patel and Goyal 2011). The compounds are viscous with good moisture retention; they are a third of the sweetness of sucrose and are stable at high temperatures and in acidic environments (Macfarlane *et al.* 2008). GOS have been extensively tested in endothermic animals (Sako *et al.* 1999; Vos *et al.* 2007; Macfarlane *et al.* 2008; Roberfroid *et al.* 2010). Knowledge about the use of GOS as a food ingredient has shown that they can enhance health-related physiological activities (production of SCFAs, energy transduction in colonocytes, growth and cellular differentiation of colonic epithelial cells, lipid and carbohydrate metabolism) and contribute to a significant increase in faecal LAB and bifidobacteria. These activities can provide protection against enteric pathogens, reduce the numbers of potentially pathogenic bacteria (*E. coli* and *Campylobacter*), facilitate the normal function of the gut, improve mineral bioavailability including magnesium, iron or zinc, and decrease blood lipid content (Newburg 1997; van den Broek *et al.* 2008; Macfarlane *et al.* 2008; Roberfroid *et al.* 2010). In human studies GOS, in combination with FOS, have been extensively investigated (Roberfroid *et al.* 2010).

7.3.3 Mannan oligosaccharides (MOS)

According to Spring (2003), the initial interest in using MOS (Figure 7.2) as feed additives for animal nutrition was adapted from studies at the end of the 1980s by Oyoyo *et al.* (1989a; 1989b). These studies evaluated the ability of mannose to inhibit the adherence/colonization of microbial pathogens, such as *Salmonella* Typhimurium, to GI epithelial cells of broiler chickens. Many studies in terrestrial animals have shown that MOS are able to reduce the

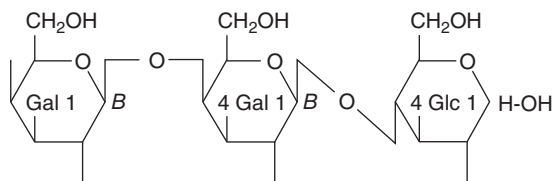


Fig. 7.1 Chemical structure of galactooligosaccharides.

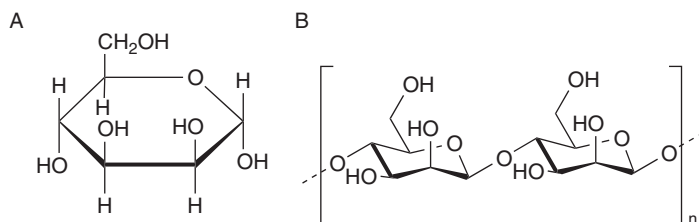


Fig. 7.2 Chemical structure of (A) mannose and (B) mannan oligosaccharides.

bacterial load and pathogenic bacteria of the intestine (of chickens, Fernandez *et al.* 2002; broiler chickens, Yang *et al.* 2008; dogs, Grieshop *et al.* 2004; pigs, Castillo *et al.* 2008). An essential step in the bacterial infection process is attachment of pathogens to epithelial cells (Swanson *et al.* 2002). Carbohydrate-binding proteins such as lectins are present on the exterior of cells and are associated with the antigen recognition and fimbrial adhesins (binding) of bacteria, especially of Gram-negatives (Engering *et al.* 1997). Lectins bind to the epithelial cells of the gut by attaching to oligosaccharide components of glycoconjugate receptors. Type-1 fimbrial adhesins, which are common on numerous potentially pathogenic bacterial species such as members of the Enterobacteriaceae, are specific for mannan residues (Oyofe *et al.* 1989a; Spring *et al.* 2000; Newman 2006). It is suggested that MOS are able to interact with these receptors by acting as a receptor analogue for type-1 fimbriae and thus preventing bacterial colonization of the GI tract (Oyofe *et al.* 1989b; Spring 2003; Newman 2006). Essentially MOS binds to type-1 fimbrial adhesins of potentially pathogenic bacteria (Enterobacteriaceae) and prevents them colonizing the GI tract. Furthermore, MOS supplementation influences immune responses such as increased antibody response against viral infection in broilers (Shashidhara and Devegowda 2003), decreased peripheral lymphocyte concentrations in dogs (Grieshop *et al.* 2004) and increased serum IgG and IgM levels and decreased peripheral blood T lymphocyte percentage in turkeys (Çetin *et al.* 2005). Later studies with MOS supplementation in terrestrial animals have shown that MOS work in several different ways within the digestive tract. MOS can improve gut function and health by increasing the villi height, uniformity and integrity (in broiler chickens, Iji *et al.* 2001; Hooze 2004; pigs, Castillo *et al.* 2008). As a result, the feed within the digestive tract may be more efficiently digested leading to a superior nutrient absorption (Spais *et al.* 2003; Sims *et al.* 2004). The effect of MOS supplementation on the intestinal morphology may explain the improved growth performance in terrestrial animals such as poultry (Shane 2001; Fritts and Waldroup 2003; Yang *et al.* 2008) and pigs (Spring and Privulesku 1998; Miguel *et al.* 2004).

7.3.4 Inulin, oligofructose and fructooligosaccharides

Inulin (IN; Figure 7.3A), oligofructose (OF) and fructooligosaccharides (FOS) belong to a larger class called IN-type fructans, which refers to all linear fructans containing β-2,1 fructosyl-fructose glycosidic bonds (Kelly 2009). These molecules differ in chain length and methods of extraction or synthesis. The nomenclature is inconsistent in the literature (Klosterbuer *et al.* 2011). In general, inulin refers to molecules with an average degree of polymerization of 2 to 60, while OF and FOS refer to shorter-chain molecules. Inulin, OF

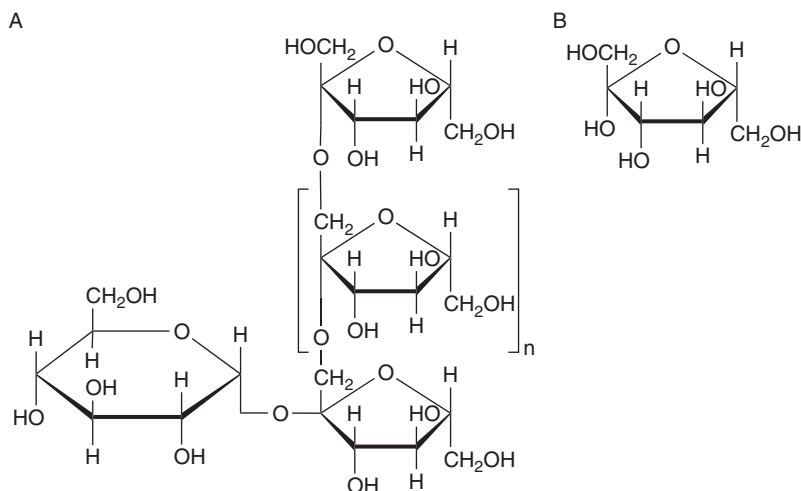


Fig. 7.3 Chemical structure of (A) inulin ($n = 2-65$) and (B) fructose.

and FOS are non-viscous, soluble fibres obtained from a number of foods, or FOS can be produced synthetically by adding fructose (Figure 7.3B) units to a sucrose molecule via β -1,2 linkages (Kelly 2009).

IN is present as a storage carbohydrate in a large number of edible grains, fruits and vegetables such as wheat, onions, leeks, garlic, asparagus, artichokes and bananas (van Loo *et al.* 1995; Ritsema and Smeekens 2003). IN-type fructans are widely researched in human and animal studies with reported beneficial impacts on the *Bifidobacterium* and *Lactobacillus* populations. IN-type fructans can also be utilized by some *Eubacterium* and *Roseburia* strains which generate high levels of SCFA and an increased molar ratio of butyric acid to total SCFA compared with other fibres (Pryde *et al.* 2002; Tremaroli and Bäckhed 2012). Although IN-type fructans are not natural fibres in fish diets, they may have interesting applications in aquaculture to stimulate colonization of the ‘good’ gut bacteria, suppress pathogens and enhance immune response.

Chemically, oligofructose (OF) and IN can be defined as polydisperse fructans. OF is always present in IN, a blend of fructose oligomers and polymers, and differs from IN in regard to its degree of polymerization. It can be produced from IN by controlled partial enzymatic hydrolysis from chicory roots, for example (Flickinger *et al.* 2003). Dietary OF supplementation can have various functional properties such as the improvement of mineral absorption, impact on bowel habit, interaction with lipid metabolism and preventive effect against colon cancer (van Loo *et al.* 1999).

FOS are compounds of vegetable origin and are reported in varying concentrations in many foods such as onions, asparagus, artichokes, garlic, wheat, bananas, tomatoes and honey (Roberfroid and Delzenne 1998). Roberfroid *et al.* (2010) presented a structural characteristic of FOS and described the degree of polymerization as between 2 and 8. FOS have been extensively tested in endothermic animals and readers with special interest are referred to the comprehensive review of Macfarlane *et al.* (2008).

7.4 SYNBIOTICS

Synbiotics are nutritional supplements that contain both a prebiotic and a probiotic that work together and beneficially affect the host by improving the survival and implantation of live microbial dietary supplements in the GI tract (Gibson and Roberfroid 1995). The consensus is that prebiotics are able to prepare the ground for probiotics to become established by modulating the endogenous microbial community and/or by directly providing a competitive advantage for the probiotic being supplied, if it is able to ferment the prebiotic. This has led to a new avenue of research which is becoming well documented in terrestrial animals, but very little information is available for aquatic species.

7.5 SUMMARY

In terms of definition, both probiotics and prebiotics are viewed as feed additives or dietary supplements providing scope for more robust nutrition without the need for antibiotic-type growth promoters, which have been banned from use within the EU since January 2006. This classification has been extended to include various unicellular algae and yeasts as well as phytobiotic chemicals and natural agents from exotic sources and products from marine biotechnology, especially from marine macroalgae such as seaweeds (Courtois 2009). Additionally, both probiotics and prebiotics are considered new ingredients in fish feed manufacturing. Their use is extended far beyond finfish, and they have been used in experimental and/or commercial diets for crustacea, molluscs, echinoderms and live feed enrichment. Hence, the aquaculture industry has great expectations from the use of such additives to produce superior quality products for human consumption.

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8 Probiotic Modulation of the Gut Microbiota of Fish

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ABSTRACT

A growing body of literature is available on the effects of probiotics on fish growth performance, digestive function, immunity, disease resistance, fecundity, oxidative stress and deformities but, perhaps surprisingly, many of these studies have not investigated the effect of probiotic applications on the gastrointestinal (GI) microbiota. Despite the debate on the precise definition of a 'probiotic' for aquaculture applications, the different definitions are consistent in that host benefits are driven, in part at least, by modulation of the host microbiota. Too often fish probiotic studies either lack a microbial investigation, or conduct the minimum assessment: enumeration of probiotic levels. Given the complexity of the gut microbiome, and its importance to the host, it is not sufficient to determine only the probiont levels and to attribute host benefits solely to the direct presence of the probiotic, which is usually only present as a minor component of the total microbial population. Changes in the indigenous populations must be examined as these potential changes are also factors involved in driving host benefits. This chapter summarizes the findings of studies which have addressed this topic and discusses the data to support the claims of probiotic 'colonization'. From the literature it is clear that a greater emphasis on understanding the effects of probiotics on the complex microecology of the GI tract of fish is essential, and future studies must incorporate quantitative techniques to determine microbial abundance as well as elucidating microbial functionality and activity.

8.1 INTRODUCTION

Despite the large variations in the definitions of the term 'probiotic' (as discussed elsewhere: Merrifield *et al.* 2010a; *Chapter 7*), the one consistency among the definitions is that the host benefits are said to be driven, in part at least, by modulations in the microbiota of the host.

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Despite this, however, historically much of the research concerning probiotic applications in aquatic animals has focused on the effects on the host at the system/whole-body level (e.g. growth performance, immune status and disease resistance) and unfortunately the effect on the gut microbiota has seldom been assessed. For example, of the 79 probiotic investigations discussed in the recent reviews on probiotic applications in salmonids (Merrifield *et al.* 2010a) and Mediterranean fish species (Dimitroglou *et al.* 2011), only 35 investigations (or 44%) assessed the impact on the gut microbiota. Since the publication of these reviews, with a greater appreciation and understanding of the importance of the gut microbes to the host, more emphasis has been placed on understanding the effects of the probiotics on the gut microbiota of aquatic animals.

In general studies which have incorporated microbiological analyses have typically adopted one of several strategies (as summarized in Table 5.2):

- (1) Most commonly, culture-dependent enumeration of the probiont levels within the digestive tract (typically digesta based) has been undertaken, with or without quantifying total viable populations. Typically, de Man, Ragoza and Sharpe (MRS) agar is used for the enumeration of LAB (e.g. Carnevali *et al.* 2006; Lv *et al.* 2007; Merrifield *et al.* 2010b; 2011), Slanetz and Bartley medium is used for the enumeration of enterococci (Merrifield *et al.* 2010c; 2010d), and *Bacillus* spp. have been assessed after heat processing on tryptone soy agar (TSA) (Ghosh *et al.* 2008) or a *Bacillus* selective medium containing polymyxin B (Daniels *et al.* 2010). Methods for determining probiont identity have included biochemical and phenotypic analysis, 16S rRNA sequencing or RAPD analysis. Some studies of this nature also enumerate the indigenous microbiota typically using a general purpose medium such as TSA or nutrient agar.
- (2) Less frequently, some studies have used culture-dependent approaches to assess probiont levels as well as enumerating and identifying the indigenous microbiota (e.g. Chang and Liu 2002; Aubin *et al.* 2005; Bagheri *et al.* 2008; Ghosh *et al.* 2008). In order to assess the indigenous microbiota after probiotic supplementation, selective media have been utilized for the enumeration of indigenous bacterial groups of particular interest (e.g. Carnevali *et al.* 2004; 2006; Rollo *et al.* 2006; Ghosh *et al.* 2008; Silvi *et al.* 2008) or selected isolates from general purpose media have been identified by traditional biochemical assays and phenotypic observations (e.g. Chang and Liu 2002; Bagheri *et al.* 2008) or 16S rRNA sequence analysis (e.g. Aubin *et al.* 2005).
- (3) More recently, culture-independent assessment of the gut microbiota after probiotic feeding using techniques such as denaturing gradient gel electrophoresis (DGGE) has become more common (Kim and Austin 2006; Sáenz de Rodríguez *et al.* 2009; Ferguson *et al.* 2010; Sun *et al.* 2011a) and to a lesser extent clone libraries (Zhou *et al.* 2012; Abid, Zhou and Merrifield unpublished), fluorescent *in situ* hybridization (FISH) (Salinas *et al.* 2008; Del'Duca *et al.* 2013) and qPCR (Avella *et al.* 2010a; 2012) have also been used. Due to the low cultivability of the gut microbiota of fish (Table 5.1), culture-independent methodologies are useful tools in furthering our understanding of fish intestinal microbiota. Therefore, methods for assessing the gut microbiota of fish, including probiotic studies, should move away from exclusive use of culture-dependent methods (i.e. approaches 1 and 2) and, in accordance with other more established areas of environmental microbiology, derive a more complete picture of the extant microbial diversity present within fish intestines by incorporating more sensitive molecular based methods, including next-generation sequencing, into probiotic studies.

Studies have demonstrated modulation of the fish gut microbiota after probiotic application of both Gram positive bacteria such as *Bacillus*, *Micrococcus* and lactic acid bacteria (LAB) (e.g. *Carnobacterium*, *Enterococcus*, *Lactobacillus* and *Weissella*) and Gram negative bacteria such as *Aeromonas*, *Photobacterium*, *Pseudomonas*, *Phaeobacter*, *Shewanella* and *Vibrio*. Additionally dietary supplementation of yeast, such as *Saccharomyces cerevisiae*, *Debaromyces hansenii* and *Rhodotorula glutinis*, has also been demonstrated to modulate the indigenous gut microbiota of fish (Gatesoupe 2007). Probiotic applications may yield populations potentially resident on the epithelium, in the intestinal mucus and/or in the digesta. The establishment of probiotic populations present in the pyloric caeca, stomach and intestine has been reported and these probiotic populations can influence the indigenous microbiota levels as well as composition. The sensitivity of the GI microbiota to probiotic modulation does not appear to be restricted by fish maturation as several investigations have demonstrated the effects of the probiotics on the gut microbiota of fish across a range of life stages, including larval (via live preys), fry, fingerlings, juvenile and adult stages. The present chapter reviews the current literature on the effect of probiotic applications on the microecology of the fish GI tract.

8.2 *BACILLUS* spp.

Bacillus subtilis and *Bacillus licheniformis* are among the most well studied probiotic species in fish. However, *Bacillus cereus* (Hidalgo *et al.* 2006), *Bacillus circulans* (Ghosh *et al.* 2004), *Bacillus pumilus* (Aly *et al.* 2008; Sun *et al.* 2011a), *Bacillus clausii* (Yang *et al.* 2012), *Bacillus toyoi* (Chang and Liu 2002; Hidalgo *et al.* 2006), *Bacillus amyloliquefaciens* (Ridha and Azad 2012) and unidentified *Bacillus* spp. (Gatesoupe 1993; Del'Duca *et al.* 2013) have also been investigated. Despite the numerous *Bacillus* probiotic applications in fish, relatively few have included comprehensive assessments of the impact on GI microecology (refer to Table 8.1). These studies have however demonstrated that probiotic *Bacillus* applications can elevate fish GI *Bacillus* levels (Newaj-Fyzul *et al.* 2007; Bagheri *et al.* 2008; Ghosh *et al.* 2008; Merrifield *et al.* 2010b; 2010c; 2010d; Sun *et al.* 2011a; Mohapatra *et al.* 2012; Del'Duca *et al.* 2013), modulate indigenous or total bacterial levels (Chang and Lui 2002; Bagheri *et al.* 2008; Ghosh *et al.* 2008; Ridha and Azad 2012; Del'Duca *et al.* 2013) and alter indigenous bacterial community composition (Chang and Lui 2002; Bagheri *et al.* 2008; Ghosh *et al.* 2008; Sun *et al.* 2011a).

Newaj-Fyzul *et al.* (2007) demonstrated that high levels of *B. subtilis* (originally isolated from the intestine of rainbow trout) dominated both the intestinal contents (allochthonous) and intestinal mucus (autochthonous) of rainbow trout fed dietary levels of 10^7 CFU g⁻¹ for a period of 14 days. *B. subtilis* was recovered at 5.3×10^4 CFU g⁻¹ in the gut contents and 8.1×10^4 CFU g⁻¹ in the gut mucus, which equated to nearly 70% of the total cultured populations in both the mucus and gut contents. The study demonstrated that the establishment of *B. subtilis* populations in the intestinal tract stimulated cellular and humoral immune responses (i.e. respiratory burst, serum and gut lysozyme, peroxidase, phagocytic killing, total and α 1-antiprotease and lymphocyte populations) which subsequently provided protection against a virulent *Aeromonas* sp. Successful establishment of intestinal *Bacillus* populations in rainbow trout was further demonstrated by Bagheri *et al.* (2008) and Merrifield *et al.* (2010b; 2010c; 2010d) who investigated the effects of the commercial product BioPlus

Table 8.1 Overview of *Bacillus* probiotic studies in fish which have assessed gut microbiota.

Probiotic	Fish	Techniques	References
<i>B. subtilis</i>	Guppy	TVC, probiotic and selected bacterial groups enumerated on selective media	Ghosh <i>et al.</i> (2008)
	Molly	TVC, probiotic and selected bacterial groups enumerated on SM	Ghosh <i>et al.</i> (2008)
	Green swordtail	TVC, probiotic and selected bacterial groups enumerated on SM	Ghosh <i>et al.</i> (2008)
	Southern platyfish	TVC, probiotic and selected bacterial groups enumerated on SM	Ghosh <i>et al.</i> (2008)
	Rainbow trout	TVC; probiotic; conventional tests and 16S rRNA of probiotic	Newaj-Fyzul <i>et al.</i> (2007)
	Koi carp	DGGE	He <i>et al.</i> (2011)
<i>B. subtilis</i> (+ <i>Lc. lactis</i> and/or <i>S. cerevisiae</i>)	Rohu	TVC; probiotic SM; conventional tests	Mohapatra <i>et al.</i> (2012)
<i>B. subtilis</i> + <i>B. licheniformis</i>	Rainbow trout	TVC; probiotic; indigenous groups identified by conventional tests	Bagheri <i>et al.</i> (2008)
<i>B. subtilis</i> + <i>B. licheniformis</i>	Rainbow trout	TVC; probiotic SM; 16S rRNA of probiotic; SEM; TEM	Merrifield <i>et al.</i> (2010b)
<i>B. subtilis</i> + <i>B. licheniformis</i>	Rainbow trout	TVC; probiotic SM; 16S rRNA of probiotic	Merrifield <i>et al.</i> (2010c)
<i>B. subtilis</i> + <i>B. licheniformis</i>	Rainbow trout	TVC; probiotic SM; 16S rRNA of probiotic	Merrifield <i>et al.</i> (2010d)
<i>B. toyoi</i>	European eel	TVC; probiotic; indigenous microbiota identified by conventional tests	Chang and Liu (2002)
<i>B. subtilis</i> + <i>B. licheniformis</i> + <i>B. pumilus</i>	Gilthead sea bream	TVC and selected bacterial group (<i>Vibrio</i>) enumerated on SM	Avella <i>et al.</i> (2010b)
<i>B. pumilus</i>	Grouper	DGGE	Sun <i>et al.</i> (2011a)
<i>B. clausii</i>	Grouper	DGGE	Yang <i>et al.</i> (2012)
<i>B. amyloliquefaciens</i>	Tilapia	TVC	Ridha and Azad (2012)
<i>Bacillus</i> sp.	Tilapia	FISH	Del'Duca <i>et al.</i> (2013)
Multiple <i>Bacillus</i> strains	Channel catfish	Probiotic; conventional tests and 16S rRNA of probiotic	Ran <i>et al.</i> (2012)

Microbial techniques: conventional tests = standard biochemical and/or phenotypic characterization; probiotic = enumeration of probiotic; SEM = scanning electron microscopy; SM = selective media; TEM = transmission electron microscopy; TVC = total viable counts.

2B (Chr. Hansen, Denmark) which contains *B. subtilis* (DSM 5750) and *B. licheniformis* (DSM 5749).

Bagheri *et al.* (2008) fed rainbow trout fry diets containing *Bacillus* levels ranging from ca. 5×10^8 to 6×10^9 CFU g⁻¹ for 2 months. This led to a significant increase of viable bacterial counts in the intestine compared to the control group at the end of the trial. Total aerobic bacterial counts were significantly different among treatments, suggesting that the indigenous microbiota was affected by dietary probiont level. The percentage of probiotic *Bacillus* spp. recovered from the intestine ranged from 65% to over 99% of the total viable culturable counts. Changes in the relative proportions of the indigenous microbial groups were also observed: *Pseudomonas* spp. and members of the Vibrionaceae seemed the least sensitive as they remained the dominant members of the non-probiotic population. However, as the probiotic supplementation level increased, Enterobacteriaceae and a group of unidentified Gram-negative spp. were gradually phased out. Merrifield *et al.* (2010c) fed rainbow trout *B. subtilis* and *B. licheniformis* (5×10^7 CFU g⁻¹) for 10 weeks and reported that *Bacillus* levels accounted for over 35% (1.42×10^3 CFU g⁻¹) and 60% (1.17×10^7 CFU g⁻¹) of the culturable microbial population on the mucosa and in the digesta, respectively (Figure 8.1A). In a second study Merrifield *et al.* (2010d) investigated the effects of *B. subtilis* and *B. licheniformis* on the gut microbiota of rainbow trout pre-treated with an antibiotic. Viable allochthonous counts after oxolinic acid administration were $<10^4$ CFU g⁻¹ but after 10 weeks feeding on probiotic supplemented diets (ca. 6×10^7 CFU g⁻¹) total culturable levels increased to 10^7 CFU g⁻¹, compared to levels of 10^6 CFU g⁻¹ in the control fed group. *Bacillus* levels accounted for 75% (5.5×10^3 CFU g⁻¹) of the culturable mucosal associated populations and over 80% (2.6×10^7 CFU g⁻¹) of the digesta populations (Figure 8.1B). Although a common strategy in mammals (Madden *et al.* 2005), to the authors' knowledge this is the first study in fish to incorporate the use of a probiotic after the use of antibiotics. Antibiotics remain an important part of the arsenal of treatments for many fish bacterial diseases and are used globally, and such applications can alter the gut microbiota in terms of total viable numbers and/or diversity of populations (Austin and Al-Zahrani 1988; Lesel *et al.* 1989; Moffitt and Mobin 2006; Bakke-McKellep *et al.* 2007; Liu *et al.* 2012; Romero *et al.* 2012). If reductions in the microbial levels, diversity or composition occur it could open a niche leaving the GI tract susceptible to secondary potential pathogens. The study of Merrifield *et al.* (2010d) demonstrates that probiotic supplementation after antimicrobial treatment increases the number of viable bacteria within the digestive tract and associated with the epithelial mucosa via repopulation with viable probionts. This is likely to create competition for potential pathogens re-entering the digestive tract and stabilize/reinforce the gut microbial defensive barrier after antibiotic treatment.

These studies demonstrate that dietary *B. licheniformis* and *B. subtilis* can survive transit through the rainbow trout digestive tract and successfully populate the intestine as both transient and potentially resident populations providing that continual dietary supplementation is applied. The evidence also suggests there is little strain specificity, as both commercial *B. subtilis* and autochthonous (strains isolated from fish) *B. subtilis* strains have displayed high intestinal recovery after dietary supplementation.

In addition to rainbow trout, it has been demonstrated that dietary *B. subtilis* can modulate the gut microbiota of a number of live bearing ornamental fish species. Ghosh *et al.* (2008) reported that intestinal *B. subtilis* levels significantly increased from being non-detectable to $>\log 6-7$ CFU g⁻¹ in the gut of *B. subtilis* fed guppy (*Poecilia reticulata*), molly (*Poecilia sphenops*), green swordtail (*Xiphophorus helleri*) and southern platyfish (*Xiphophorus*

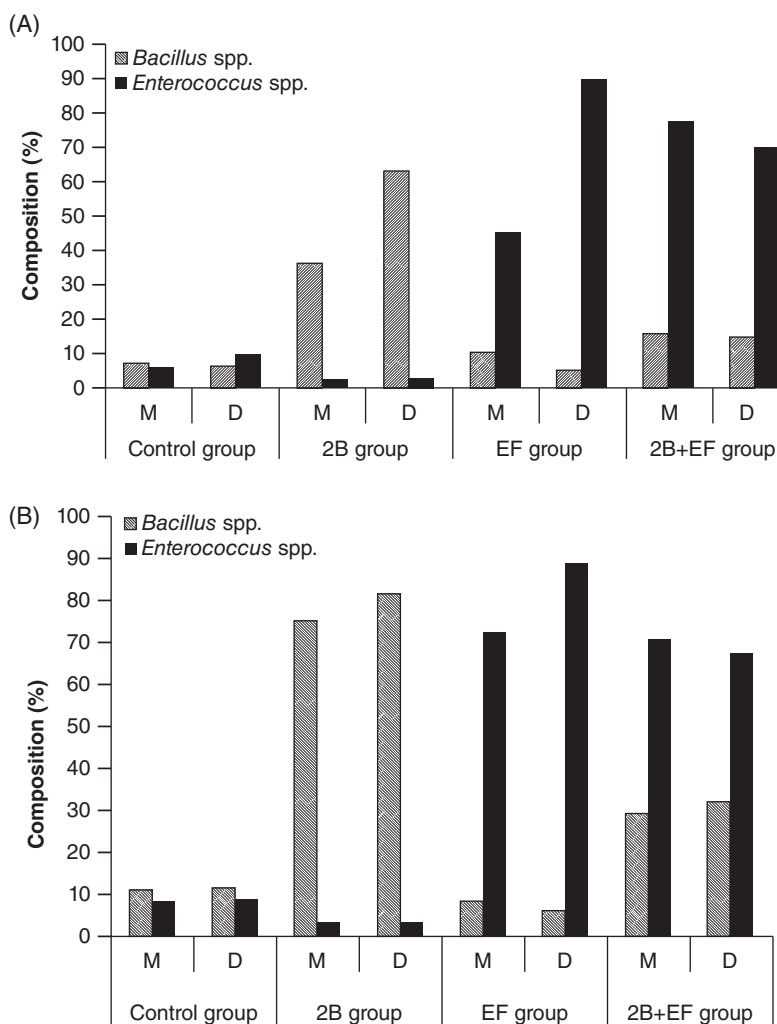


Fig. 8.1 Proportion (%) of *Bacillus* spp. and enterococci from the culturable rainbow trout intestinal microbiota after 10 weeks feeding on the experimental diets, without (A) and with (B) an oxolinic acid pre-treatment prior to probiotic feeding. Intestinal regions: M, mucosa; D, digesta. Dietary treatments: 2B, *B. licheniformis* + *B. subtilis*; EF, *E. faecium*; 2B + EF, *B. licheniformis* + *B. subtilis* + *E. faecium*. (Source: Adapted from Merrifield *et al.* 2010c and Merrifield *et al.* 2010d.)

maculatus) fry. Furthermore, the total heterotrophic bacterial levels generally decreased in all probiotic fed molly, swordtail and southern platyfish groups while levels in the control groups increased with the trial duration. Motile aeromonad counts increased in the control fed fish but generally decreased in all species of the probiotic fed fish. The presumptive pseudomonad levels of all four fish species investigated decreased after 90 days of feeding on the probiotic diets by an average of ca. $\log 1.5 \text{ CFU g}^{-1}$ but only by ca. $\log 0.5 \text{ CFU g}^{-1}$ in the control fed group. The total coliform count remained either stable or significantly lower in all fish

groups after 90 days feeding on the probiotic diets. Contrary to this, an increase of coliforms (by ca. $\log 1.0 \text{ CFU g}^{-1}$) was observed in the control fish. As the dietary concentration of *B. subtilis* in the study was insignificant with regards to the general reduction of intestinal bacterial counts, the authors suggested that a continuous administration of 10^6 – 10^7 CFU g^{-1} is sufficient to yield a healthy balance between *B. subtilis* and the indigenous gut microbiota.

Although *Bacillus* spp. are well equipped to make similar impacts upon the gastric microbial ecosystems of other fish species, the present data are somewhat ambiguous. Chang and Lui (2002) demonstrated that after dietary provision of *B. toyoi* to European eel (*Anguilla anguilla*) for 14 days it was not possible to recover viable *B. toyoi* isolates from the digestive tract using a culture based approach. Despite this, however, a clear effect on the indigenous microbiota was observed as the total viable heterotrophic population was reduced by 90% compared to initial levels even though the bacterial community composition was not affected. However, as the authors starved the fish for 24 h prior to sampling one cannot draw a full conclusion. Similarly, after feeding dietary *B. subtilis* C-3102 (Calsporin®, Calpis Co. Ltd, Tokyo, Japan) to koi carp *Cyprinus carpio* for five weeks, He *et al.* (2011) reported, using DGGE, that the probiont was not present at detectable levels in the intestine of probiotic fed fish at any sampling time point. In spite of this the probiotic treatment could impact the intestinal microbial community, particularly at the early stages of the trial. Yang *et al.* (2012) reported from DGGE analysis that the *B. clausii* was not detectable in the fore-, mid- or hindgut of grouper *Epinephelus coioides* juveniles after dietary provision at $10^8 \text{ cells g}^{-1}$ for 60 days. The microbial fingerprints revealed complex, and generally similar, bacterial communities in the respective GI regions of the two treatment groups. Differences in the communities in the different gut regions were observed, which were characterized by regional specialization of some operational taxonomic units (OTUs) and clustering of hindgut replicates, irrespective of treatment, together in the dendrogram. No differences in the species richness or diversity were observed between the treatments. However, four OTUs (closest known relatives identified as *Enterococcus* sp. (92% similarity), *Photobacterium* sp. (100%), *Bacillus pumilus* (90%) and an uncultured bacterium (97%)) were observed to be present only in the probiotic group and three OTUs (closest known relatives identified as *Staphylococcus* sp. HB4 (90%), uncultured Gammaproteobacterium clone (93%) and *Vibrio ponticus* (99%)) were present only in the control group (though not in all replicates of all gut regions).

The same research group fed grouper juveniles *B. pumilus* (originally isolated from the GI tract of juvenile grouper: Sun *et al.* 2009) at $10^8 \text{ cells g}^{-1}$ for 60 days (Sun *et al.* 2011a). Complex DGGE patterns were observed but no significant difference in the total number of OTUs or bacterial diversity was detected between the probiotic group and the control group. Despite this, UPGMA analysis showed clustering into three groups, one for each gut region investigated (fore-, mid- and hindgut), and each region contained two sub-clusters representing the two dietary treatments. Most observed OTUs, including an OTU at the same migration point as the probiotic, were common to all groups. Four OTUs (closest known relatives identified as uncultured *Bacillus* sp. clone (100% similarity), uncultured bacterium clone LL14117 (89%), *Nitratireductor* sp. YCSC5 (100%) and an uncultured bacterium clone G2-66 (95%)) were present only in the probiotic group, and two OTUs (*Staphylococcus saprophyticus* (99%) and uncultured bacterium clone SW-12 (98%)) were only present in the control group (though not in all replicates). Furthermore, the relative abundance (determined by the intensity of the band as a proportion of the total band intensities) of one OTU, identified as uncultured bacterium clone D3T-094 (95%), was significantly higher in the probiotic samples. Although the

probiotic was present in all samples, suggesting it to be indigenous to the fish in the experiment (indeed, it was originally isolated from the GI tract of juvenile grouper), the authors concluded that dietary administration stimulated further establishment of *B. pumilus* in each compartment of the digestive tract, which was based on their observation that the abundance, as a proportion of the total band intensities, of the probiotic was higher in the probiotic replicates than the control replicates.

Taken together, it is interesting to note that several studies have failed to observe detectable levels of *Bacillus* probionts in the GI tract of fish after dietary exposure: *B. toyoi* fed to European eel (Chang and Lui 2002), *B. subtilis* C-3102 fed to koi carp (He *et al.* 2011) and *B. clausii* fed to grouper (Yang *et al.* 2012). Despite this, however, these studies reported to some extent that probiotic administration modulated the gut microbiota and/or induced host benefits. This therefore suggests that low (i.e. non-detectable using the methods so far employed: DGGE and plate counts on general purpose media), or non-viable, populations of *Bacillus* cells/spores in the GI tract of fish can be sufficient to affect the host microbiota to some extent. Further studies, using more sensitive methods such as qPCR and metagenomics, are warranted to more accurately determine the level of probiotic presence and impact on the host when using *Bacillus* strains as probiotics for fish. It is evident from the literature that probiotic viability, and levels, within the GI tract of fish can be important factors and thus it is recommended that well documented *Bacillus* strains which can populate the GI tract be considered as preferential candidate probiotics.

Recently, Ran *et al.* (2012) investigated the persistence of 21 *Bacillus* strains, isolated from the intestine of channel catfish which displayed antibacterial activity against fish pathogens, and a *B. subtilis* type strain, on the intestine tissue of channel catfish using a culture based approach. Catfish were fed *Bacillus* spore-supplemented feeds (ca. 10^9 CFU g⁻¹) for 7 days followed by a normal non-supplemented feed for 3 days. No probiotic *Bacillus* were detected in the control fed fish but relatively high ($>\log 5$ CFU g⁻¹) *Bacillus* levels were observed in all of the probiotic fed fish 3 days after the cessation of dietary provision. For six probiotic strains (AB01, AP76, AP77, AP79, AP143 and AP254), $>10^7$ CFU g⁻¹ of introduced *Bacillus* was observed in the gut.

8.3 LACTIC ACID BACTERIA (LAB)

LAB are a group of Gram-positive rods and cocci that are non-sporing, lacking catalase and oxidase (cytochrome c), and are fermentative in Hugh–Leifson medium. Readers with a specific interest in LAB in the gut of fish are referred to the reviews of Ringø and Gatesoupe (1998), Ringø and Birkbeck (1999), Ringø (2004), Ringø *et al.* (2005; 2010), Michel *et al.* (2007), Gatesoupe (2008), Lauzon and Ringø (2012) and Chapter 6. Bacteria belonging to this group often produce bacteriocins and other chemical compounds that may inhibit colonization of pathogenic bacteria in the GI tract (for review see Ringø *et al.* 2005; Ringø 2008). The most commonly used LAB probionts for applications with fish so far belong to the *Carnobacterium*, *Enterococcus*/*Streptococcus*, *Pediococcus*, *Lactobacillus*, *Lactococcus* and *Leuconostoc* genera (Table 8.2) but some information on *Vagococcus fluvialis* is also available (Roman *et al.* 2012; Sorroza *et al.* 2012).

Table 8.2 Overview of LAB probiotic studies in fish which have assessed gut microbiota.

Probiotic	Fish	Microbial techniques	References
<i>Lb. rhamnosus</i>	Rainbow trout	TVC; probiont SM; 16S rRNA of probiont	Panigrahi <i>et al.</i> (2004)
	Rainbow trout	TVC; probiont SM; 16S rRNA of probiont	Panigrahi <i>et al.</i> (2005)
	Rainbow trout	Probiont SM	Nikoskelainen <i>et al.</i> (2003)
	Common clown fish	Probiont qPCR	Avella <i>et al.</i> (2010a)
	Zebrafish	DGGE	Gioacchini <i>et al.</i> (2012)
	European sea bass	TVC; probiont SM	Frouël <i>et al.</i> (2008)
<i>Lb. sakei</i>	Rainbow trout	Probiont SM; RAPD profiles of probiont	Balcázar <i>et al.</i> (2007b)
	Brown trout	Probiont SM; RAPD profiles of probiont	Balcázar <i>et al.</i> (2007a)
<i>Lb. plantarum</i>	Rainbow trout	Probiont SM; RAPD profiles of probiont	Vendrell <i>et al.</i> (2008)
	Rainbow trout	DGGE	Pérez-Sánchez <i>et al.</i> (2011)
	Tilapia	Probiont SM	Bucio Galindo <i>et al.</i> (2009)
	Tilapia	TVC, SM	Jatoba <i>et al.</i> (2011)
	European sea bass	Probiont SM; API 50 CHL identification of the probiont	Carnevali <i>et al.</i> (2004)
<i>Lb. fructivorans</i>	European sea bass	Probiont SM; API 50 CHL identification of the probiont	Carnevali <i>et al.</i> (2004)
<i>Lb. farciminis</i>	European sea bass	TVC; probiont SM	Frouël <i>et al.</i> (2008)
<i>Lb. curvatus</i>	Beluga and Persian sturgeon	TVC, probiont SM and conventional tests	Askarian <i>et al.</i> (2011)
<i>Lb. brevis</i>	Tilapia	DGGE	Liu <i>et al.</i> (2013)
<i>Lb. acidophilus</i>	Tilapia	DGGE	Liu <i>et al.</i> (2013)
<i>Lactobacillus</i> sp.	Tilapia	TVC	Ridha and Azad (2012)
Lactobacilli (multiple species)	Zebrafish	DGGE, clone libraries, probiont SM; 16S rRNA of probiont; SEM	Zhou <i>et al.</i> (2012)
<i>E. faecium</i> ^a	Rainbow trout	TVC; probiont SM; 16S rRNA of probiont; SEM; TEM	Merrifield <i>et al.</i> (2010b)
	Rainbow trout	TVC; probiont SM; 16S rRNA of probiont	Merrifield <i>et al.</i> (2010c)
	Rainbow trout	TVC; probiont SM; 16S rRNA of probiont	Merrifield <i>et al.</i> (2010d)
	Common carp	Indigenous groups identified by SM and conventional tests	Bogut <i>et al.</i> (1998)
	Common carp	Probiont SM; conventional tests	Gopalakannan and Arul (2011)
	Sheat fish	Indigenous groups identified by SM and conventional tests	Bogut <i>et al.</i> (2000)

Table 8.2 (continued)

Probiotic	Fish	Microbial techniques	References
	European eel	TVC; probiotic enumerated; indigenous microbiota identified by conventional tests	Chang and Liu (2002)
	Grouper	DGGE	Sun <i>et al.</i> (2012a)
	Senegalese sole	TVC; probiotic SM; RAPD profiles of probiotic; SM of groups of interest	Avella <i>et al.</i> (2011)
<i>Enterococcus</i> sp.	Atlantic cod	TVC, <i>Vibrio</i> and LAB SM; 16S rRNA sequencing	Lauzon <i>et al.</i> (2010a)
<i>Enterococcus</i> sp.	Atlantic cod	TVC, <i>Vibrio</i> and LAB SM	Lauzon <i>et al.</i> (2010b)
<i>Enterococcus</i> sp.	Tilapia	FISH	Del'Duca <i>et al.</i> (2013)
<i>Lc. lactis</i>	Turbot	Probiotic SM	Villamil <i>et al.</i> (2002)
	Brown trout	Probiotic SM; RAPD profiles of probiotic	Balcázar <i>et al.</i> (2007a)
	Brown trout	Probiotic SM; RAPD profiles of probiotic; <i>A. salmonicida</i> was determined using qPCR	Balcázar <i>et al.</i> (2009)
	Rainbow trout	Probiotic SM; RAPD profiles of probiotic	Balcázar <i>et al.</i> (2007b)
	Rainbow trout	DGGE	Pérez-Sánchez <i>et al.</i> (2011)
	Grouper	DGGE	Sun <i>et al.</i> (2012b)
	Rohu	TVC; probiotic SM; conventional tests	Mohapatra <i>et al.</i> (2012)
<i>Leu. mesenteroides</i>	Brown trout	Probiotic SM; RAPD profiles of probiotic	Balcázar <i>et al.</i> (2007a)
	Brown trout	Probiotic SM; RAPD profiles of probiotic; <i>A. salmonicida</i> was determined using qPCR	Balcázar <i>et al.</i> (2009)
	Rainbow trout	Probiotic SM; RAPD profiles of probiotic	Balcázar <i>et al.</i> (2007b)
	Rainbow trout	Probiotic SM; RAPD profiles of probiotic	Vendrell <i>et al.</i> (2008)
	Rainbow trout	DGGE	Pérez-Sánchez <i>et al.</i> (2011)
	Beluga and Persian sturgeon	TVC, probiotic SM and conventional tests	Askarian <i>et al.</i> (2011)
<i>P. acidilactici</i>	Pollock	SM, conventional tests	Gatesoupe (2002)
	Rainbow trout	TVC; probiotic SM; 16S rRNA of probiotic; 16S rRNA of indigenous microbiota	Aubin <i>et al.</i> (2005)
	Rainbow trout	TVC; probiotic SM; 16S rRNA of probiotic; SEM; TEM	Merrifield <i>et al.</i> (2010b)
	Rainbow trout	TVC; probiotic SM; 16S rRNA of probiotic; SEM; TEM	Merrifield <i>et al.</i> (2011)
	Rainbow trout	SM; 16S rRNA of probiotic; SEM; TEM	Harper <i>et al.</i> (2011)

Table 8.2 (continued)

Probiotic	Fish	Microbial techniques	References
<i>C. inhibens</i> ^b	Tilapia	Probiotic SM; 16S rRNA of probiont; DGGE	Ferguson <i>et al.</i> (2010)
	Tilapia	TVC; probiont SM; 16S rRNA of probiont	Standen <i>et al.</i> (2013)
	Turbot	Probiotic SM	Villamil <i>et al.</i> (2010)
	Rainbow trout	TVC; probiont SM; conventional tests	Jöborn <i>et al.</i> (1997)
	Atlantic salmon	Probiotic, conventional tests	Robertson <i>et al.</i> (2000)
	Rainbow trout	Probiotic, conventional tests	Robertson <i>et al.</i> (2000)
<i>C. divergens</i>	Rainbow trout	Probiotic; conventional tests, API 50CH and API 20E	Irianto and Austin (2002)
	Atlantic salmon	TVC; probiont SM; indigenous microbiota identified by conventional tests	Gildberg <i>et al.</i> (1995)
	Atlantic cod	TVC; probiotics and indigenous bacteria identified by conventional tests	Strøm and Ringø (1993) ^c
	Atlantic cod	TVC; probiont; indigenous microbiota identified by conventional tests	Gildberg <i>et al.</i> (1997)
	Atlantic cod	Probiotic, ELISA; histochemistry	Gildberg and Mikkelsen (1998)
	Atlantic cod	TVC, <i>Vibrio</i> and LAB SM; 16S rRNA sequencing	Lauzon <i>et al.</i> (2010a)
	Turbot	ELISA	Ringø (1999)
	Rainbow trout	TVC; probiont SM; 16S rRNA of probiont; DGGE	Kim and Austin (2006)
<i>C. maltaromaticum</i>	Common carp	Probiotic SM; conventional tests	Gopalakannan and Arul (2011)
	Rainbow trout	TVC; probiont SM; 16S rRNA of probiont; DGGE	Kim and Austin (2006)
<i>Carnobacterium</i> spp. (isolate BA211)	Rainbow trout	Probiotic SM; conventional tests, API 50CH and API 20E	Irianto and Austin (2002)
LAB isolate (identified as <i>Lb. plantarum</i> or <i>Carnobacterium</i> sp.)	Turbot	TVC, <i>Vibrio</i> and LAB SM	Gatesoupe (1994)
Protexin ^d	Gilthead sea bream	TVC; probiont SM	Suzer <i>et al.</i> (2008)

^aPreviously *Streptococcus faecium* (Schleifer and Kilpper-Balz 1984).

^bPreviously known as *Carnobacterium* strain K1 (Joborn *et al.* 1999).

^cThe probiotic was described as *Lb. plantarum* in this study but was later identified as *C. divergens* by Ringø *et al.* (2001).

^dProtexin (solid form): Novartis, Saúde Animal Ltda, São Paulo, Brasil. Contains: *Lb. plantarum*, *Lb. bulgaricus*, *Lb. acidophilus*, *Lb. rhamnosus*, *Str. thermophilus*, *Bifidobacterium bifidum* and *E. faecium*.

Genera abbreviations: C. = *Carnobacterium*, E. = *Enterococcus*, Lb. = *Lactobacillus*, Lc. = *Lactococcus*, Leu. = *Leuconostoc*, P. = *Pediococcus*, S. = *Saccharomyces*.

Microbial techniques: conventional tests = standard biochemical and/or phenotypic characterization; probiont = enumeration of probiont; SEM = scanning electron microscopy; SM = selective media; TEM = transmission electron microscopy; TVC = total viable counts.

8.3.1 *Carnobacterium* spp.

In most of the early investigations on the intestinal LAB in fish, *Carnobacterium* and *Lactobacillus* were not easily distinguished (Ringø and Gatesoupe 1998). Today, however, based on new information and technological advances, it is easy to distinguish *Carnobacterium* from *Lactobacillus* by the presence of meso-diaminopimelic acid in the cell wall, the isomers of lactic acid produced, production of citrulline from arginine, growth on acetate and CTAS agar plates and 16S rRNA sequencing (Ringø and Gatesoupe 1998).

During the last 15 years, several strains of *Carnobacterium* have shown potential for successful probiotic applications with fish species. These include *Carnobacterium maltaromaticum* (Kim and Austin 2006) (*C. maltaromaticum* was previously *Carnobacterium piscicola*, but this was reclassified in 2003: Mora *et al.* 2003), *Carnobacterium divergens* (Gildberg *et al.* 1995; 1997; Gildberg and Mikkelsen 1998; Ringø 1999; Kim and Austin 2006; Gopalakannan and Arul 2011), *Carnobacterium inhibens* (previously *Carnobacterium* strain K1) (Jöborn *et al.* 1997; Robertson *et al.* 2000; Irianto and Austin 2002) and unidentified *Carnobacterium* spp. (Irianto and Austin 2002). Furthermore, some studies have suggested that carnobacteria have the potential to colonize the digestive tract of cold water fish such as salmonids (Gildberg *et al.* 1995; Jöborn *et al.* 1997; Robertson *et al.* 2000; Irianto and Austin 2002; Kim and Austin 2006) and Atlantic cod *Gadus morhua* (Gildberg *et al.* 1997; Gildberg and Mikkelsen 1998) (Table 8.2).

The early study by Jöborn *et al.* (1997) demonstrated that *C. inhibens* (originally isolated from the gut of Atlantic salmon and designated as strain K1, later identified as *C. inhibens*: Jöborn *et al.* 1999) grew well in rainbow trout intestinal mucus *in vitro* and showed in *in vivo* experiments that *C. inhibens* was able to survive gastric passage and persist within the digestive tract of rainbow trout fingerlings. *C. inhibens* was administered via the feed at levels of 4×10^7 CFU g⁻¹ and faecal *Carnobacterium* levels were reported at 10^6 – 10^7 CFU g⁻¹ during the supplemented feeding phase. Despite this, and the observed *in vitro* adhesion to and growth within intestinal mucus, *C. inhibens* cells were not detected within the intestinal mucus, and so the authors suggested that *in vivo* *C. inhibens* populates the lumen rather than the mucosa of the intestinal tract. Following this, Robertson *et al.* (2000) fed rainbow trout fry, rainbow trout fingerlings and Atlantic salmon fingerlings *C. inhibens*, at 5×10^7 cells g⁻¹ for up to 28 days, and reported that *C. inhibens* remained viable within the digestive tract of the salmonids during feeding and that a clear time-dependent relationship was evident. For example, in rainbow trout fingerlings, *Carnobacterium* reached maximum levels (7.4×10^6 g⁻¹ intestine) after 28 days of probiotic feeding. Similar results were reported by Irianto and Austin (2002) where high levels of carnobacteria were detected in fish fed *Carnobacterium inhibens* and an unidentified *Carnobacterium* spp. (isolate BA211) for 7 days. The recovery of *C. inhibens* displayed a clear relationship with the duration of feeding; intestinal levels increased fourfold over the 7 day period from 5.5×10^6 to 2.1×10^7 CFU g⁻¹ after 1 and 7 days feeding, respectively. This time-dependent response was not observed for *Carnobacterium* spp. isolate BA211 which remained present at high levels (ca. 5×10^6 CFU g⁻¹) within the intestine with no evident relationship with feeding duration.

Despite the reported inability of *C. inhibens* to populate the intestinal mucosa of trout (Jöborn *et al.* 1997) it has been reported that *C. divergens* and *C. maltaromaticum* (originally isolated from rainbow trout) displayed exceptional qualities for the successful population of the rainbow trout intestine (Kim and Austin 2006). Dietary application for 14 days at 10^7 CFU g⁻¹ led to probiont levels of >90% and >99% of the total culturable populations

in the intestinal contents and intestinal mucus, respectively. Furthermore, *C. divergens* strains (originally isolated from the intestines of Atlantic cod and Atlantic salmon: Strøm 1988) have been reported to populate the mucus/mucosa of Atlantic cod fry (Gildberg and Mikkelsen 1998). *C. divergens* were fed to fry at 10^8 CFU g⁻¹, with and without the addition of a fish protein hydrolysate immuno-stimulating peptide, for 3 weeks. Both *C. divergens* strains successfully survived transit through the entire GI tract as LAB levels were recovered in the faeces; however, these levels were only approximately 1% of initial dietary levels. LAB levels in the pyloric caeca samples were observed at log 3–4 CFU g⁻¹ tissue and histochemistry verified the presence of *C. divergens* in the internal mucus layer. Levels in the intestine however were lower and less frequently detected in the samples. The study suggests that the pyloric caeca may be a more favourable site for colonization than the intestine, and the authors suggested that there seemed to be no host specificity (i.e. specificity of probionts derived from the host) with preference for GI colonization as the strain previously isolated from Atlantic salmon displayed better colonization than the strain previously isolated from Atlantic cod.

Two studies have investigated the effect of *C. divergens* application, via rearing water, on the gut microbiota of fish larvae. In a preliminary study with 5-day-old Atlantic cod larvae, Strøm and Ringø (1993) evaluated the effect of inoculating rearing water with *C. divergens* (described as *Lb. plantarum* in this study but later reclassified as *C. divergens* by Ringø *et al.* 2001) which was originally isolated from the gut of adult Atlantic cod and produced a substance that inhibited the *in vitro* growth of the fish pathogens *Vibrio* (*Listonella*) *anguillarum*, *Vibrio salmonicida* and *Vibrio* sp. (Strøm 1988). By exposing the cod larvae to *C. divergens* the cultivable bacterial population level was significantly reduced and the composition was also affected. In larvae exposed to the probiotic, *C. divergens* accounted for 70% of the cultivable microbiota and *Pseudomonas* spp. accounted for 20%, while *Aeromonas* spp. and bacteria belonging to the *Cytophaga*/*Flexibacter* group were detected in the control group but were not detected in larvae exposed to *C. divergens*. Whether this modulation in the gut microbiota had any effect on larval growth or health was however not investigated. Following this, Ringø (1999) investigated if *C. divergens* isolated from the gut of Atlantic salmon (Strøm 1988) was able to attach to the mucosal surface and colonize the gut of turbot (*Scophthalmus maximus* L.) larvae. *Vibrio pelagius*, originally isolated from turbot larvae, was used as a control and an ELISA was used to determine bacterial levels. When larvae were exposed to *V. pelagius* the bacteria was detected in larvae 3 days post hatching (dph), while *C. divergens* was detected in larvae exposed to *C. divergens*, and when in combination with *V. pelagius*, at 6 dph. Based on these results the author concluded that *C. divergens*, originally isolated from the GI tract of Atlantic salmon, was able to attach to and colonize the gut of turbot larvae at the time of hatching.

The present studies indicate that *Carnobacterium* spp. are resistant to specific conditions within the GI of salmonids, Atlantic cod and turbot (i.e. proteolytic enzymes, low pH, bile acids and pancreatic juice) and appear to be effective at competing with indigenous microbes within the gut. However, there appear to be differences in the efficacy of strains to populate the mucosa, and the reasons for this have not been comprehensively determined as specificity of strains does not necessarily correlate to their original isolation source (i.e. fish species). Carnobacteria, particularly *C. divergens* strains, appear to be good candidates to populate the intestinal mucus and modulate the gut microbiota of cold water fish species; they are likely to be far less effective in warm water species where their slower growth rates are likely to render them unable to outcompete many of the faster growing components of the indigenous microbiota. However, Gopalakannan and Arul (2011) reported that during probiotic feeding

C. divergens could populate the intestinal tract of common carp *Cyprinus carpio*, with levels in line with that observed for *E. faecium*.

8.3.2 *Lactobacillus* spp.

Lactobacilli have frequently been investigated as probiotics for aquaculture applications. Commonly used species include: *Lactobacillus rhamnosus* (Nikoskelainen *et al.* 2001; 2003; Panigrahi *et al.* 2004; 2005; 2007; Pirarat *et al.* 2006; Suzer *et al.* 2008), *Lactobacillus sakei* (Balcázar *et al.* 2007a; 2007b), *Lactobacillus delbrueckii* (Carnevali *et al.* 2006; Salinas *et al.* 2006; 2008; Silvi *et al.* 2008; Picchietti *et al.* 2009), *Lactobacillus acidophilus* (Lara-Flores *et al.* 2003; Aly *et al.* 2008; Suzer *et al.* 2008; Liu *et al.* 2013) and *Lactobacillus plantarum* (Carnevali *et al.* 2004; Rollo *et al.* 2006; Picchietti *et al.* 2007; Suzer *et al.* 2008; Vendrell *et al.* 2008; Bucio Galindo *et al.* 2009; Jatoba *et al.* 2011; Perez-Sanchez *et al.* 2011), but *Lactobacillus fructivorans* (Carnevali *et al.* 2004; Picchietti *et al.* 2007), *Lactobacillus curvatus* (Askarian *et al.* 2011), *Lactobacillus bulgaricus* (Suzer *et al.* 2008), *Lactobacillus brevis* (Liu *et al.* 2013), *Lactobacillus farciminis* (Frouël *et al.* 2008) and *Lactobacillus* sp. (Ridha and Azad 2012) have also been studied. It has been suggested that the successful application of *Lactobacillus* strains as microbial supplements for fish appears to be linked with high viability during processing and storage and after GI transit (Robertson *et al.* 2000).

Several studies have provided valuable information regarding lactobacilli colonization of the fish GI tract and persistence within the GI tract after reverting to non-supplemented feeds, and thus *Lactobacillus* spp. are perhaps the most well investigated probiotics in fish with regards to their impact on ingenious GI microbial ecology (Table 8.2). Aside from the information on salmonids (rainbow trout, brown trout and Atlantic salmon) and Mediterranean teleosts (European sea bass *Dicentrarchus labrax* and gilthead sea bream *Sparus aurata*), some information is also available for Atlantic cod, clownfish *Amphiprion ocellaris*, Nile tilapia *Oreochromis niloticus*, Persian sturgeon *Acipenser persicus*, beluga *Huso huso* and zebrafish *Danio rerio*.

Lb. fructivorans (AS17B), isolated from the gut of adult sea bream (Carnevali *et al.* 2004), was simultaneously administered with another probiotic, *Lb. plantarum*, isolated from human faeces, during the early development of sea bream. Using rotifers (*Brachionus plicatilis*) and/or *Artemia salina* and dry feed as vectors, it was observed that the probiotics significantly decreased GI tract cultivable heterotrophic bacterial levels (both anaerobic and aerobic) and elevated LAB levels (Carnevali *et al.* 2004). Interestingly it was observed that the two lactobacilli seemed to vary in their efficacy as *Lb. plantarum* was the dominant LAB present in the early stages of the study (35 dph) and *Lb. fructivorans* dominated in the post metamorphosis stages (66 and 90 dph). *Lb. delbrueckii* subsp. *delbrueckii* has also been reported to show good capability to colonize the gut of sea bass larvae and modulate the gut microbiota (Silvi *et al.* 2008). *Lb. delbrueckii* subsp. *delbrueckii*, isolated from the European sea bass intestine, was provided to larvae via live feeds either from an early stage (11 dph; group A) or at a later stage (30 dph; group B) and the GI microbiota was assessed at multiple time points. The probiotic administration modulated the cultivable aerobic and anaerobic levels as well as LAB levels and composition. In brief, the probiotic *Lb. delbrueckii* subsp. *delbrueckii* was present in group A from 11 dph and increased from 17% (of the LAB population) at the beginning of the study to 96% at the end of the study (70 dph). Group B presented low percentages of *Lb. delbrueckii* subsp. *delbrueckii* before the probiotic treatment, and after treatment the percentage increased to 35% at 50 dph and then to 95% of the total LAB at 70 dph. During the whole

period the control group showed percentages of *Lb. delbrueckii* subsp. *delbrueckii* lower than 32%, with the contemporaneous presence of other LAB species. In contrast, the application of dietary lactobacilli initially isolated from the rumen of goat (*Lb. farciminis* CNCM MA27/6R and *Lb. rhamnosus* CNCM MA27/6B) did not influence the cultivable heterotrophic bacterial levels or LAB levels in the intestinal tract of European sea bass (Frouël *et al.* 2008).

Recently, the effects of the *Lb. rhamnosus* on the gut microbiota of a popular ornamental species, the clownfish, was studied by Avella *et al.* (2010a). Avella and colleagues exposed clownfish larvae and juveniles to *Lb. rhamnosus* ICM501 at 10^6 CFU ml⁻¹ by two methods: via live preys and addition to rearing water (group 2) or via live preys only (group 3). GI *Lactobacillus* levels were estimated by qPCR; the microbiota of clownfish administered with *Lb. rhamnosus* was affected in both larval and juvenile stages in both treatment groups, as evidenced by the changes of *Lb. rhamnosus* 16S rDNA levels. On the contrary, in the clownfish control group (group 1; no addition of probiotics) no *Lb. rhamnosus* rDNA was detected. Although this might not be so surprising, the interesting observation was that *Lb. rhamnosus* levels were significantly higher in treatment group 2 (probiotic provision via feed and rearing water) than in group 3 (probiotic provision via feed only). As a marine species, clownfish have an internal osmotic concentration lower than that of the surrounding seawater, and therefore they drink large quantities of rearing water to replace water loss. The study suggests that addition of probiotics to the rearing water of marine fish, perhaps more so than that of freshwater fish, may be a promising way to effectively boost probiotic intake of dietary sources. Although this is likely difficult or unfeasible on large fish, particularly in sea cages or flow through systems, this method may be a viable alternative at the larval rearing stage where small scale, controlled, recirculation facilities are used (refer to Chapter 16). Indeed, Avella *et al.* (2010a) observed that the best probiotic effects on the host were observed in fish provided with *Lb. rhamnosus* via both live preys and additions to the rearing water.

The zebrafish has become an important model for assessing the gut microbiota of vertebrates (Rawls *et al.* 2004; 2006; Carnevali *et al.* 2013) and this model has also been used to assess the efficacy of potential probiotic colonization and GI microbial modulation. In a recent study, Zhou *et al.* (2012) tested the capabilities of 10 *Lactobacillus* strains to adhere to zebrafish intestine using partial *rpoB* gene DGGE analysis, clone libraries and scanning electron microscopy. The 10 strains were simultaneously applied via the feed for a period of 4 weeks. The probiotic application led to significantly elevated lactobacilli levels in the GI tract of probiotic treated fish (1.9×10^5 CFU g⁻¹ of gut) compared to those of the control fish (no probiotic provision; 1.4×10^4 CFU g⁻¹). The composition of the lactobacilli in the probiotic group revealed that the adhesive capabilities of lactobacilli varied greatly; *Lactobacillus brevis* CGMCC 1.2028, *Lactobacillus buchneri* BU 222, *Lb. plantarum* subsp. *plantarum* JCM 1149T, *Lb. rhamnosus* CICC 6141, and *Lb. rhamnosus* JCM 20300 were identified as highly adhesive gut strains. Two further studies have also verified that *Lb. rhamnosus* (strain IMC 501®) has good capacity to populate the GI tract of zebrafish at multiple life stages (Avella *et al.* 2012; Gioacchini *et al.* 2012). Using qPCR Avella *et al.* (2012) verified the presence of *Lb. rhamnosus* in the GI tract of zebrafish larvae (6 days post fertilization) and juveniles (9 weeks post fertilization) after administration at 10^6 CFU g⁻¹/ml⁻¹ via live feed and rearing water. Gioacchini *et al.* (2012) fed adult zebrafish a commercial diet containing *Lb. rhamnosus* (10^6 CFU g⁻¹) for 10 days. DGGE revealed the presence of *Lb. rhamnosus* in all replicates of the probiotic group and subsequently these populations influenced the microbial communities. DGGE fingerprints from the two treatment groups formed two clusters with approximately

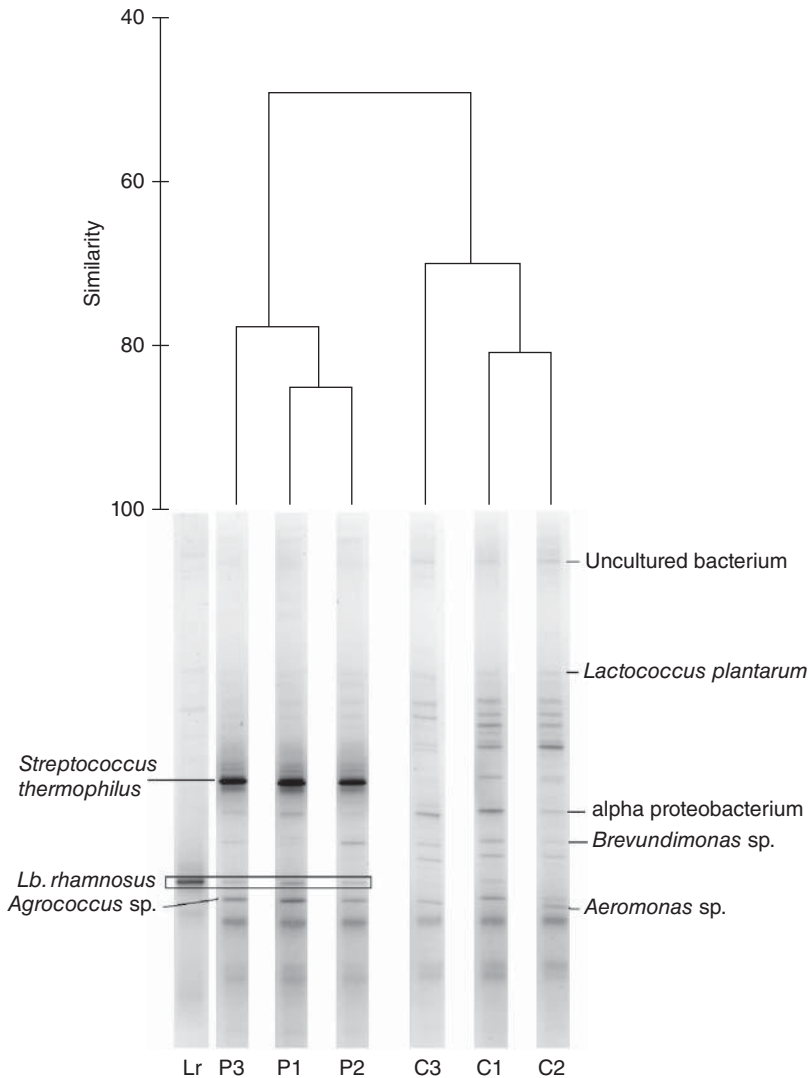


Fig. 8.2 Dendrogram and DGGE profiles of the GI bacterial populations of zebrafish. Each lane represents replicates of the control (C1–C3) or the *Lb. rhamnosus*-treated (P1–P3) fish. Lane Lr represents the migration point of the known *Lb. rhamnosus* culture. (Source: Adapted from Gioacchini *et al.* 2012.)

50% dissimilarity to one another, and trends towards reduced bacterial richness, evenness and diversity were observed in the probiotic fed group (see Figure 8.2). Sequence analysis of isolated bands revealed that the abundance of a strain of *Streptococcus thermophilus*, which was not detected in the control replicates, was clearly stimulated by the presence of the probiotic.

The administration of *Lactobacillus* strains in teleosts has shown varying degrees of success. Studies which have successfully modulated the GI microbiota of fish with *Lactobacillus*

strains have demonstrated that these changes can often lead to the improvement of general animal welfare in terms of survival, immune status, growth performance and/or stress response (Merrifield *et al.* 2010a; Dimitoglou *et al.* 2011).

8.3.3 *Enterococcus* spp.

To the authors' knowledge the investigation of enterococci for probiotic applications in fish is restricted to *Enterococcus faecium* (previously *Streptococcus faecium*: Schleifer and Kilpper-Balz 1984) strains (Bogut *et al.* 1998; 2000; Chang and Liu 2002; Panigrahi *et al.* 2007; Shelby *et al.* 2007; Wang *et al.* 2008; Merrifield *et al.* 2010b; 2010c; 2010d; Avella *et al.* 2011; Palermo *et al.* 2011; Gopalakannan and Arul 2011; Sun *et al.* 2012a) and an unidentified *Enterococcus* sp. (Del'Duca *et al.* 2013) (Table 8.2). Several of these studies have investigated the interactions with the indigenous fish gut microbiota.

Bogut *et al.* (1998) assessed the effect of *Enterococcus faecium* (M74; described in this study as *Streptococcus faecium*) on the cultivable microbiota of common carp. Relative to the control, Enterobacteriaceae, *Streptococcus faecalis* and *Staphylococcus aureus* levels were considerably lower in the probiotic fed group and *Escherichia coli* was no longer present after 14 days of probiotic feeding. Later, Bogut *et al.* (2000) fed sheat fish dietary *E. faecium* at 10^5 CFU g⁻¹ for 58 days. Probiotic application of *E. faecium* resulted in a clear alteration of the culturable intestinal microbiota including reductions of Enterobacteriaceae (including *E. coli*), *Staphylococcus aureus* and *Clostridium* spp. Subsequently, significantly improved weight gain was observed in *E. faecium* fed fish compared to the control group.

Later, Chang and Liu (2002) fed European eel diets supplemented with *E. faecium* SF68 for 14 days and investigated the gut microbiota every second day, up to day 14, following a 24 h starvation period. *E. faecium* began to successfully populate the intestine after 4 days; levels generally increased with feeding duration and maximal levels were recovered between 10 and 14 days (log 5.1–5.25 CFU g⁻¹). *E. faecium* was not detected in the control fed eels but at day 14 *E. faecium* represented the dominant cultivable intestinal population of the probiotic fed eels, accounting for 73% of the total culturable microbiota. Modulation of the indigenous microbiota was demonstrated by alterations of both the levels and the composition of *Aeromonas* spp. Furthermore, the level of *Vibrio* spp. was reduced from 6% in the control to non-detectable levels in the probiotic fed fish, and *Pasteurella multocida* and *Plesiomonas shigelloides* levels were also considerably lower in the probiotic fed fish (at 2% and 4%, respectively) than in the control fed fish (at 37% and 12%, respectively). Chang and Lui (2002) demonstrated *in vitro* antagonism of *E. faecium* against *Edwardsiella tarda*; subsequently *in vivo* protection was demonstrated in fish fed *E. faecium* (compared to both the control group and a group fed *B. toyoi*) during challenge experiments. As no detectable levels of *B. toyoi* were observed in the intestine of *B. toyoi* fed fish, the study demonstrates the importance of successful establishment of gastric probiotic populations for protection against infection.

More recently the effect of dietary *E. faecium* (10^8 CFU g⁻¹ diet) was observed on intestinal microbiota of rainbow trout with (Merrifield *et al.* 2010d) and without (Merrifield *et al.* 2010c) an antibiotic pre-treatment (Figure 8.1). In the initial study without antibiotic treatment, GI tract levels of *E. faecium* were ca. 2×10^2 CFU g⁻¹ (accounting for 45% of the cultured population) and 3.26×10^7 CFU g⁻¹ (89% of the cultured population) on the mucosa and in the digesta, respectively, after 10 weeks of dietary administration (Figure 8.1A) (Merrifield *et al.* 2010c). In a similar study Merrifield *et al.* (2010d) investigated the effects of *E. faecium* colonization of the GI tract of rainbow trout pre-treated with dietary oxolinic acid at 20 mg kg⁻¹

for 10 days. Viable counts after oxolinic acid administration were $<10^4$ CFU g⁻¹, but after 10 weeks feeding on *E. faecium* supplemented diets the total culturable counts increased to 10^7 CFU g⁻¹ compared to 10^6 CFU g⁻¹ in the control fed fish. *E. faecium* levels in the probiotic fed fish accounted for over 70% (6.88×10^2 CFU g⁻¹) of the epithelial mucosal populations and nearly 90% (6.07×10^7 CFU g⁻¹) of the digesta populations (Figure 8.1B). Re-population of the GI tract with probiotics after antibiotic administration is likely to create competition for potential pathogens re-entering the digestive tract and stabilize/reinforce the gut microbial defensive barrier, and therefore it is suggested that this topic be further investigated.

Recently Avella *et al.* (2011) administered *E. faecium* IMC 511 to common sole (*Solea solea* Linnaeus, 1758) larvae from the onset of the opening of the mouth (2 dph) to 50 dph. At day 50, *Vibrio* counts were significantly lower than in the control group and LAB levels were significantly higher in the probiotic groups at all time points (10, 30 and 50 dph). RAPD analyses of isolates demonstrated that the *E. faecium* component of cultivable LAB population increased in the probiotic groups with trial duration, peaking at 80–100% at 50 dph. The probiotic was not detected in the control group at any time point. The modulation of GI microbiota was associated with a better growth and a beneficial effect on animal welfare supported by lower levels of 70 kDa Heat Shock Protein.

All of the aforementioned studies have used culture-dependent approaches; to the authors' knowledge only two studies have used a culture-independent approach to assess the effect of enterococci probiotics on the gut microbiota of fish (Sun *et al.* 2012a; Del'Duca *et al.* 2013). Sun *et al.* (2012a) assessed the autochthonous microbiota in the fore-, mid- and hindgut of juvenile grouper after the dietary administration of *E. faecium* MM4, previously isolated from the whole intestine of juvenile grouper (Sun *et al.* 2009), at 10^8 cells g⁻¹ for 60 days. DGGE analysis revealed that the *E. faecium* MM4 successfully populated all three gut regions, of all replicates, but was absent in the control fish. Subsequently, bacterial richness and diversity in the midgut and hindgut were significantly elevated in the probiotic fed fish. Four OTUs (closest known relatives identified as uncultured Betaproteobacterium (82% similarity), uncultured Alphaproteobacterium (85%), *Comamonas* sp. MPI12 (90%) and *Dietzia psychrhalcaliphila* (94%)) were present only in the probiotic group. Del'Duca *et al.* (2013) observed that dietary provision of *Enterococcus* sp. ($>10^6$ bacteria g⁻¹) elevated the total bacterial levels and enterococci levels in the gut of tilapia. In addition, the application led to a reduction in the abundance of putative *Pseudomonas fluorescens*.

Taken together, these studies reveal that *E. faecium* strains possess the capabilities to populate the gut, and modulate the gut microbiota, of rainbow trout, carp, grouper, European eel, common sole and sheet fish.

8.3.4 *Lactococcus* spp.

To the authors' knowledge the only lactococci strains which have been investigated with regards to intestinal colonization and microbial modulation of fish *in vivo* belong to the species *Lactococcus lactis* (Villamil *et al.* 2002; Balcázar *et al.* 2007a; 2007b; 2009; Perez-Sanchez *et al.* 2011; Mohapatra *et al.* 2012; Sun *et al.* 2012b; Table 8.2).

Villamil *et al.* (2002) reported that the *in vivo* adhesion to, and colonization of, the turbot intestine by *Lc. lactis* was low. Despite demonstrating that *Lc. lactis* was able to adhere to turbot intestinal mucus *in vitro*, after the *in vivo* experiment the bacteria were recovered from only one fish (of five sampled, at 1.5×10^3 cells ml⁻¹). It was suggested that the adherence capabilities of *Lc. lactis* could have been reduced *in vivo* by environmental factors such

as GI acids and competition with the indigenous microbiota. Another suggested factor was salinity, which may partially explain the contrary results later observed in freshwater trout (Balcázar *et al.* 2007a; 2007b; 2009) and the marine species turbot (Villamil *et al.* 2002) and grouper (Sun *et al.* 2012b). Balcázar *et al.* (2007a) studied the effect of feeding *Lc. lactis* subsp. *lactis* at 10^6 CFU g^{-1} to brown trout for 2 weeks before reverting to non-supplemented feeds for a further 2 weeks. A clear relationship was evident between the duration of feeding and the recovery of the *Lc. lactis* within the intestinal contents. Peak levels (approaching $\log 8$ CFU g^{-1}) were observed after 2 weeks feeding. *Lc. lactis* displayed an ability to persist within the gut for at least 2 weeks after reverting to non-supplemented diets. A later study by the same research group reported that *Lc. lactis* populations colonized the intestinal mucosa of brown trout (Balcázar *et al.* 2009), and Balcázar *et al.* (2007b) reported that feeding rainbow trout *Lc. lactis* at 10^6 CFU g^{-1} for two weeks led to the successful recovery of the probiont from the intestinal contents at ca. $\log 7.5$ CFU g^{-1} . Sun *et al.* (2012b) assessed the autochthonous microbiota in the fore-, mid- and hindgut of juvenile grouper after the dietary administration of *Lc. lactis* MM1, previously isolated from the whole intestine of juvenile grouper (Sun *et al.* 2009), at 10^8 cells g^{-1} for 60 days. None of the DGGE bands sequenced were identified as *Lc. lactis*, and no unidentified bands were present only in the probiotic group, which may be suggestive that *Lc. lactis* does not colonize the mucosa of this marine fish species under the experimental conditions used. Despite this, bacterial richness and diversity were significantly elevated in all gut regions of the probiotic fed fish, and dendrogramatic analysis revealed that the bacterial profiles of the probiotic fed fish were generally clustered into one group, distinctly different from the corresponding control replicates, in each GI section. In addition, the abundance of some unidentified bacteria was elevated in the probiotic fish and some potential harmful species, like *Staphylococcus saprophyticus*, were decreased.

The topic of salinity and its potential impact on probiotic colonization, particularly of *Lc. lactis*, is worthy of further investigation; to the authors' knowledge no data are currently available regarding the effect of salinity on the efficacy of probiotics to modulate the gut microbiota of fish. The direct effect of salinity on the probiont itself is not the only consideration; the indigenous gut microbiota of marine and freshwater fish are often distinctly different (Sakata 1990; Ringø and Strøm 1994; Hansen and Olafsen 1999). For example, Ringø and Strøm (1994) demonstrated the effect of salinity on the indigenous gut microbiota of Arctic charr (*Salvelinus alpinus*) when reared in freshwater and seawater. Arctic charr reared in seawater presented lower (by 1–2 log scales) cultivable bacterial populations, a lower proportion of *Aeromonas* spp. and a higher proportion of *Vibrio* spp. Future studies should include probiotic feeding administered pre-, during and post-transfer to seawater of anadromous species to help to elucidate the extent of the effect of salinity on the gut microbiota of fish and the subsequent interactions with probiotics.

8.3.5 *Leuconostoc* spp.

To the authors' knowledge the only *Leuconostoc* spp. which has been investigated *in vivo* with regards to the intestinal modulation and colonization of fish are *Leuconostoc mesenteroides* strains (Balcázar *et al.* 2007a; 2007b; 2009; Vendrell *et al.* 2008; Pérez-Sánchez *et al.* 2011; Askarian *et al.* 2011; Table 8.2).

The studies of Balcázar *et al.* (2007a; 2007b) both demonstrated the positive effects of *Leu. mesenteroides* in terms of intestinal presence and subsequent health benefits for trout. Vendrell *et al.* (2008) demonstrated that the presence of high gastric levels of *Leu. mesenteroides*, after

dietary provision, reduced mortalities induced by *Lc. garvieae* in a cohabitation challenge. As it has been reported that the GI tract is a preferred target for *La. garvieae* colonization and a possible route of infection (Vendrell *et al.* 2006), the authors suggested that gastric *Leu. mesenteroides* provided protection by competitive exclusion, hypothesizing that this was mediated, to some extent, by an acidification of fish intestine by LAB administration. However, recent *in vitro* data suggest that endogenous trout gut *Leu. mesenteroides* also produces extracellular proteinaceous compounds which antagonize *Lc. garvieae* (Pérez-Sánchez *et al.* 2011).

A recent study reported that a *Leu. mesenteroides* strain, originally isolated from the GI tract of Persian sturgeon, showed good capacity to populate the GI tract of Persian sturgeon fry when supplemented via the diet; in contrast, however, this was not observed in beluga fry fed a *Leu. mesenteroides* supplemented diet (Askarian *et al.* 2011). Indeed, generally improved growth performance and digestive enzyme activities were observed in Persian sturgeon fry fed *Leu. mesenteroides*, compared with fry fed the control diet and a strain of *Lb. curvatus* (originally isolated from beluga). The authors suggested that these findings presented some evidence of host-specific efficacy of *Leu. mesenteroides* in sturgeons.

8.3.6 *Pediococcus* spp.

To the authors' knowledge the only pediococci which has been investigated with regards to intestinal colonization of fish *in vivo* is *Pediococcus acidilactici* (Gatesoupe 2002; Aubin *et al.* 2005; Shelby *et al.* 2007; Merrifield *et al.* 2010b; 2011; Ferguson *et al.* 2010; Villamil *et al.* 2010; Harper *et al.* 2011; Abid *et al.* 2012; Standen *et al.* 2013; Table 8.2).

Gatesoupe (2002) first demonstrated the potential abilities of *P. acidilactici* to populate the fish intestinal tract *in vivo*. After feeding larval pollack (*Pollachius pollachius*) *Artemia* cysts and nauplii, while introducing *P. acidilactici* into the enrichment process, LAB levels were recovered at ca. $1-2 \times 10^5$ CFU larva⁻¹ in the *P. acidilactici* fed larvae while they were not detected in the control group. The effect on the indigenous microbial populations was not fully elucidated but petrifilm counts and *Vibrio* levels remained unaffected by the introduction of the probiont. More recently it has been reported that uptake of *P. acidilactici* into live feeds is time dependent, with *P. acidilactici* recovery from rotifer cultures supplemented with *P. acidilactici* for 24 h significantly lower than in cultures supplemented for 1 h (Villamil *et al.* 2010). Furthermore, *P. acidilactici* recovery from the GI tract of turbot (*Psetta maxima*) larvae was higher when larvae were fed with 1 h supplemented rotifer cultures than 24 h supplemented rotifers. As demonstrated by these studies, live feeds are a good vector for *P. acidilactici* delivery at the larval stages of fish, which is particularly relevant as the digestive tract is not fully developed and functionality is limited (as further discussed elsewhere: Dimitroglou *et al.* 2011; Chapter 16).

A number of studies have investigated the efficacy of *P. acidilactici* to modulate the microbiota and improve the growth and health status of rainbow trout. A comprehensive study by Aubin *et al.* (2005) not only enumerated probiotic levels within the intestinal tract of rainbow trout but also utilized 16S rRNA sequencing to identify the indigenous, non-probiotic, components of the rainbow trout intestinal tract. Rainbow trout were fed diets containing *P. acidilactici* (1.5×10^6 CFU g⁻¹) for up to 5 months. After 20 days feeding on the experimental diets, epithelium associated *P. acidilactici* levels were recorded at log 2.5 CFU g⁻¹. The indigenous aerobic autochthonous bacteria (ca. log 5–6 CFU g⁻¹) and yeast (ca. log 3–5 CFU g⁻¹) levels in the intestine were not affected by treatment. However, changes in the composition of the microbial populations were evident; of particular interest was the large increase in

the level of *Pseudomonas* spp. and the observation that *Carnobacterium* sp. PT220 was only isolated in fish fed *P. acidilactici*. After 5 months feeding on the experimental diets, *P. acidilactici* levels declined to ca. $\log 0.9 \pm 0.6 \text{ CFU g}^{-1}$ in the probiotic group. The indigenous bacterial levels were significantly lower in the *P. acidilactici* fed fish than in the control, and autochthonous yeast levels were detected at low levels ($\log 0.6 \pm 0.6 \text{ CFU g}^{-1}$) in the *P. acidilactici* fed fish but were not detected in the control group. The composition of the indigenous autochthonous microbiota was distinctly different and less diverse than that sampled from fish after 20 days experimental feeding, with *Carnobacterium* sp. PT220 dominating both fish groups at 5 months. However, as the fish were starved for 24 h prior to sampling the probiotic values reported may not be a true representation of the precise levels during the feeding periods.

Merrifield *et al.* (2011) fed rainbow trout either vegetative or lyophilized *P. acidilactici* cells incorporated into a basal diet at either 10^7 or 10^8 CFU g^{-1} for a period of 10 weeks. Total viable counts of indigenous microbiota associated with the posterior mucosa increased marginally from $\log 3.60 \text{ CFU g}^{-1}$ in the control fish to $\log 3.75\text{--}4.63 \text{ CFU g}^{-1}$ in the probiotic fed fish. Total viable levels of posterior allochthonous microbiota did not differ significantly between groups, ranging from ca. $\log 8 \text{ CFU g}^{-1}$ in the control group to ca. $\log 7.5\text{--}8.5 \text{ CFU g}^{-1}$ in probiotic fed groups. *P. acidilactici* were absent in the control group but successfully populated the posterior intestine (as both epithelium associated and transient lumen populations) in all probiotic groups with no significant differences between vegetative or lyophilized treatments. Digesta based *P. acidilactici* levels were considerably higher than the epithelium based populations (levels approaching $\log 6 \text{ CFU g}^{-1}$) but again no significant differences were observed between dietary levels or supplementation forms. Scanning electron microscopy revealed the presence of *P. acidilactici*-like cells on the intestinal epithelium, particularly at the bases between mucosal folds. Similar results were reported in another rainbow trout study which demonstrated *P. acidilactici* populations on the mucosa of the stomach, proximal intestine and distal intestine (Merrifield *et al.* 2010b) as well as elevated microvilli length in the proximal intestine and increased endocytic activity in the proximal intestine and distal intestine. The effect of this probiotic on the rainbow trout epithelial structure was further studied using the *ex vivo* intestinal sac method, with or without the co-incubation of *Vibrio anguillarum* (Harper *et al.* 2011). DGGE and sequence analyses revealed the presence of these bacteria on the intestinal epithelium after exposure. Although the probiotic was not shown to antagonize the pathogen directly, it did outcompete it, reduce the epithelial pathogen induced damage to some extent, and elevate levels of goblet cells and intraepithelial leukocytes.

The effect of *P. acidilactici* on the gut microbiota of tilapia was reported by Ferguson *et al.* (2010). After feeding dietary *P. acidilactici* at $\log 7 \text{ CFU g}^{-1}$ to tilapia for 32 days, DGGE fingerprints revealed that dietary *P. acidilactici* significantly affected the composition of the intestinal microbial communities as microbial profiles of the probiotic fed fish clustered into a group distinctly different to that of the gut microbiota of the control group and the microbiota of the initial fish (Figure 8.3). The probiotic fed fish displayed lower bacterial species richness and diversity which indicated that the probiont outcompeted some indigenous bacterial populations. Although total culturable aerobic and anaerobic levels within the gut were not affected, the LAB levels were significantly higher in the probiotic group ($\log 7.5 \text{ CFU g}^{-1}$, ca. 10% of the cultured population) than in the control group ($\log 4 \text{ CFU g}^{-1}$, <0.01% of the cultured population). 16S rRNA sequence analysis confirmed the isolates from the probiotic group as *P. acidilactici*. The probiotic was observed to be present for at least 17 days after reverting the probiotic fish to a control diet, and during this period DGGE revealed

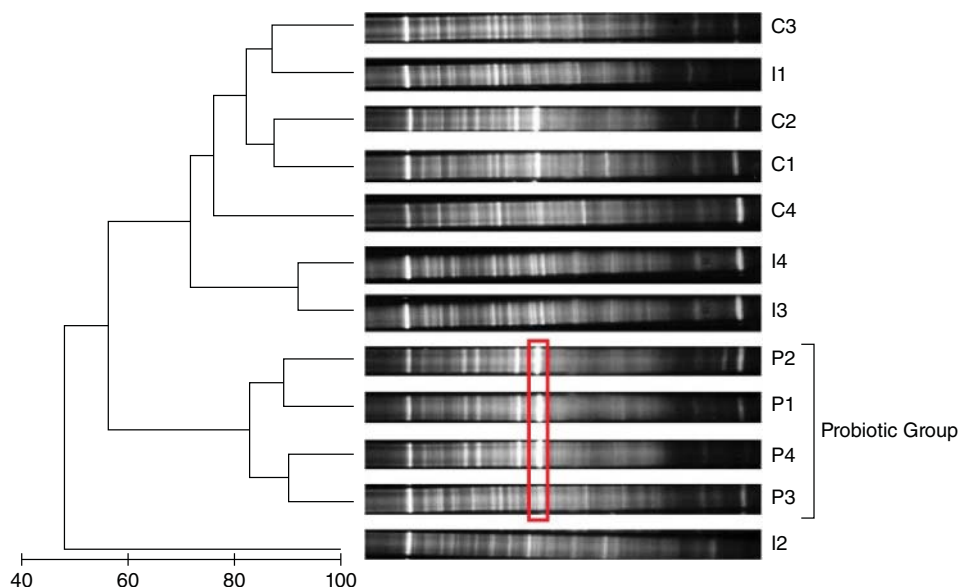


Fig. 8.3 DGGE profiles and dendrogram of red Nile tilapia allochthonous gut microbiota showing resemblance and similarity of the initial (I1–I4; day 0), control (C1–C4; day 32) and *P. acidilactici* (P1–P4; day 32) fed fish. The probiotic is indicated by the box. (Source: Adapted from Ferguson *et al.* 2010. Reproduced with permission of John Wiley & Sons.)

direct antagonism between *P. acidilactici* and an indigenous unidentified bacterial clone (most similar to an unidentified uncultured bacterium from the gut of Atlantic salmon: Liu *et al.* 2008) (Figure 8.4). Low levels of this bacterium were present whilst high levels of the probiotic were present in the intestines, but as the abundance of the probiotic slowly declined with time a clear inverse relationship was observed as the abundance of this phylotype increased. Additionally, an unidentified bacterium from the Actinomycetales order showed a general decline in abundance concomitantly as the probiotic declined and the salmon gut phylotype increased.

8.4 OTHER PROBIOTICS

A number of other bacterial genera have been investigated for use as potential probiotics in fin-fish: for example *Pseudomonas* (Gram *et al.* 1999; 2001; Spanggaard *et al.* 2001; Korkea-aho *et al.* 2011; 2012), *Shewanella* (Sáenz de Rodríguez *et al.* 2009; de La Banda *et al.* 2010), *Phaeobacter* (Hjelm *et al.* 2004; Planas *et al.* 2006) and *Psychrobacter* (Sun *et al.* 2011b; Yang *et al.* 2011). In addition, some early studies investigated members of the *Aeromonas* (Irianto and Austin 2002) and *Vibrio* (Austin *et al.* 1995; Gatesoupe 1997; Ringø and Vadstein 1998; Ringø *et al.* 1996; Irianto and Austin 2002) genera as probiotic candidates; using such strains may not be wise and, even if efficacy is shown to be high, is questionable in terms of safety and regulatory authorization (see Chapter 13).

Some of the aforementioned studies have investigated potential GI colonization or the impact on the endogenous microbiota (refer to Table 8.3). These studies, mostly focused on

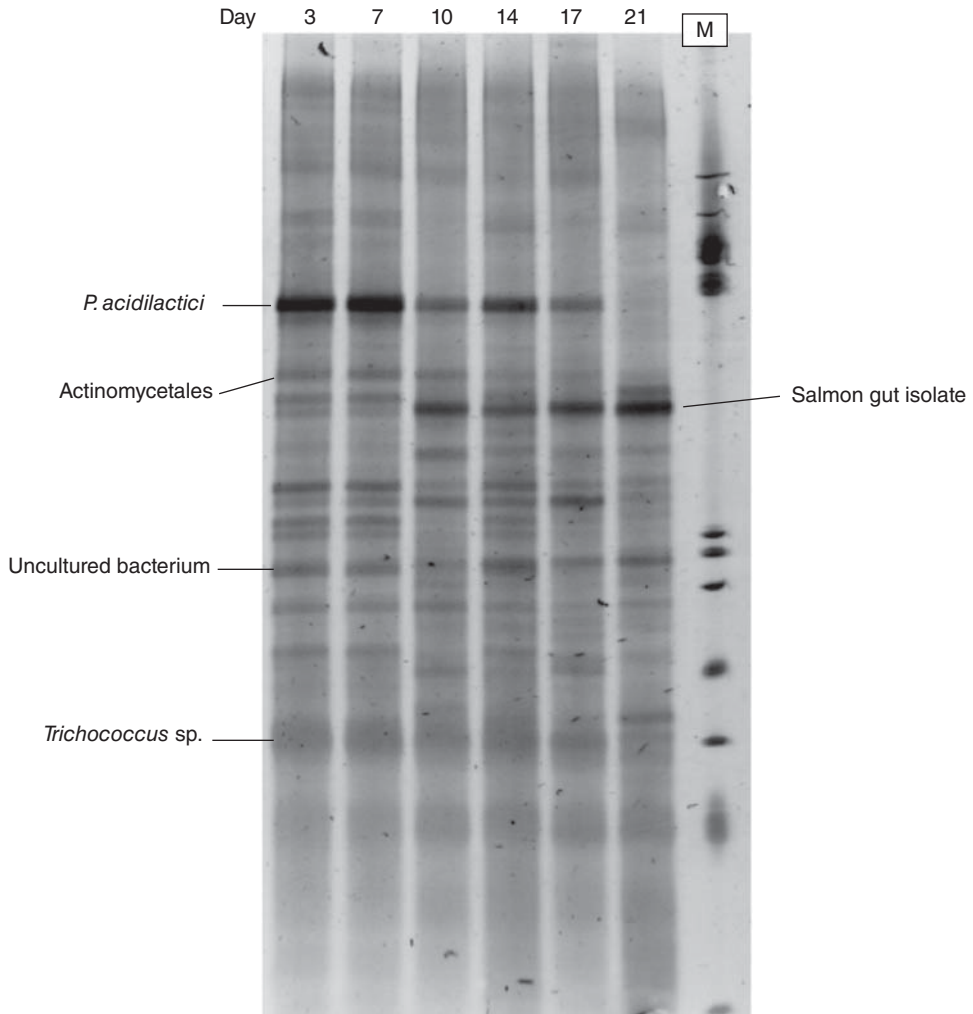


Fig. 8.4 DGGE fingerprints demonstrating the persistence of *P. acidilactici* within the red Nile tilapia intestinal tract after reverting to a non-supplemented control diet. Each lane represents a pooled sample from two fish. Lane M shows arbitrary markers. (Source: Adapted from Ferguson *et al.* 2010. Reproduced with permission of John Wiley & Sons.)

Mediterranean teleosts and salmonid species, have been discussed in detail by Merrifield *et al.* (2010a) and Dimitroglou *et al.* (2011) and thus will not be repeated in this chapter.

However, the bacterial probiotic strains Ppd11 and Pdp13, from the Alteromonadaceae family, are among the most well researched Gram-negative bacterial probiotics in fish and therefore some attention will be paid to these strains (Díaz-Rosales *et al.* 2006; 2009; Seaez de Rodríguez *et al.* 2009; de La Banda *et al.* 2010; Tapia-Paniagua *et al.* 2012). These strains were originally isolated from gilthead sea bream skin and later identified as *Shewanella putrefaciens* and *Shewanella baltica* (Díaz-Rosales *et al.* 2009). These probiotic species have been extensively studied in Senegalese sole and gilthead sea bream and varying benefits have been

Table 8.3 Overview of other bacterial probiotic studies in fish which have investigated gut microbiota.

Probiotic	Fish	Techniques	References
<i>Phaeobacter</i> strain 27-4 ^a	Turbot	Probiotic; plate counts and immunohistochemistry	Planas <i>et al.</i> (2006)
<i>V. alginolyticus</i>	Rainbow trout	Probiotic; conventional tests, API 50CH and API 20E	Irianto and Austin (2002)
Isolate A3-47S (preliminarily ID <i>V. fluvialis</i>)	Rainbow trout	Probiotic; conventional tests, API 50CH and API 20E	Irianto and Austin (2002)
Isolate A3-51 (preliminarily ID <i>A. hydrophila</i>)	Rainbow trout	Probiotic; conventional tests, API 50CH and API 20E	Irianto and Austin (2002)
<i>Arthrobacter</i> sp.	Atlantic cod	TVC, <i>Vibrio</i> and LAB SM; 16S rRNA sequencing	Lauzon <i>et al.</i> (2010a)
<i>Arthrobacter</i> sp.	Atlantic cod	TVC, <i>Vibrio</i> and LAB SM	Lauzon <i>et al.</i> (2010b)
<i>Vibrio</i> sp. (2J18), <i>Shewanella</i> sp. (2J27) and an unidentified Gram-positive (J84)	Senegalese sole	TVC and immunocolony blot ID of probiotics	Makridis <i>et al.</i> (2008)
<i>Pseudomonas</i> M174	Rainbow trout	Probiotic SM; conventional tests and PCR analysis	Korkea-aho <i>et al.</i> (2011)
<i>Pseudomonas</i> M162	Rainbow trout	Probiotic SM; conventional tests and API 20E tests	Korkea-aho <i>et al.</i> (2012)
<i>V. pelagius</i>	Turbot	ELISA	Ringø (1999)
<i>Sh. putrefaciens</i> (Pdp11) and <i>Sh. baltica</i> (Pdp13)	Senegalese sole	DGGE	Sáenz de Rodríguez <i>et al.</i> (2009)
<i>Sh. putrefaciens</i> (Pdp11) and <i>Sh. baltica</i> (Pdp13)	Senegalese sole	DGGE	de La Banda <i>et al.</i> (2010)
<i>Sh. putrefaciens</i> (Pdp11)	Senegalese sole	DGGE	Tapia-Paniagua <i>et al.</i> (2010)

^aDesignated as *Roseobacter* 27-4 in the original article.

Genera abbreviations: *A.* = *Aeromonas*, *Sh.* = *Shewanella*, *V.* = *Vibrio*.

Microbial techniques: conventional tests = standard biochemical and/or phenotypic characterization; probiotic = enumeration of probiotic; SM = selective media; TVC = total viable counts.

documented for growth performance, immunomodulation and disease resistance (for a review see Tapia-Paniagua *et al.* 2012). Some information is also available regarding their ability to modulate the gut microbiota (Seanz de Rodríguez *et al.* 2009; de La Banda *et al.* 2010; Tapia-Paniagua *et al.* 2010). Seanz de Rodríguez and co-authors (2009) used DGGE to investigate dietary administration of *Sh. putrefaciens* (Pdp11) and *Sh. baltica* (Pdp13). After feeding

Table 8.4 Overview of yeast probiotic studies in fish which have investigated gut microbiota.

Probiotic	Fish	Techniques	References
<i>S. cerevisiae</i>	Rainbow trout	TVC; probiont SM; 16S rRNA of probiont; 16S rRNA of indigenous microbiota	Aubin <i>et al.</i> (2005)
	European sea bass	Fluorescent staining; culture based (yeast)	Tovar <i>et al.</i> (2002)
	Pollock	TVC; probiotic SM; SM groups of interest	Gatesoupe (2002)
	Rainbow trout	Culture based (total and yeast); SEM	Andlid <i>et al.</i> (1995)
	Gibel carp	DGGE	He <i>et al.</i> (2011)
<i>S. cerevisiae</i> + (<i>Lc. lactis</i> and/or <i>B. subtilis</i>)	Rohu	TVC, probiont SM; conventional tests	Mohapatra <i>et al.</i> (2012)
<i>D. hansenii</i>	European sea bass	Fluorescent staining; culture based (yeast)	Tovar <i>et al.</i> (2002)
	Gilthead sea bream	DGGE	Tapia-Paniagua <i>et al.</i> (2011)
	Rainbow trout	Culture based (total and yeast); SEM	Andlid <i>et al.</i> (1995)
<i>R. glutinis</i>	Rainbow trout	Culture based (total and yeast); SEM	Andlid <i>et al.</i> (1995)

Senegalese sole supplemented diets for 60 days, DGGE fingerprints revealed intestinal microbial profiles which were clearly distinct from fish receiving the different diets; dendrogrammatic analysis of the DGGE profiles showed that the bacterial communities in the probiotic fed fish clustered together and displayed ca. 50% dissimilarity to the control profiles. Tapia-Paniagua *et al.* (2010) reported that dietary administration of both fresh and lyophilized *Sh. putrefaciens* (Pdp11) modulated the gut microbiota of Senegalese sole: DGGE profiles from both probiotic treatments clustered away from the control replicates, similarity coefficients of within treatment replicates were significantly elevated in both probiotic treatments, and probiotic supplementation led to an increase of the predominant species related to the *Vibrio* genus. Modulation of the Senegalese sole gut bacterial communities by these probiotic strains was confirmed by de La Banda *et al.* (2010), although this study revealed that the extent of the modulation may be dependent on the probiotic strain administered, with *Sh. baltica* inducing a clearer and more consistent effect. Unfortunately, no quantitative data are yet available on the impact of *Shewanella* probiotics on the gut microbiota of fish.

Yeasts have also been investigated for their properties as probiotics for fish and some of these studies have investigated the mechanisms of action and the extent of GI microbial modulation for *Saccharomyces cerevisiae*, *Debaryomyces hansenii* and *Rhodotorula glutinis* (Table 8.4). Readers with a specific interest in yeast are referred to the comprehensive review on yeast in the gut of fish (indigenous and probiotic) by Gatesoupe (2007) and elsewhere (Merrifield *et al.* 2010a; Dimitoglou *et al.* 2011).

8.5 PROBIOTIC COLONIZATION?

Within this book we, like so many others, are perhaps guilty of using the term ‘colonize’ somewhat erroneously or frivolously. According to Conway (1996) a microorganism is able to colonize the GI tract when it can persist there for a long time, by possessing a multiplication rate higher than the expulsion rate. As a result of continual fluxing of environmental conditions, stochastic factors and a high influx of a microbe laden aqueous medium (in particular in brackish and marine fish), the GI tract of fish is far more dynamic than that of terrestrial animals. This leads us to ask the question: *is it possible for a probiont to truly colonize the digestive tract of fish?* It is likely possible with the use of a probiont originally isolated as an indigenous microbe from the host species and administered under favourable and stable conditions, but *has it been achieved?* From the present literature available, the answer is probably no.

Many studies, as have been discussed within this chapter, have recovered the probiont from the GI tract of fish after dietary supplementation. This does not however necessarily constitute colonization. Certainly being present in observable numbers associated with the digesta in the lumen cannot be described as true colonization unless the probiont levels are substantially higher within the GI tract than the level administered in the feed. This would at least indicate that the probiont could multiply at a greater rate than it is expelled and/or killed by the harsh gastric conditions, host immunological parameters and antagonism from indigenous microbial components. A limited number of studies have reported probiotic levels in the GI tract in excess of the dietary level of provision; for example Balcázar *et al.* (2007a; 2007b) reported LAB probiotic levels 1–2 log scales higher in the intestine than the dietary probiotic provision. From the present literature this seems to be the exception rather than the rule; levels recorded in the GI tract or faeces are typically a mere fraction of those initially administered. Bucio Galindo *et al.* (2009) conducted a comprehensive study to investigate the depletion of probiotic presence in fish GI tract utilizing a quantitative method to study the kinetics of the excretion of *Lb. plantarum* 44a in the faeces of tilapia. The study of the kinetic passage of *Lb. plantarum* 44a from feed to faeces was performed on tilapia using 10^{11} , 10^8 , 10^5 and 0 CFU per single feed ration or 10^{11} CFU as follows: 14 times in 14 days, five times in 14 days, once in 14 days and zero times in 14 days, in order to study the number of *Lactobacillus* cells excreted. Using selective media, the kinetics of *Lactobacillus* in the faeces was described: the persistence of *Lb. plantarum* 44a in the GI tract was relatively short and did not appear to be dose related. Peak levels of lactobacilli in faeces were observed between 3 and 5 h after the single dose, and after 72 h the recovery level was generally below 6% of the ingested dose. The authors concluded that the probiont was expelled in short pulses which could be described by a mathematical model which allowed the calculation of the survival rate (Bucio Galindo *et al.* 2009). Further studies are required to quantitatively analyse the kinetic responses of probiotic bacteria within different fish hosts as well as to observe the localized populations possibly present in close association with the mucosal epithelium and within pockets of less fluidic spaces such as the pyloric caeca. By ascertaining such data a more effective feeding strategy conscious of dose, timing and duration can be devised.

Probiotic presence within the mucus layer or in intimate association with the epithelial surface is something that one might describe as colonization with a higher degree of confidence. Such a finding demonstrates an active process whereby positive chemotactic responses to

mucus, and adhesion to and proliferation within mucus, could provide a platform for subsequent probiont interaction and attachment with the host epithelium. The adhesion of probiotic bacteria to fish intestinal epithelial cells has been frequently studied but the mechanisms by which this process is achieved have received little direct attention. According to Ringø *et al.* (2007), bacterial adhesion to epithelial cells involves a number of different types of interactions which depend on the proximity of bacteria to the host cell surface; attraction is initially by van der Waal's forces operating at distances >50 nm but electrostatic interactions become more significant at closer distances. As the cells are usually negatively charged, electrostatic repulsion can hinder and prevent closer association. A closer interaction may occur in regions of lower ionic strength, allowing hydrophobic interaction and specific receptor–ligand binding. Information on specific adhesins and receptors of probiotics in fish is partly described; that is, a number of adhesins have been identified on potential fish probiotics and receptors or receptor analogues have been identified on the cell surface of fish epithelial cells. The interactions of these molecules and their importance in initiating bacterial–host interactions and the subsequent epithelial (and lymphoid) cell responses are inferred from studies with bacterial cells and higher vertebrates. This topic is further discussed elsewhere (Hansen and Olafsen 1999; Ringø *et al.* 2007). Although further research into the mechanisms is warranted, a number of studies report that probionts can populate the intestinal mucus or epithelium of fish.

Can population of the mucus or mucosa be described as colonization? In order to be sure, extensive investigations in the absence of dietary probiotic provision, to assess probiotic persistence, must be conducted. This has been the topic of several studies which have shown that some dietary probiotics have a temporal ability to persist within the GI tract of fish after dietary administration of the probiont ceases (Jöborn *et al.* 1997; Robertson *et al.* 2000; Panigrahi *et al.* 2005; Kim and Austin 2006; Balcázar *et al.* 2007a; Ferguson *et al.* 2010; Abid *et al.* 2012; Ran *et al.* 2012; Table 8.5). To our knowledge, the first study to address this topic was that of Jöborn *et al.* (1997), where culture-dependent methods indicated that *C. inhibens* persisted in the intestinal tract of juvenile rainbow trout for >4 days after reverting to non-supplemented diets. Robertson *et al.* (2000) reported that the same *C. inhibens* strain could be re-isolated from the intestine of rainbow trout fingerlings and fry for up to 4 and 10 days, respectively, after reverting to feeding with control diets. A more recent study using a culture-independent approach, DGGE, demonstrated that *C. divergens* (and *C. maltaromaticum*) could persist in the intestine of rainbow trout for at least 3 weeks (Kim and Austin 2006).

It is clear that the efficacy of probiotics to persist within the GI tract of fish differs among fish species, with differing host selective pressures, and different rearing conditions. For example, Ferguson *et al.* (2010) reported that *P. acidilactici* could remain present in the GI tract of tilapia for at least 17 days after the cessation of dietary administration (Figure 8.4), whereas the same probiotic strain persisted in the intestinal tract of rainbow trout for less than 3 days after reverting to a control diet (Abid *et al.* 2012). This large discrepancy is likely to be due to a range of factors, including the different microbial communities present in the GI tracts of the respective species and the variation in the GI selection pressures, but the most obvious factor is the large difference in rearing temperatures (15 °C in trout versus ca. 25–26 °C in tilapia); optimum growth for pediococci is at 25–40 °C (Holt and Bergey 1994) and so lower temperature applications are likely to reduce the bacterial multiplication rates

Table 8.5 Probiotic persistence in the GI tract of fish after the cessation of dietary provision.

Probiotic	Fish species	Persistence duration	Methodology and notes	Reference
<i>C. divergens</i> ^a	Rainbow trout	>4 days	Culture based; probiont decreased from ca. log 8 CFU g ⁻¹ at the end of the feeding period to log 5 CFU g ⁻¹ after cessation of probiotic feeding for 4 days	Jöborn <i>et al.</i> (1997)
<i>C. divergens</i>	Rainbow trout	>3 weeks	DGGE	Kim and Austin (2006)
<i>C. maltaromaticum</i>	Rainbow trout	>3 weeks	DGGE	Kim and Austin (2006)
<i>C. inhibens</i>	Rainbow trout	<6 days (fingerlings); <10 days (fry)	Culture based	Robertson <i>et al.</i> (2000)
<i>Lb. rhamnosus</i>	Rainbow trout	<15 days	Culture based approaches revealed no detectable probiotic levels after 15 days cessation of provision, no further data at other time points	Panigrahi <i>et al.</i> (2005)
<i>Lb. sakei</i>	Brown trout	>2 weeks	Culture based	Balcázar <i>et al.</i> (2007a)
<i>Lb. plantarum</i>	Tilapia	<3 days	Culture based	Bucio Galindo <i>et al.</i> (2009)
<i>Leu. mesenteroides</i>	Brown trout	>2 weeks	Culture based	Balcázar <i>et al.</i> (2007a)
<i>Lc. lactis</i>	Brown trout	>2 weeks	Culture based	Balcázar <i>et al.</i> (2007a)
<i>P. acidilactici</i>	Tilapia	>17, <21 days	DGGE detected the presence of the probiont 17 days post cessation of provision, but not at day 21. Culture based data revealed that LAB levels decreased to levels comparable to the control after 14 days	Ferguson <i>et al.</i> (2010)
<i>P. acidilactici</i>	Rainbow trout	>1, <3 days	Culture based	Abid <i>et al.</i> (2012)

Table 8.5 (continued)

Probiotic	Fish species	Persistence duration	Methodology and notes	Reference
<i>B. amyloliquefaciens</i>	Tilapia	n.d.	Culture based. Probiotic not identified but TVC remained higher than the control and <i>Lactobacillus</i> sp. treatments after 40 or 61 days cessation of probiotic provision	Ridha and Azad (2012)
Multiple <i>Bacillus</i> strains	Channel catfish	>3 days	Culture based	Ran <i>et al.</i> (2012)
<i>Pseudomonas</i> sp. M174	Rainbow trout	2 weeks	Culture based approach identified one probiont colony present 2 weeks after cessation of provision	Korkea-aho <i>et al.</i> (2011)
<i>Pseudomonas</i> M162	Rainbow trout	>1 week	Culture based approaches revealed that during the feeding phase the probiont accounted for 23.7% of the cultivable counts; it accounted for 0.8% of the isolates after 1 week of non-supplemented feeding	Korkea-aho <i>et al.</i> (2012)

^aPreviously designated as *Carnobacterium* strain K1.
n.d. = not determined.

and likely the persistence post cessation of probiotic supplementation. To our knowledge the longest recorded period for recovery of probiotics after the cessation of dietary provision reported in the present literature is 3 weeks.

Current literature demonstrates the ability of some probionts to show a temporal level of colonization both during, and shortly after, dietary supplementation. In addition it has recently been reported that application of dietary *B. amyloliquefaciens* significantly elevated tilapia GI tract TVC levels, compared to the control and *Lactobacillus* sp. fed groups, and these levels remained higher 61 days after the cessation of probiotic provision (Ridha and Azad 2012). Unfortunately the probiont levels were not presented in this study. Future studies should seek to investigate whether true colonization is possible, and terms such as ‘colonization’, when used to describe probiont levels isolated from the gut (especially in the context of gut contents), should be replaced with terms such as ‘populate’ or ‘temporal colonization’ which are more appropriate phrases to use when discussing probiotic persistence and presence within the GI tract.

8.6 CONCLUSION AND FUTURE PERSPECTIVES

It is clear that more emphasis in future studies should be placed on elucidating the extent of probiotic modulation of the gut microbiota of fish, either as a direct implantation of the probiotic

population and/or via changes in the indigenous microbial populations, in order to be able to apply probiotic applications with greater efficacy. We suggest that future studies assessing the effects of probiotics on fish production and health must, as a golden rule, conduct investigations of the gut microbiota. The very minimum that should be expected is the enumeration of the probiotic and an overview of the impact on the indigenous microbes. However, it is hoped and indeed expected that the application of next-generation sequencing approaches to quantify the impact on indigenous populations and scanning confocal laser microscopy to observe potential probiotic colonization on mucosal folds will become more common in future investigations.

The present literature is heavily focused on the bacterial microbiota and considerably less information is available on indigenous yeast, bacteriophages, Archaea, microalgae and protozoans. In particular bacteriophage levels and their influence on indigenous and probiotic bacteria must be the subject of future studies. The ‘kill the winner’ hypothesis could shape the gastric microbiome (Mills *et al.* 2013) and may also have important implications for probiotics should the probiotic bacteria become dominant members of the microbiota. Surveys for probiotic-specific bacteriophages should be undertaken in the fish environment, water and gut contents in order to ascertain the potential danger to the probiont.

Furthermore, it is evident that the present studies have focused mainly on microbial abundance, with functionality and activity all but unaddressed. Metagenomic and metabolomic studies should be used to ascertain bacterial activities and proteomic studies should be used to ascertain the host mucosal responses. These approaches will build on the foundation of studies which have thus far only partly described the host mucosal response to probiotics, and modulated microbial populations, using genomics, histochemistry and electron microscopy. Indeed, it is this primary host benefit, the improvement of microbial balance and activity at the host mucosal interface, which effectively drives the secondary host benefits in respect to systemic health, disease resistance and growth performance. This point must not be forgotten or overlooked.

8.7 ACKNOWLEDGEMENTS

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9 Probiotic Applications in Cold Water Fish Species

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ABSTRACT

Cold water fish species are an important contributor to aquaculture production in many countries. However, in large-scale production facilities, where aquatic animals are exposed to stressful conditions, problems related to disease and deterioration of environmental conditions often occur and result in serious economic losses. Concerted research efforts have therefore been focused on optimizing production through environmentally friendly alternatives to the use of chemotherapeutic agents. Probiotics offer a potential alternative because they may confer benefits to the host by providing both nutritional benefit and protection against pathogens. Some benefits linked to the administration of probiotics include the production of inhibitory compounds, competition for essential nutrients and adhesion sites, inhibition of virulence gene expression or disruption of quorum sensing, the supply of essential nutrients and enzymes resulting in enhanced nutrition, and the modulation of interactions with the environment and the development of beneficial immune responses. Although many *in vivo* studies suggest that probiotics may confer benefits to cold water fish species, future studies should be focused on providing practical industrial-scale applications, including a cost–benefit analysis. Additionally, a better understanding of microbe–host and microbe–microbe interactions in the gastrointestinal (GI) tract and surrounding environment is needed in order to achieve an optimal utilization.

9.1 INTRODUCTION

To achieve a sustainable aquaculture industry, diversification of fish species may be considered as the first step towards success. Several cold water fish species (loosely defined

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herein as fish with optimal growth performance at sub 20 °C) have been selected for rearing activity. These include members of the Salmonidae, Percidae and Gadidae as well as flatfish and some other marine and freshwater species. Although the industry has expanded and intensified, the emergence of a large variety of pathogens is considered to be a major limiting factor. Concerted research efforts have therefore been focused on optimizing production through environmentally friendly alternatives to the use of antibiotics (Merrifield *et al.* 2010a; Pérez-Sánchez *et al.* 2013).

The use of animals raised under germ-free conditions has revealed that the endogenous microbiotas promote the development and maturation of the host immune system (Rawls *et al.* 2004; Galindo-Villegas *et al.* 2012). Evidence obtained through these studies suggests that the gut microbiota has an important impact on immune regulatory functions, which has resulted in the promotion of the use of probiotics. Probiotics have been defined by the Food and Agriculture Organization (FAO) and World Health Organization (WHO) as ‘live microorganisms which, when administered in adequate amounts, confer a health benefit on the host’ (FAO/WHO 2001). Intake of probiotics has been demonstrated to modulate the gut microbiota, and therefore assist in returning a microbiota that has been disturbed (by antibiotics or other risk factors) to a beneficial composition. Mechanisms by which this is achieved include the production of inhibitory compounds against pathogens, competition for essential nutrients and adhesion sites, inhibition of virulence gene expression or disruption of quorum sensing, the supply of essential nutrients and enzymes resulting in enhanced nutrition, and the modulation of interactions with the environment and the development of beneficial immune responses (Balcázar *et al.* 2006; Merrifield *et al.* 2010a; Pérez-Sánchez *et al.* 2013).

Current research suggests that probiotics may confer benefits on several cold water fish species. Evaluation of candidate probiotics in cold water fish has been carried out at various rearing stages and by different approaches. Several probiotic microorganisms tested in fish aquaculture have been selected based on their effects in humans (Nikoskelainen *et al.* 2001a; Panigrahi *et al.* 2004) and terrestrial livestock (Gatesoupe 2002; El-Haroun *et al.* 2006; Suzer *et al.* 2008). These solutions may prove safe for human consumption but their efficiency in an unrelated environment, such as cold water fish, may be expected to be poor due to the different requirements of mesophilic microbes. Other research has focused on the selection of autochthonous microorganisms, that is, those originating from the respective aquatic animals, or on those isolated from the host’s environment (Balcázar *et al.* 2008; Azad and Al-Marzouk 2008; Pérez *et al.* 2010). Potential probiotics tested in cold water fish include both Gram-positive (e.g. lactic acid bacteria (LAB), *Brochothrix thermosphacta*, *Arthrobacter*, *Bacillus* and *Kocuria* spp.) and Gram-negative (e.g. *Aeromonas*, *Gluconobacter*, *Paracoccus*, *Phaeobacter*, *Phenylobacterium*, *Photobacterium*, *Pseudomonas*, *Pseudoalteromonas*, *Psychrobacter*, *Shewanella* and *Vibrio* spp.) bacteria as well as yeasts (*Saccharomyces cerevisiae*, *Debaryomyces hansenii* and *Rhodotorula glutinis*).

The aim of the present chapter is to present information on the use of probiotic bacteria in salmonids and other relevant cold water aquaculture fish species. The results cited in the present review include studies published in peer-reviewed journals and abstracts presented at national and international conferences. The information presented is organized according to fish families following the current recognized taxonomical structure (FishBase 2013).

9.2 SALMONIDAE

Salmonids are one of the most important farmed fish groups; in 2011 salmonid aquaculture production exceeded 2.7 million tonnes and was valued at over \$14 billion. As such, considerable information is available on the applications of probiotics for the main aquacultured salmonid species (refer to Tables 9.1 and 9.2). Readers with a specific interest in salmonids, beyond the information presented here, are referred to the review on probiotics in salmonids of Merrifield *et al.* (2010a).

9.2.1 Rainbow trout (*Oncorhynchus mykiss*)

The production of rainbow trout has grown exponentially since the 1950s, especially in Europe and more recently in Chile. The largest producers are currently Chile, Norway, and to a lesser extent Turkey, Denmark, Iran, France, Italy, USA, Spain, Germany, Poland, Finland and the UK (Stevenson 2007). However, diseases have become a major limiting factor for trout production. Traditional control strategies are based on the use of chemotherapeutic agents and vaccination; however, the use of antibiotics should be limited due to the emergence of antibiotic-resistant bacteria (Balcázar *et al.* 2006; Cabello 2006; Romero *et al.* 2012). Although the prevention of disease by vaccination is increasing, the process is impractical and often ineffective when applied to juvenile fish because they are not fully immunocompetent and do not always respond to vaccination (Gram *et al.* 1999).

Probiotics have been considered a more environmentally friendly approach to disease management in these species, as they could compete with pathogenic bacteria. Different probiotic bacteria have been tested for the control of the most important cold water bacterial pathogens, including *Flavobacterium psychrophilum* (Ström-Bestor and Wiklund 2011; Burbank *et al.* 2011; 2012), *Lactococcus garvieae* (Brunt and Austin 2005; Vendrell *et al.* 2008), *Aeromonas salmonicida* (Irianto and Austin 2002; Balcázar *et al.* 2007a), *Vibrio* (*Listonella*) *anguillarum* (Brunt *et al.* 2007; Harper *et al.* 2011) and *Yersinia ruckeri* (Raida *et al.* 2003; Brunt *et al.* 2007). A summary of probiotic studies assessing disease resistance in rainbow trout is shown in Table 9.2.

The most common mechanisms by which probiotics may prevent bacterial diseases are the creation of a hostile environment for pathogens by the production of inhibitory compounds, by competing for essential nutrients and adhesion sites, or by modulating the host immune responses (Balcázar *et al.* 2006; Merrifield *et al.* 2010a). Previous studies have suggested that competition for nutrients and adhesion receptors may provide protection against *A. salmonicida* and *Lc. garvieae* after the administration of *Leuconostoc mesenteroides* in rainbow trout (Balcázar *et al.* 2007b; Vendrell *et al.* 2008). The ability to colonize mucosal surfaces is an important criterion when selecting potential probiotics (Balcázar *et al.* 2006; Vine *et al.* 2006). Burbank *et al.* (2012) isolated two potential probiotics able to provide an alternative strategy for managing *F. psychrophilum* infections, mainly mediated through mechanisms based on colonization of fish skin and competitive exclusion at the site of infection. Similarly, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Lactobacillus sakei* reduced the adhesion of *A. salmonicida* and *Y. ruckeri* to intestinal mucus from rainbow trout (Balcázar *et al.* 2007c). Brunt *et al.* (2007) demonstrated that *Aeromonas sobria* GC2 and *Bacillus*

Table 9.1 Summary of potential probiotic applications for salmonids.

Potential probiotic (fish species)	Parameters investigated	References
Rainbow trout (<i>Oncorhynchus mykiss</i>)		
<i>Lc. lactis</i> , <i>Leu. mesenteroides</i> , <i>Lb. sakei</i>	DR, IR, GM	Balcázar <i>et al.</i> (2007b) ^a
<i>Leu. mesenteroides</i> , <i>Lb. plantarum</i>	DR, GM, GP	Vendrell <i>et al.</i> (2008) ^a
<i>Lb. rhamnosus</i>	DR, GP	Nikoskelainen <i>et al.</i> (2001a) ^a
<i>Lb. rhamnosus</i> , <i>Lb. casei</i> , <i>Lb. bulgaricus</i> , <i>Lb. johnsonii</i> , <i>Bifidobacterium lactis</i> , <i>E. faecium</i>	PA	Nikoskelainen <i>et al.</i> (2001b) ^b
<i>Lb. rhamnosus</i>	GM, IR	Nikoskelainen <i>et al.</i> (2003) ^a
<i>Lb. rhamnosus</i>	GM, IR	Panigrahi <i>et al.</i> (2004) ^a
<i>Lb. rhamnosus</i>	GM, IR	Panigrahi <i>et al.</i> (2005) ^a
<i>Lb. rhamnosus</i> , <i>B. subtilis</i> , <i>E. faecium</i>	IR	Panigrahi <i>et al.</i> (2007) ^{a,b}
<i>Lb. rhamnosus</i>	IR	Panigrahi <i>et al.</i> (2010) ^a
<i>Lb. rhamnosus</i>	IR, GM, PA	Panigrahi <i>et al.</i> (2011) ^a
<i>Lb. plantarum</i>	DR, IR	Pérez-Sánchez <i>et al.</i> (2011) ^a
<i>Lb. casei</i>	DR, GM, IR	Min <i>et al.</i> (2012) ^a
<i>E. faecalis</i>	BC, DR, GP, IR	Rodriguez-Estrada <i>et al.</i> (2009) ^a
<i>B. subtilis</i>	DR, GM, IR	Newaj-Fyzul <i>et al.</i> (2007)
<i>B. subtilis</i> + <i>B. licheniformis</i>	DR, IR	Raida <i>et al.</i> (2003) ^a
<i>B. subtilis</i> + <i>B. licheniformis</i>	BC, FU, GM, GP	Bagheri <i>et al.</i> (2008) ^a
<i>B. subtilis</i> + <i>B. licheniformis</i> , <i>E. faecium</i>	BC, FU, GM, GP, IR	Merrifield <i>et al.</i> (2010b; 2010c) ^a
<i>B. subtilis</i> + <i>B. licheniformis</i> , <i>E. faecium</i> , <i>P. acidilactici</i>	GH, GM	Merrifield <i>et al.</i> (2010d) ^a
<i>Bacillus</i> sp., <i>A. sobria</i>	DR, IR	Brunt <i>et al.</i> (2007) ^a
<i>Bacillus</i> sp., <i>A. sobria</i>	IR	Brunt <i>et al.</i> (2008) ^a
<i>P. acidilactici</i>	BC, FU, GM, GP, IR	Merrifield <i>et al.</i> (2011) ^a
<i>P. acidilactici</i>	DR, GH, GM	Harper <i>et al.</i> (2011) ^a
<i>P. acidilactici</i>	GH, GM, IR	Abid <i>et al.</i> (2012) ^a
<i>P. acidilactici</i> , <i>S. cerevisiae</i>	BC, FU, GM, GP, SM	Aubin <i>et al.</i> (2005a; 2005b) ^a
<i>P. acidilactici</i> , <i>S. cerevisiae</i>	DR, IR	Quentel <i>et al.</i> (2004) ^a
<i>C. divergens</i> , <i>C. maltaromaticum</i>	IR	Kim and Austin (2006a) ^a
<i>C. divergens</i> , <i>C. maltaromaticum</i>	IR	Kim and Austin (2006b) ^b
<i>C. inhibens</i> ^c	DR, GM	Robertson <i>et al.</i> (2000) ^a
<i>A. sobria</i>	DR, IR	Brunt and Austin (2005) ^{a,b}
<i>A. sobria</i> , <i>Brochothrix thermosphacta</i>	DR, IR	Pieters <i>et al.</i> (2008) ^a
<i>A. sobria</i>	DR, IR, PA	Arijo <i>et al.</i> (2008) ^{a,b}
<i>A. sobria</i> , <i>B. subtilis</i>	DR	Abbass <i>et al.</i> (2010) ^a
<i>A. hydrophila</i> , <i>V. fluvialis</i> , <i>Carnobacterium</i> spp., <i>C. inhibens</i> , <i>V. alginolyticus</i> and an unidentified Gram-positive coccus	DR, IR, GM	Irianto and Austin (2002) ^a
<i>A. hydrophila</i> , <i>V. fluvialis</i> , <i>Carnobacterium</i> spp., unidentified Gram-positive coccus	DR, IR	Irianto and Austin (2003) ^a
<i>Ps. fluorescens</i> , <i>Pseudomonas</i> strains, <i>Carnobacterium</i> strains	DR, PA	Spanggaard <i>et al.</i> (2001) ^{a,b}
<i>Ps. fluorescens</i>	DR, PA	Gram <i>et al.</i> (1999) ^{a,b}
<i>Pseudomonas</i> M174	DR, IR, PA	Korkea-aho <i>et al.</i> (2011) ^{a,b}

Table 9.1 (continued)

Potential probiotic (fish species)	Parameters investigated	References
<i>Pseudomonas</i> M162	DR, GM, IR, PA	Korkea-aho <i>et al.</i> (2012) ^{a,b}
<i>Pseudomonas</i> sp. MSB1	PA	Ström-Bestor and Wiklund (2011) ^b
<i>Kocuria</i> sp. SM1	DR, IR	Sharifuzzaman and Austin (2009) ^a
<i>Enterobacter cloacae</i> , <i>Bacillus mojavensis</i>	DR, IR	Capkin and Altinok (2009) ^a
<i>Enterobacter amnigenus</i> , <i>Enterobacter</i> sp.	DR, GM	Burbank <i>et al.</i> (2011) ^a
<i>A. sobria</i> , <i>A. caviae</i> , <i>Plesiomonas shigelloides</i> , <i>Hafnia alvei</i> , <i>Enterobacter</i> sp., <i>Citrobacter freundii</i> , <i>Lysinibacillus fusiformis</i> , <i>Staphylococcus equorum</i>	PA	Burbank <i>et al.</i> (2012) ^b
<i>S. cerevisiae</i> , <i>D. hansenii</i> , <i>R. glutinis</i>	GM	Andlid <i>et al.</i> (1995) ^a
<i>S. cerevisiae</i>	EA, GM	Waché <i>et al.</i> (2006) ^a
<i>S. cerevisiae</i>	DR, GM, GP, IR	Tukmechi <i>et al.</i> (2011) ^a
<i>S. cerevisiae</i>	BC, EA, GP, IR	Sheikhzadeh <i>et al.</i> (2012) ^a
Brown trout (<i>Salmo trutta</i>)		
<i>Lc. lactis</i> , <i>Leu. mesenteroides</i> , <i>Lb. sakei</i>	GM, IR	Balcázar <i>et al.</i> (2007a) ^a
<i>Lc. lactis</i> , <i>Leu. mesenteroides</i>	DR, IR	Balcázar <i>et al.</i> (2009) ^a
Autochthonous trout bacteria	PA	Carbajal-González <i>et al.</i> (2013) ^b
Brook trout (<i>Salvelinus fontinalis</i>)		
<i>Luteimonas aestuarii</i> , <i>Rhodococcus cercidiphylli</i> , <i>Rhodococcus qingshengii</i> M. oxydans, <i>Sphingopyxis bauzanensis</i> , <i>Leucobacter luti</i> , <i>Dietzia maris</i>	DR, PA	Boutin <i>et al.</i> (2012) ^{a,b}
Atlantic salmon (<i>Salmo salar</i>)		
<i>C. inhibens</i> ^c	DR	Robertson <i>et al.</i> (2000) ^a
<i>C. divergens</i> ^d	DR, GM, GP	Gildberg <i>et al.</i> (1995) ^a
<i>Lb. delbrueckii</i>	GH, DR, GM	Salinas <i>et al.</i> (2008) ^a
<i>C. divergens</i>	GH, DR, GM	Ringø <i>et al.</i> (2007a) ^b
<i>C. divergens</i>	GH, GM, PA	Kristiansen <i>et al.</i> (2011) ^{a,b}
<i>Ps. fluorescens</i>	DR, PA	Gram <i>et al.</i> (2001) ^{a,b}
<i>Ps. fluorescens</i>	DR	Smith and Davey (1993) ^a
<i>V. alginolyticus</i>	DR, PA	Austin <i>et al.</i> (1995) ^{a,b}

Source: Adapted from Merrifield *et al.* 2010.^aIn vivo studies.^bIn vitro experiments.^cPreviously *Carnobacterium* strain K1, reclassified as *Carnobacterium inhibens* (Jöborn *et al.* 1999).^dPreviously *Lb. plantarum*, reclassified as *Carnobacterium divergens* (Ringø *et al.* 2001).Genera abbreviations: A. = *Aeromonas*, B. = *Bacillus*, C. = *Carnobacterium*, Ct. = *Citrobacter*, D. = *Debaryomyces*, E. = *Enterococcus*, Lb. = *Lactobacillus*, Lc. = *Lactococcus*, Leu. = *Leuconostoc*, P. = *Pediococcus*, Ps. = *Pseudomonas*, R. = *Rhodotorula*, S. = *Saccharomyces*, V. = *Vibrio*.

Parameters investigated: BC = body composition, DR = disease resistance, EA = enzyme activities, FU = feed utilization, GH = gut histology, GM = gut microbiota (inclusive of probiotic colonization), GP = growth performance, IR = immunological/haematological response, PA = pathogen antagonism/competition, SM = skeletal malformation.

Table 9.2 Probiotic applications for improving disease resistance of salmonids.

Disease	Causative agent	Species	Challenge method	Potential probiotic	Reduced mortalities	Notes	Reference
Streptococcosis	<i>Str. iniae</i>	<i>O. mykiss</i>	IP	<i>A. sobria</i>	Yes	Live cells, disrupted cells and cell free supernatant effective. Formalized cells ineffective	Brunt <i>et al.</i> (2005)
Lactococcosis	<i>Str. iniae</i>	<i>O. mykiss</i>	IP	<i>A. sobria</i> , <i>Bacillus</i> sp.	Yes	Live cells, disrupted cells and cell free supernatant effective. Formalized cells ineffective	Brunt <i>et al.</i> (2007) Brunt and Austin (2005)
	<i>Lc. garvieae</i>			<i>A. sobria</i>	Yes		
	<i>Lc. garvieae</i>			<i>A. sobria</i> , <i>Bacillus</i> sp. <i>Leu. mesenteroides</i> , <i>Lb. plantarum</i> <i>Lb. plantarum</i>	Yes Yes Yes		
Furunculosis	<i>Lc. garvieae</i>	<i>O. mykiss</i>	IP	<i>A. sobria</i> , <i>Bacillus</i> sp.	Yes	Reduced fry and fingerling mortalities	Brunt <i>et al.</i> (2007) Vendrell <i>et al.</i> (2008) Pérez-Sánchez <i>et al.</i> (2011) Brunt <i>et al.</i> (2007) Irianto and Austin (2002)
	<i>Lc. garvieae</i>			<i>Leu. mesenteroides</i> , <i>Lb. plantarum</i>	Yes		
	<i>Lc. garvieae</i>			<i>Lb. plantarum</i>	Yes		
	<i>A. salmonicida</i>			<i>A. sobria</i> , <i>Bacillus</i> sp.	Yes		
	<i>A. salmonicida</i>			<i>A. hydrophila</i> , <i>Vibrio</i> sp., an unidentified	Yes		
	<i>A. salmonicida</i>			<i>Carnobacterium</i> sp., an unidentified	Yes		
	<i>A. salmonicida</i>	<i>O. mykiss</i>	CO	Gram-positive coccus	Yes	Dead probiotic cells used	Irianto and Austin (2003)
				<i>A. hydrophila</i> , <i>V. fluvialis</i> , <i>Carnobacterium</i> sp., an unidentified			
	<i>A. salmonicida</i>			Gram-positive coccus			
				<i>Lc. lactis</i> , <i>Leu. mesenteroides</i> and <i>Lb. sakei</i>			
	<i>A. salmonicida</i>			<i>Lb. rhamnosus</i>			
	<i>A. salmonicida</i>			<i>C. inhibens</i> ^a			
					Yes		Balcázar <i>et al.</i> (2007b)
					Yes		Nikoskelainen <i>et al.</i> (2001a)
					Yes		Robertson <i>et al.</i> (2000)

<i>A. salmonicida</i>	<i>S. salar</i>	CO	<i>C. inhibens^a</i>	Yes	Robertson <i>et al.</i> (2000)
<i>A. salmonicida</i>		CO	<i>C. divergens</i>	No	Gildberg <i>et al.</i> (1995)
<i>A. salmonicida</i>		CO	<i>Ps. fluorescens</i>	No	Gram <i>et al.</i> (2001)
<i>A. salmonicida</i>		-	<i>Ps. fluorescens</i>	Yes	Smith and Davey (1993)
<i>A. salmonicida</i>		IMM	<i>V. alginolyticus</i>	Yes	Austin <i>et al.</i> (1995)
<i>A. salmonicida</i>		Ex vivo	<i>C. divergens</i>	NA	Ringø <i>et al.</i> (2007a)
<i>A. salmonicida</i>		Ex vivo	<i>Lb. delbrueckii</i>	NA	Salinas <i>et al.</i> (2008)
<i>A. salmonicida</i>	<i>S. trutta</i>	Asymptomatic carrier	<i>Lc. lactis</i> , <i>Leu. mesenteroides</i>	Yes	Balcázar <i>et al.</i> (2009)
<i>V. anguillarum</i>	<i>O. mykiss</i>	IMM	<i>Pseudomonas</i> sp., <i>Carnobacterium</i> sp.	Yes/no	Spanggaard <i>et al.</i> (2001)
<i>V. anguillarum</i>		IMM	<i>Ps. fluorescens</i>	Yes	Gram <i>et al.</i> (1999)
<i>V. anguillarum</i>		IP	<i>A. sobria</i> , <i>Bacillus</i> sp.	Yes	Brunt <i>et al.</i> (2007)
<i>V. anguillarum</i>		IP	<i>E. faecalis</i>	Yes	Rodriguez-Estrada <i>et al.</i> (2009)
<i>V. anguillarum</i>		Ex vivo	<i>P. acidilactici</i>	NA	Harper <i>et al.</i> (2011)
<i>V. ordalii</i>		IP	<i>A. sobria</i> , <i>Bacillus</i> sp.	Yes	Brunt <i>et al.</i> (2007)
<i>V. harveyi</i>		IP	<i>A. sobria</i>	Yes	Arijo <i>et al.</i> (2008)
<i>V. anguillarum</i>	<i>S. salar</i>	CO	<i>C. inhibens^a</i>	No	Robertson <i>et al.</i> (2000)

(continued overleaf)

Table 9.2 (continued)

Disease	Causative agent	Species	Challenge method	Potential probiotic	Reduced mortalities	Notes	Reference
	<i>V. anguillarum</i>		IM	<i>V. alginolyticus</i>	Yes	Added to tank water	Austin <i>et al.</i> (1995)
	<i>V. anguillarum</i>		Ex vivo	<i>C. divergens</i>	NA	Prevent to some extent pathogen-induced gut damage	Ringø <i>et al.</i> (2007b)
	<i>V. ordalii</i>		CO	<i>C. inhibens</i> ^a	Yes		Robertson <i>et al.</i> (2000)
	<i>V. ordalii</i>		IMM	<i>V. alginolyticus</i>	Yes	Added to tank water	Austin <i>et al.</i> (1995)
ERM	<i>Y. ruckeri</i>	<i>O. mykiss</i>	IP	<i>A. sobria</i> , <i>Bacillus</i> sp.	Yes		Brunt <i>et al.</i> (2007)
	<i>Y. ruckeri</i>		IP	<i>A. sobria</i> , <i>B. subtilis</i>	Yes	Live cells and cellular components	Abbass <i>et al.</i> (2010)
	<i>Y. ruckeri</i>		IP	<i>B. subtilis</i> + <i>B. licheniformis</i>	Yes		Raida <i>et al.</i> (2003)
	<i>Y. ruckeri</i>		CO	<i>C. inhibens</i> ^a	Yes		Robertson <i>et al.</i> (2000)
	<i>Y. ruckeri</i>		IMM	<i>Enterobacter cloacae</i> , <i>B. mojavensis</i>	Yes		Capkin and Alinok (2009)
	<i>Y. ruckeri</i>		IP	<i>P. acidilactici</i> ; <i>S. cerevisiae</i> var. <i>boulardii</i>	Yes		Aubin <i>et al.</i> (2005b), Quentel <i>et al.</i> (2004)
	<i>Y. ruckeri</i>		IP	<i>S. cerevisiae</i>	Yes		Tukmechi <i>et al.</i> (2011)
	<i>Y. ruckeri</i>	<i>S. salar</i>	CO	<i>C. inhibens</i> ^a	Yes		Robertson <i>et al.</i> (2000)
Fin rot	<i>A. bestiarum</i>	<i>O. mykiss</i>	ID	<i>A. sobria</i> , <i>Brochothrix thermosphacta</i>	Yes		Pieters <i>et al.</i> (2008)
White spot	<i>Ichthyophthirius multifiliis</i>	<i>O. mykiss</i>	IMM	<i>A. sobria</i> , <i>Brochothrix thermosphacta</i>	Yes	<i>Brochothrix thermosphacta</i> not effective	Pieters <i>et al.</i> (2008)

Bacterial coldwater disease	<i>F. psychrophilum</i>	<i>O. mykiss</i>	ID	<i>Enterobacter amnigenus</i> , <i>Enterobacter</i> sp.	Yes	Burbank <i>et al.</i> (2011)
	<i>F. psychrophilum</i>		IM	<i>Pseudomonas</i> M174	Yes	Korkea-aho <i>et al.</i> (2011)
	<i>F. psychrophilum</i>		IM	<i>Pseudomonas</i> M162	Yes	Korkea-aho <i>et al.</i> (2012)
	<i>F. psychrophilum</i> <i>F. columnare</i>	<i>Salvelinus fontinalis</i>	NO	Multiple indigenous skin bacterial isolates	Yes	Boutin <i>et al.</i> (2012)
IPNV	Infectious pancreatic necrosis virus	<i>O. mykiss</i>	Injection	<i>Lb. casei</i> and recombinant IPNV cDNA	N/A	Min <i>et al.</i> (2012)
	<i>Aeromonas</i> sp.	<i>O. mykiss</i>	IP	<i>B. subtilis</i>	Yes	Newaj-Fyzul <i>et al.</i> (2007)

Source: Adapted from Merrifield *et al.* 2010.

^aPreviously *Carnobacterium* strain K1, reclassified as *Carnobacterium inhibens* (Jöbörn *et al.* 1999).

Pathogen/probiotic genera abbreviations: A. = *Aeromonas*, B. = *Bacillus*, C. = *Carnobacterium*, F. = *Flavobacterium*, Lb. = *Lactobacillus*, Lc. = *Lactococcus*, Lev. = *Leuconostoc*, P. = *Pedococcus*, Ps. = *Pseudomonas*, S. = *Saccharomyces*, Str. = *Streptococcus*, V. = *Vibrio*, Y. = *Yersinia*.

Challenge method abbreviations: CO = cohabitation, ID = intradermal injection, IM = intramuscular injection, IMM = immersion, IP = intraperitoneal injection, NO = natural outbreak. NA = not applicable

subtilis JB-1 conferred protection against *A. salmonicida*, *Lc. garvieae*, *Streptococcus iniae*, *V. (L.) anguillarum*, *V. ordalii* and *Y. ruckeri* in rainbow trout. The mode of action reflected nutrition, production of inhibitory substances and stimulation of the innate immune responses.

The modulation of rainbow trout immune responses with probiotic bacteria has been the subject of much attention and this property has been recognized as a key mode of action protecting fish against bacterial infections (Sharifuzzaman and Austin 2009). Sakai *et al.* (1995) demonstrated that oral administration of *Clostridium butyricum* enhanced rainbow trout resistance to vibriosis by increasing the phagocytic activity of leukocytes. The supplementation of *Aeromonas hydrophila* A3-51, *Vibrio fluvialis* A3-47S, *Carnobacterium* sp. BA211 or *Micrococcus luteus* A1-6 was able to increase serum lysozyme activity in rainbow trout (Irianto and Austin 2002). Moreover, higher serum lysozyme and total protein levels were recorded after rainbow trout were fed *Kocuria* sp. SM1 and then challenged with *V. anguillarum* (Sharifuzzaman and Austin 2009). Some LAB strains that are generally used as human probiotics, such as *Lactobacillus rhamnosus*, have been considered for use in rainbow trout (Nikoskelainen *et al.* 2001a; Panigrahi *et al.* 2005) and have stimulated respiratory burst activity (Nikoskelainen *et al.* 2003) and superoxide anion production of rainbow trout head kidney (HK) leukocytes (Panigrahi *et al.* 2005). The production of pro-inflammatory and anti-inflammatory cytokines is another immune mechanism that probiotics can modulate. In fact, administration of *Enterococcus faecium*, *Lb. rhamnosus* or *B. subtilis* was able to up-regulate interleukin-1 β (IL-1 β) and transforming growth factor β (TGF- β) in rainbow trout (Panigrahi *et al.* 2007). Similarly, Pérez-Sánchez *et al.* (2011) demonstrated that administration of *Lactobacillus plantarum*, isolated from the intestinal mucosa of rainbow trout, was able to up-regulate pro-inflammatory cytokine gene expression in the HK and intestine. Dietary *Pediococcus acidilactici* treatment may also potentiate the immune responsiveness of the rainbow trout intestine. Abid *et al.* (2012) observed an up-regulation of the gene expression of the pro-inflammatory cytokines IL-1 β and IL-8 and down-regulation of the anti-inflammatory IL-10, compared to control fed fish. In addition, the mRNA levels for the mucosal antibody IgT was also elevated in probiotic fed fish.

Probiotics have also been suggested to have a beneficial effect in the digestive processes by promoting nutrient uptake or providing nutrients and vitamins (Ringø and Gatesoupe 1998), and also improving digestibility and weight gain. For instance, enhanced growth was observed in rainbow trout after the supplementation of *Saccharomyces cerevisiae* (Tukmechi *et al.* 2011). Similar results have been reported using the same yeast species by Sheikhzadeh *et al.* (2012), suggesting that yeast supplementation may promote lysozyme, protease, alkaline phosphatase and esterase activities in rainbow trout. Dietary provision of *S. cerevisiae* var. *boulardii* has also been reported to enhance rainbow trout intestinal enzymatic activities (Waché *et al.* 2006). Although information on the effect of bacterial probiotics on intestinal enzymatic function in trout is not presently available, limited studies have reported improved digestive morphology (Merrifield *et al.* 2010d), feed utilization (Bagheri *et al.* 2008) and growth performance (Bagheri *et al.* 2008). The majority of bacterial probiotic studies in trout which have assessed growth performance, as listed in Table 9.1, report no benefits in growth performance relative to control fed fish.

The present studies indicate that probiotic treatments offer a promising alternative to the use of chemotherapeutic agents in the trout industry, but further studies are required to elucidate the exact mechanisms of action involved, as well as the limitations in the use of these microorganisms as a prophylactic or therapeutic measure in these production systems.

9.2.2 Brown/sea trout (*Salmo trutta*)

According to the Food and Agriculture Organization (FAO), the main producers (>100 tonnes/year) of brown/sea trout in seawater or freshwater in 2010 were the Russian Federation (accounting for 80% of the global total, almost exclusively in freshwater), Italy, Romania, France, the UK, Germany, Denmark and Bosnia and Herzegovina (FAO FIGIS 2013). Moreover, it is well known that native species of brown/sea trout are important in terms of their contribution to recreational fisheries and angling (Merrifield *et al.* 2010a).

In contrast to rainbow trout, few probiotic studies have been conducted on brown trout (Table 9.1). Balcázar *et al.* (2007a) observed a correlation between colonization with probiotic strains (*Lc. lactis* subsp. *lactis* or *Leu. mesenteroides*) and non-specific humoral responses such as alternative complement pathway activity and lysozyme activity. Further studies demonstrated that administration of these probiotic strains enhanced the phagocytic activity of the HK leukocytes and conferred protection against furunculosis in brown trout (Balcázar *et al.* 2009). Further, some probiotic organisms may help to reduce brown trout fungal infections (Carbajal-González *et al.* 2013). The capacity of potential probiotics to adhere to brown trout skin mucus and to reduce the adhesion of *Saprolegnia parasitica* cysts under exclusion, competition and displacement conditions were analysed *in vitro* by Carbajal-González *et al.* (2013). Almost all of the tested bacterial isolates reduced fungal cyst adhesion ratios significantly; the bacterial isolates which most effectively reduced the adhesion of the pathogen were identified as belonging to the species *A. sobria* and *Pseudomonas fluorescens*.

9.2.3 Atlantic salmon (*Salmo salar* L.)

Compared to the depth of information available on probiotic applications in rainbow trout, considerably less information is available for Atlantic salmon. The available information, summarized in Tables 9.1 and 9.2, can be divided into *in vivo* studies (Smith and Davey 1993; Austin *et al.* 1995; Gildberg *et al.* 1995; Robertson *et al.* 2000; Gram *et al.* 2001) and *ex vivo* studies (Ringø *et al.* 2007a; Salinas *et al.* 2008; Kristiansen *et al.* 2011).

To our knowledge, the first study on probiotics in Atlantic salmon was carried out by Smith and Davey (1993). The authors isolated 100 bacteria from brown trout and tested their ability to inhibit the *in vitro* growth of *A. salmonicida* and suggested that the observed inhibition was caused by competition of free iron. The most promising isolate, a fluorescent pseudomonad, *Ps. fluorescens* F19/3, was further tested to compete against *A. salmonicida* in asymptotically infected pre-smolt Atlantic salmon. The results from the four experiments revealed a significant reduction in the frequency of stress-induced furunculosis after a bath treatment. Based on their results, the authors suggested that *Ps. fluorescens* F19/3 excluded *A. salmonicida* on the external surfaces. In contrast, Gram *et al.* (2001) reported that *Ps. fluorescens* (strain AH2) was not effective at reducing Atlantic salmon mortalities when challenged by cohabitation with *A. salmonicida* infected fish. However, the strain showed strong *in vitro* inhibitory activity towards *A. salmonicida*. Based on their results, the authors concluded that a strong *in vitro* growth inhibition alone cannot be used to predict possible *in vivo* effects and suggested that the lack of effect in the *in vivo* experiment might be due to the infection route of the pathogen or that the level of the probiont may not have been sufficient to outcompete the pathogen.

Austin *et al.* (1995) used a potential probiont, *Vibrio alginolyticus*, obtained from a commercial shrimp hatchery in Ecuador, in an Atlantic salmon pathogen challenge. Salmon were

bathed in probiotic suspensions ($\sim 10^7$ cells ml^{-1}) for 10 minutes, 7 days prior to the pathogen challenge. The challenge was conducted by bathing salmon in suspensions containing 10^7 cells ml^{-1} of *A. salmonicida*, *V. anguillarum*, *Vibrio ordalii* or *Y. ruckeri* for 10 minutes. The probiotic treatment led to a significant reduction in mortalities after challenge with *A. salmonicida* (18% mortality in the probiotic treated fish versus 100% in the control). The probiotic effect was less clear in the challenge studies using *V. anguillarum* and *V. ordalii* and no probiotic effect was observed in the *Y. ruckeri* challenge where all of the probiotic treated and the control fish died. Gildberg *et al.* (1995) carried out a probiotic study on Atlantic salmon juveniles where *Lb. plantarum*, later reclassified as *Carnobacterium divergens*, was incorporated into feeds at 2.5×10^9 cells g^{-1} . The results of a cohabitation challenge with fish infected with *A. salmonicida* revealed a clear tendency towards increased mortality of fish fed diets supplemented with *C. divergens*. Prior to the challenge, *C. divergens* completely dominated the culturable autochthonous gut microbiota of fish fed *C. divergens*; however, in surviving fish at the end of the challenge *A. salmonicida* outcompeted *C. divergens* as the population level of LAB (presumed *C. divergens*) was reduced. In contrast to the lack of protecting effect of *C. divergens* reported by Gildberg *et al.* (1995), Robertson *et al.* (2000) reported a protective effect of *Carnobacterium inhibens*, isolated from the GI tract of Atlantic salmon (Jöborn *et al.* 1999), towards *A. salmonicida*, *V. ordalii* and *Y. ruckeri* challenges. However, *C. inhibens* was not effective against *V. anguillarum*.

The European Union has imposed restrictions on scientists in the use of experimental animals including fish in *in vivo* studies (Revision of the EU Directive for the Protection of Animals Used for Scientific Purposes, Directive 86/609/EEC, 8 September 2010) and, in light of this imposition, *in vitro* and *ex vivo* methods have been suggested as alternatives. With regards to *ex vivo* methods, three papers have used an intestinal sac method to evaluate adherence of probiotics in the GI tract of Atlantic salmon (Ringø *et al.* 2007a; Salinas *et al.* 2008; Kristiansen *et al.* 2011). Ringø *et al.* (2007a) and Salinas *et al.* (2008) evaluated the interactions between LAB (*C. divergens* and *Lb. delbrueckii* subsp. *lactis*, respectively) and pathogens in the intestine and concluded that the LAB used was able to outcompete the pathogen to some extent. In a more recent study, Kristiansen *et al.* (2011) showed that *C. divergens* adhered to the epithelium or mucus in the proximal intestine of Atlantic salmon but this effect was not apparent in the distal part of the intestine. However, by using this method one should bear in mind that the method is restricted to short term experiments as the viability of intestinal tissue is limited once it is excised from the host and permanent adherence merits further investigations.

9.3 GADIDAE

9.3.1 Atlantic cod (*Gadus morhua*)

Atlantic cod is an important economic commodity in international markets, and declining stocks over the last decades have motivated the commercial development of cod aquaculture, mainly in Norway but also in the UK, Iceland and Canada to some extent (Lauzon 2010). However, biological and technological constraints have hampered the expansion of cod culture (FAO 2009; Rana 2007). Health and disease management at early cod stages has relied on the use of antibiotics since vaccination is first conducted on 1 g juveniles (Lauzon 2010). The need for faster growing and healthy juveniles has prompted the search for novel prophylactic methods.

Applications of potential probiotics in cod rearing have been beneficial in many different ways (Table 9.3). Microbiota control and changes in its composition have resulted following probiotic treatments at larval and juvenile stages. Addition of an autochthonous LAB strain (initially identified as *Lb. plantarum*, but later reclassified as *Carnobacterium divergens*) into the rearing water of 5 days post hatching (dph) cod larvae at an initial dose of 10^5 colony forming units (CFU) ml^{-1} influenced the gut bacterial composition and resulted in strain colonization, accounting for 70% of the cultivable microbiota (9 days post-treatment), as well as the reduction of opportunistic bacteria (Strøm and Ringø 1993).

At early larval stages, the GI tract is poorly developed. The microbiota established may therefore reflect that of the environment and live feed which often carries high bacterial load (Olafsen 2001; Chapter 16). Hence, early and frequent probiotic treatments should be applied for maximum effect. The regular addition of two bacterial strains (*Arthrobacter* sp. and *Enterococcus* sp.) via rearing water (10^{5-7} CFU ml^{-1}) from early post-fertilization of cod through the larval stage contributed to gut microbiota control and lower *Vibrio* levels, as well as enhanced larval survival, growth, development and vitality (Lauzon *et al.* 2010a; 2010b; 2010c). Indeed, enhancement of the gut microbiota in the probiotic-treated groups (36 dph) was suggested to have played a role in better feed utilization of cod larvae, enabling their faster weight gain despite the same feeding ration given to all groups. The presence of the potential probiotics in cod larval gut was confirmed 8 days post-treatment (Lauzon *et al.* 2010c). Further, the properties of the two prospective probiotics, isolated at the cod hatchery, were confirmed in an intervention study of early cod juveniles (Lauzon *et al.* 2010d). Dry feed coated with single or mixed probiotics was administered for 54 or 27 days, respectively. Microbiological stability of the probiotics added to the feed was demonstrated. Juveniles administered the *Enterococcus*-probiotic feed grew faster and had lower gut *Vibrio* loads than control juveniles. Overall, the results suggest that both probiotics, especially the *Enterococcus* strain, modified the gut microbiota and contributed to enhanced juvenile growth and survival. During a larval study involving the application of a commercial product (Remus®, Avecom), via supplementation of rearing water and enriched rotifers, up-regulation of proteins related to growth and development as well as down-regulation of stress-related proteins were detected in cod larvae (Sveinsdottir *et al.* 2009).

Cod juvenile trials dealing with administration of probiotics via feeding were initiated more than a decade ago and aimed mainly at assessing their disease reducing potential. Gildberg *et al.* (1997) investigated the effect of *C. divergens* administered to Atlantic cod juveniles via feeding (10^{7-9} CFU g^{-1}) for six weeks, and reported some protection upon challenging with *V. anguillarum* at the midterm and observed *C. divergens* among the intestinal microbiota of surviving fish. A similar feeding experiment conducted for three weeks by Gildberg and Mikkelsen (1998) showed a significantly reduced cumulative mortality 12 days post-challenge while the effect was lost 4 weeks post-infection. Nevertheless, colonization of the probiotic in the cod intestine was again observed.

More recently, the emphasis has been towards the elucidation of the different properties of probiotic strains. The *ex vivo* study of Martinsen *et al.* (2011) indicated the modulating effect of an autochthonous probiotic strain of *Carnobacterium maltaromaticum* on the cod midgut microbiota following exposure (10^7 CFU ml^{-1}) for 30–60 min, as well as its ability to outcompete *V. anguillarum*. The *in vitro* study of D'Alvise *et al.* (2012) showed the ability of *Phaeobacter gallaeciensis* BS107 (10^7 CFU ml^{-1}), a producer of the antibacterial compound tropodithietic acid (TDA), to reduce cod larval mortality in challenge trials with

Table 9.3 Probiotic applications at different rearing stages of Gadidae and the main effects observed.

Rearing stage	Probiotic used	Administration mode	Main effects observed	Reference
Atlantic cod (<i>Gadus morhua</i>) Larval (9 days)	<i>C. divergens</i> -like (AUT)	Unfed larvae bathed in RW containing the probiotic (10^5 CFU ml ⁻¹) for 9 days	Controlled bacterial load and influenced microbiota composition	Strøm and Ringø (1993)
(24 days)	Commercial product Remus® (Avecom)	Enriched rotifers (12 g 500 000 rotifers ⁻¹ ; daily) and larval RW (40 ppm every 2 days) for 24 days	Down-regulation of proteins related to stress and immune function; most of up-regulated proteins linked to growth and development	Sveinsdóttir <i>et al.</i> (2009)
(27 days)	<i>C. divergens</i> V41, <i>Arthrobacter</i> and <i>Enterococcus</i> spp. (ALL)	Ova and larvae bathed in RW containing bacterial mixture (10^6 – 7 CFU ml ⁻¹)	Enhanced survival, growth, vitality and microbiota control in larvae retaining both <i>Arthrobacter</i> and <i>Enterococcus</i> spp.	Lauzon <i>et al.</i> (2010c)
(36 days)	<i>Arthrobacter</i> and <i>Enterococcus</i> spp.	Ova and larvae bathed in RW containing bacterial mixture (10^5 – 6 CFU ml ⁻¹)	Enhanced larval development, growth and gut microbiota, with proportionally lower <i>Vibrio</i> load	Lauzon <i>et al.</i> (2010d)
(0–14 dph)	<i>Phaeobacter gallaeciensis</i> BS107 (DSM17395) (ALL)	<i>In vitro</i> test (10^7 CFU ml ⁻¹ of probiotic $\pm$ 10^6 CFU ml ⁻¹ of <i>V. anguillarum</i>) on cod embryos distributed singly in 2 ml seawater	Harmless and beneficial to cod embryos/larvae; increased larval survival of treated and <i>Vibrio</i> -challenged embryos compared to positive and negative controls	D'Alvise <i>et al.</i> (2012)
(0–14 dph)	<i>Phaeobacter gallaeciensis</i> BS107, <i>Phaeobacter</i> sp. 27–4, and <i>Phaeobacter</i> sp. M23-3.1 (ALL)	<i>In vitro</i> test (10^7 CFU ml ⁻¹ of probiotic $\pm$ 10^6 CFU ml ⁻¹ of <i>V. anguillarum</i>) on cod embryos distributed singly in 2 ml seawater	Better survival of probiotic-treated larvae compared to control; enhanced disease resistance, especially when added 48 h before pathogen	D'Alvise <i>et al.</i> (2013)
Juvenile (6 wks)	<i>C. divergens</i> (AUT)	Dry feed (10^{7-9} CFU g ⁻¹) given for 6 weeks, with <i>V. anguillarum</i> (7×10^6 CFU ml ⁻¹ , 1 h) challenge at mid-term	Certain improvement of disease resistance; LAB dominated the intestinal microbiota of surviving fish	Gildberg <i>et al.</i> (1997)

[7 wks]	<i>C. divergens</i> (from cod or salmon intestine)	Dry feed (10^8 CFU g ⁻¹) given for 3 weeks; fish challenged with <i>V. anguillarum</i> (10^7 CFU ml ⁻¹ , 1 h)	LAB colonization in intestines and/or pyloric caeca after 3 weeks; reduced cumulative mortality 12 days post-infection using the salmon strain, but not after 24 days	Gildberg and Mikkelsen (1998)
[28 or 55 days]	<i>Arthrobacter</i> and <i>Enterococcus</i> spp. (All)	Dry feed (10^{7-9} CFU g ⁻¹), single or mixed probiotics	Faster growth of <i>Enterococcus</i> fed juveniles and lower <i>Vibrio</i> gut counts than control; both probiotics modified gut microbiota, enhanced juvenile growth and survival	Lauzon et al. (2010d)
[20 or 40 days]	<i>Psychrobacter</i> GP12 or <i>Pseudomonas</i> GP21 spp. (AUT)	Dry feed (10^6 CFU g ⁻¹) for 40 days	Increased amyolytic and cellulolytic activities in cod intestine only after 40 days for both strains; enhanced chitinase and protease activity in GP12 fed fish	Lazado et al. (2012)
<i>In vitro</i> test (24 h)	<i>Psychrobacter</i> sp., <i>Shewanella</i> sp., <i>Photobacterium</i> sp. or <i>Vibrio</i> sp. (AUT)	Incubation of HK cells with heat-inactivated gut-derived bacteria (10^6 CFU ml ⁻¹)	Differential expression of immune response genes related to bacterial defence and inflammation; the <i>Photobacterium</i> strain did not elicit much response	Caipang et al. (2010)
<i>In vitro</i> test (24 h)	<i>Psychrobacter</i> GP 12 and <i>Pseudomonas</i> GP21 spp. (AUT)	10^7 cells (live or heat inactivated) added to the primary cultures of HK leukocytes	Cod intestinal bacteria regulated some genes responsible for immune and antioxidant defences in cod HK leukocytes; bacterial viability influenced the extent of genes expression obtained	Lazado et al. (2010)

(continued overleaf)

Table 9.3 (continued)

Rearing stage	Probiotic used	Administration mode	Main effects observed	Reference
Ex vivo study	<i>C. maltaromaticum</i> (AUT)	Midgut exposed for 30 min or 1 h to bacterial solutions, 10^7 CFU ml ⁻¹ for <i>C. maltaromaticum</i> and/or 10^6 CFU ml ⁻¹ for <i>V. anguillarum</i>	Both bacteria could adhere to the midgut and influenced microbiota composition; <i>C. maltaromaticum</i> apparently out-competed <i>V. anguillarum</i> ; <i>C. maltaromaticum</i> did not affect midgut morphology	Martinsen <i>et al.</i> (2011)
Atlantic pollack (<i>Pollachius pollachius</i>) Larval		Enriched <i>Artemia</i> with <i>Saccharomyces cerevisiae</i> (10^9 CFU l ⁻¹) and/or <i>P. acidilactici</i> (10^{10} CFU l ⁻¹)	Use of <i>Saccharomyces cerevisiae</i> and/or <i>P. acidilactici</i> significantly enhanced growth.	Gatesoupe (2002)
Haddock (<i>Melanogrammus aeglefinus</i>) Larval (60 days)		<i>Arthrobacter davidanieli</i> RSXII (ALL)	Higher increase in larval dry mass and lipid accumulation compared to control; similar survival observed.	Plante <i>et al.</i> (2007)

Genera abbreviations: *C.* = *Carnobacterium*, *P.* = *Pediococcus*, *V.* = *Vibrio*.
Abbreviations: AUT = autochthonous strain; ALL = allochthonous strain; RW = rearing water.

V. anguillarum (10^6 CFU ml⁻¹) to a level beneath the uninfected controls. In a similar study (D'Alvise *et al.* 2013), this *Phaeobacter* strain along with two others were shown to reduce cod larval mortality, but the extent of this effect was influenced by the time of introduction of the probiotic with respect to the pathogen (48 h before, simultaneously or 48 h after). Since earlier addition of probiotics proved to be most efficient, use of *Phaeobacter* spp. as a prophylactic treatment against bacterial infections may be the most promising application for larviculture. The capability of TDA production by the different *Phaeobacter* strains was correlated with their probiotic impact, suggesting that TDA production is among the key mechanisms of their probiotic action. Finally, Lazado *et al.* (2012) demonstrated that two bacterial strains (*Psychrobacter* sp. and *Pseudomonas* sp.) isolated from cod GI tract could be orally delivered by feed (10^6 CFU g⁻¹) to juveniles and could improve their digestive capacity after 40 days of feeding, but not 20 days. Few studies have evaluated the cod immunomodulative properties of probiotics, but the potential of autochthonous cod strains was confirmed by *in vitro* studies (Caipang *et al.* 2010; Lazado *et al.* 2010), the latter study emphasizing the importance of probiotic viability for enhanced response.

In summary, probiotic applications in cod larviculture have been mainly administered via rearing water (10^{5-7} CFU ml⁻¹) and proved to be beneficial by enhancing survival, development, vitality and microbiota control as well as disease resistance. Most probiotics evaluated were allochthonous strains. The study of D'Alvise *et al.* (2012) demonstrated that *Phaeobacter gallaeciensis* can reduce *V. anguillarum* in axenic live feed cultures, which could be another probiotic administration vector to larvae. Juvenile probiotic treatments have been achieved by dry feed supplementation (10^{6-9} CFU g⁻¹), generally using autochthonous strains. Few studies have considered the use of multispecies probiotics, while a minimal beneficial treatment appears to be 4–6 weeks of daily feeding. LAB colonization, microbiota control and enhanced enzymatic activity, growth, survival and disease resistance were the main effects observed. Overall, the findings indicate a broad activity spectrum for probiotics, benefiting multiple or all rearing stages.

9.3.2 Atlantic pollack (*Pollachius pollachius*)

High demand for fresh fillets of white fish has stimulated the development of other farmed gadoid species. Pollack aquaculture was initiated almost two decades ago in France and Spain. As for cod, its sensitivity to pathogens and low survival at early phases of rearing has delayed culture developments. Further, its slow growth compared to Mediterranean fish species is another major weakness. Nevertheless, some information on probiotics is available. Gatesoupe (2002) investigated the effects of two commercial probiotic strains supplemented to *Artemia* nauplii and then fed to pollack larvae. The products contained *P. acidilactici* and *S. cerevisiae*. This study was the first attempt to evaluate the synergistic effect of LAB and yeast. Formaldehyde was used to disinfect *Artemia* cysts and nauplii, combat the dominating Gram-negative bacteria and reduce the high bacterial load inherent to *Artemia* culture, while the probiotics were introduced in the enrichment process. In this study, live feed was shown to be an appropriate vector for probiotic transmission to larvae and the probiotics applied enhanced larval growth, especially when applied together. However, the disinfection method used affected *P. acidilactici* and after three months led to the emergence of a resistant microbiota. It was concluded that *P. acidilactici* was a promising probiotic for fish larvae, while a careful application of *S. cerevisiae* may be valuable if bacterial imbalance can be avoided.

9.3.3 Haddock (*Melanogrammus aeglefinus*)

Marine fish species, including haddock as well as Atlantic cod, have been considered as an alternative to salmonid aquaculture. However, variability in production and heavy losses observed at early ontogenic stages may be due to both nutritional deficiencies and disease outbreaks (Olafsen 2001). Indeed, growth and survival of fish larvae are dependent upon the availability of essential fatty acids for incorporation into membrane phospholipids. Based on preliminary findings, Plante and coworkers (2007) suggested that the microbial environment of marine invertebrate species as well as the presence of pathogens may influence their lipid class and fatty acid composition, which may serve as biochemical indicators of health if similar relationships exist in fish larvae. In that respect, they evaluated the effect of adding a non-pathogenic *Arthrobacter* sp., licensed as a vaccine for protecting salmonids against selected pathogens, during rearing of haddock larvae. Larval bacterial community profiles, nutritional condition (assessing lipid class and fatty acid composition) and performance (growth and survival) were assessed. Their approach considered two pathways of probiotic administration (10^6 CFU ml⁻¹), rearing water (1 dph) and rotifers (7 dph), as one treatment. Larvae treated with the probiotic showed a superior rate of increase in dry mass and lipid accumulation compared to the control, suggesting the *Arthrobacter* strain enhanced the general health of haddock larvae, by modifying their bacterial microbiota. Detection of the probiotic in larvae by molecular analysis (DGGE) was only confirmed one day post-treatment (2 and 8 dph, 18 h and 12 h post bathing and feeding, respectively). Nevertheless, differences of larval microbiota compared to the control were observed in treated larvae from 1 dph up to 43 dph. Therefore, the study demonstrated the potential application of this probiotic in haddock hatcheries, based on improved larval health and growth rate.

9.4 PLEURONECTIFORMES

9.4.1 Atlantic halibut (*Hippoglossus hippoglossus*)

Atlantic halibut is another valuable candidate for commercial marine cold water farming. However, high mortality rates are observed during intensive larval production (Bjornsdottir *et al.* 2010). As for many other fish species, larvae are confronted with late maturation of the GI tract and the specific immune system as well as the presence of opportunistic and pathogenic bacteria. It is therefore anticipated that manipulation of the rearing and larval bacterial community by probiotics may contribute to a production success. The benefit of a probiotic treatment through bathing of ova and resulting larvae was evaluated by Ottesen and Olafsen (2000) and Bjornsdottir *et al.* (2010).

Ottesen and Olafsen (2000) evaluated the effect of allochthonous bacteria on larval survival following pre-incubation of halibut eggs and resulting larvae (at 10^6 CFU ml⁻¹). Higher survival was obtained 32 dph with an apathogenic *Aliivibrio* (*Vibrio*) *salmonicida* strain (72.8%) and *C. divergens* (previously *Lb. plantarum*) (68.4%) compared to the control (58.2%). Further, the proliferation of mucous cells in Atlantic halibut larvae, treated with *C. divergens*, was suggestive of a stimulation of cellular defences but unfortunately no immunological assays were conducted. Bjornsdottir *et al.* (2010) performed a repeated application of a three-strain autochthonous probiotic mixture, containing Gram-negative bacteria, in egg incubators ($\sim 10^2$ CFU ml⁻¹ at days 0, 6 and 13 dpf) complemented by a

higher dose (10^6 CFU ml⁻¹ for 5 min) on sterilized eggs prior to hatch. Despite the frequency of the treatment, no benefits in survival rate were observed, along with similar bacterial load compared to the control. This could be due to the rather low dose applied in the first part of the treatment. However, addition of the probiotic mixture via live feed (10^6 CFU ml⁻¹ for 30 min on days 0, 1, 17 and 18) was conducted in two other trials, resulting in higher survival in treated larvae (60 days post-feeding) and enhanced growth in one of the two trials. Therefore, live feed was shown to be an appropriate vector for probiotic transmission to halibut larvae (Bjornsdottir *et al.* 2010).

9.4.2 Turbot (*Scophthalmus maximus*/Psetta maxima)

Turbot is a valuable commercial species for aquaculture production and although commercial-scale production faces many challenges production output has rapidly increased over the last decade, reaching 75,000 tonnes in 2011 (FAO FIGIS 2013). Increasing commercial interest in turbot production has also resulted in an increasing effort to study the gut microbiota and methods for microbial fortification. Probiotic applications of *Lb. plantarum*/*Carnobacterium* sp. (Gatesoupe 1994), *Vibrio pelagius* (Ringø 1999), *C. divergens* (Ringø 1999), *Lc. lactis* (Villamil *et al.* 2002), *Phaeobacter* strain 27-4 (Planas *et al.* 2006; formerly *Roseobacter* sp. 27-4) and *P. acidilactici* (Villamil *et al.* 2010) have been reported, predominantly, in larviculture via rearing water or live feed (Table 9.4).

Colonization success of probiotic strains can be influenced by the application method (rearing stage, administration vector, dose and frequency of treatment) and host specificity. It has been reported that the addition of probiotics early post hatch in turbot larviculture led to a greater colonization success than when applied a few days later (Ringø and Vadstein 1998) and an autochthonous probiotic strain (*V. pelagius*) was shown to colonize the turbot larval gut better than an allochthonous strain (*C. divergens*) (Ringø 1999). Further, the work of Makridis *et al.* (2000) demonstrated that a strain originating from turbot larvae colonized turbot larvae better than a strain isolated from an adult. The authors also compared the addition of probiotics via rearing water and/or rotifers' supplementation. They reported a similar effect of the treatments, as discharging rotifers to the tanks influenced the water microbiota similarly with respect to the probiotics added to the rearing water.

The benefit of a probiotic treatment through bathing of ova, and resulting larvae, has been evaluated in turbot (Hjelm *et al.* 2004). An allochthonous strain, *Phaeobacter* sp., enhanced larval survival when applied at a dose of 10^7 CFU ml⁻¹ prior to hatch. The dose required for improved disease resistance by an autochthonous *Vibrio* strain transmitted via enriched rotifer to larvae challenged with *V. splendidus* ranged from 10^6 to 5×10^7 CFU ml⁻¹ (Gatesoupe 1997). Several other allochthonous strains, mainly LAB and *Bacillus* spp., have been beneficial in turbot larviculture when supplemented (10^{3-7} CFU ml⁻¹) via live feed (Gatesoupe 1991; 1993; Daga *et al.* 2013) and even when challenged by pathogenic *Vibrio* strains (Gatesoupe 1994; Planas *et al.* 2006). Nevertheless, probiotic supplementation may lead to unclear benefits under normal rearing conditions (Gatesoupe 1994; Ringø and Vadstein 1998; Ringø 1999; Makridis *et al.* 2000; Villamil *et al.* 2010).

Rearing water treated with *V. pelagius* (10^5 bacteria ml⁻¹), an autochthonous strain of turbot, also influenced the gut microbiota of turbot larvae (Ringø *et al.* 1996). Moreover, better larval uptake of the probiotic *P. acidilactici* was achieved via rearing water compared to rotifer supplementation (Villamil *et al.* 2010). Nevertheless, encapsulation of an allochthonous mixture of *Lactobacillus* and *Pediococcus* spp. in *Artemia* ($\sim 10^6$ CFU ml⁻¹ per day) fed to larvae

Table 9.4 Probiotic applications in Pleuronectiformes and the main effects observed.

Rearing stage	Probiotic used	Administration mode	Main effects observed	Reference
Atlantic halibut (<i>Hippoglossus hippoglossus</i>)				
Larval (32 days)	<i>C. divergens</i> -like (<i>Lactobacillus plantarum</i>) or <i>Alivibrio (Vibrio) salmonicida</i> (ALL)	Ova and resulting larvae incubated in RW containing bacteria (10^6 CFU ml ⁻¹)	Significantly higher survival 32 dph compared to control	Ofteisen and Olafsen (2000)
Ova (14 days)	<i>Vibrio</i> sp., <i>V. splendidus</i> , and <i>Pseudocalteromonas elyakovii</i> (AUT)	Ova treated three times in incubators (10^{1-2} CFU ml ⁻¹) and once (10^6 CFU ml ⁻¹ , 5 min) after disinfection prior to hatch; <i>Artemia</i> treated in bacterial mixture (10^5 CFU ml ⁻¹) at 30 °C for 30 min (1/3 as morning ration); the rest used as a second ration (8 h later), treated again in 10^4 CFU ml ⁻¹	Ova treatment had no effect on ova survival Larval treatment performed four times (days 0, 1, 17, 18) led to higher survival, and sometimes enhanced growth	Bjornsdottir <i>et al.</i> (2010)
Larval (60 days)				
Turbot (<i>Scophthalmus maximus</i>)				
Larval (20 days)	<i>Lb. plantarum</i> , <i>Lb. helveticus</i> or <i>Str. thermophilus</i> (ALL)	Daily addition of 10^7 CFU ml ⁻¹ rotifer culture for 6–7 days; feeding trial of turbot larvae for 20 days	<i>Lb. plantarum</i> enhanced the rotifer production rate significantly and reduced their bacterial load; all LAB fed larvae were heavier than control; no increase in survival	Gatesoupe (1991)
(10 days)	<i>Bacillus</i> sp. IP 5832 (Paciflor 9) (ALL)	<i>Bacillus</i> -enriched rotifers (4×10^3 CFU ml ⁻¹ per day); larval feeding	<i>Bacillus</i> treatment of rotifers for 5 days led to microbiota modulation; significant growth and survival increase after 10 days in <i>Bacillus</i> -fed turbot larvae	Gatesoupe (1993)
(9 days)	<i>Carnobacterium</i> -like sp. (ALL)	LAB-enriched rotifers; larvae challenged with <i>Vibrio</i> sp. (10^6 CFU ml ⁻¹ , 9 dph)	No effect on growth or survival under normal conditions; increased survival upon <i>Vibrio</i> challenge. LAB dose effect observed (10^7 CFU ml ⁻¹)	Gatesoupe (1994)

(14 days)	<i>V. pelagius</i> (AUT)	Addition to RW at 0 dph (4×10^5 bacteria ml ⁻¹); water flow after 6 days	<i>V. pelagius</i> detected in larval gut after 24 h (log 4 ml ⁻¹) and up to 14 days (log 5 ml ⁻¹); influenced larval gut microbiota	Ringø <i>et al.</i> (1996)
(10 days)	<i>Vibrio</i> E sp. (AUT)	<i>Vibrio</i> E-enriched rotifers (5×10^5 - 7 CFU ml ⁻¹); larvae challenged with <i>Vibrio splendidus</i>	Higher larval survival upon <i>V. splendidus</i> challenge with <i>Vibrio</i> E doses 1.5×10^6 to 5×10^7 CFU ml ⁻¹	Gatesoupe (1997)
(16 days)	<i>V. pelagius</i> , <i>A. caviae</i> (AUT)	Addition to RW at 2-5 or 8 dph (10^5 bacteria ml ⁻¹) separately or in combination	Colonization of added bacteria was greater if added earlier; <i>V. pelagius</i> improved larval survival compared to control or addition of <i>A. caviae</i>	Ringø and Vadstein (1998)
(15 days)	<i>C. divergens</i> (ALL), <i>V. pelagius</i> (AUT)	Addition to RW at 0 dph (10^5 bacteria ml ⁻¹) separately or in combination	<i>C. divergens</i> (from Atlantic salmon) able to attach/colonize turbot gut but <i>V. pelagius</i> attained higher levels, alone or in combination; survival of <i>C. divergens</i> treated larvae was highest but similar to control	Ringø (1999)
(12 days)	two strains from turbot (AUT, adult and larvi)	Bacteria added via RW (10^4 - 5 CFU ml ⁻¹) on day 0 and/or rotifers (10^4 - 5 rotifer ⁻¹) from day 2	Both strains colonized the larval gut, the larvi strain more efficiently; survival and growth not improved by added bacteria	Makridis <i>et al.</i> (2000)
(7 days)	Autochthonous turbot isolates and <i>V. mediterranei</i> (ALL)	Small-scale turbot confrontation tests with 10^5 CFU ml ⁻¹ added 1 dph to sterile seawater; survival evaluated at 5 dph	Cluster A (non- <i>Vibrio</i> strains) and the <i>V. mediterranei</i> had a distinct positive and reproducible effect on larval survival	Huys <i>et al.</i> (2001)
(6 days)	<i>Phaeobacter</i> (formerly <i>Roseobacter</i>) sp. 27-4 (ALL)	Applied (10^3 - 5×10^7 CFU ml ⁻¹) prior to hatch in 24-well dishes	<i>Phaeobacter</i> not lethal to egg and larvae; dose 10^7 CFU ml ⁻¹ significantly decreased mortality	Hjelm <i>et al.</i> (2004)

(continued overleaf)

Table 9.4 (continued)

Rearing stage	Probiotic used	Administration mode	Main effects observed	Reference
(10 days)	<i>Phaeobacter</i> sp. 27-4 (ALL)	Given on days 3, 5, 7 via rotifers (3×10^2 CFU ml ⁻¹); challenged via rotifers with <i>V. anguillarum</i> (2.5×10^3 CFU ml ⁻¹) on days 4, 6, 8	Addition of <i>Phaeobacter</i> significantly reduced mortalities caused by <i>V. anguillarum</i> . No indication of damages to larval gut or intestine by <i>Phaeobacter</i>	Planas <i>et al.</i> (2006)
(10 days)	<i>P. acidilactici</i> (ALL)	Supplementation (10^8 CFU ml ⁻¹) of: rotifers for 1 h vs. 24 h; rearing water 3 dph	Better larval uptake of probiotic via rearing water. Larval survival and growth not improved. Higher levels of probiotic in rotifers after 1 h	Villamil <i>et al.</i> (2010)
(64 days)	FAA: <i>Lactobacillus</i> and <i>Enterococcus</i> spp. FAB: <i>Lactobacillus</i> and <i>Pediococcus</i> spp. (ALL)	Bioencapsulated (2.5×10^5 CFU ml ⁻¹) in <i>Artemia</i> given three times a day from 16 to 34 dph	Control of gut microbiota; increased larval survival and growth, especially FAB	Daga <i>et al.</i> (2013)
Juvenile (7 days)	<i>Lc. lactis</i> ssp. <i>lactis</i> CECT 539 (ALL)	Force-fed daily with a bacterial dose of 100 µl at 10^5 CFU ml ⁻¹	Increased HK macrophage CL response and serum NO concentration after 7 days of daily administration	Villamil <i>et al.</i> (2002)
Juvenile (3 weeks)	<i>V. proteolyticus</i> (ALL)	Twice daily by oral intubations of a liquid diet containing 10^{10} <i>V. proteolyticus</i> ml ⁻¹	Improved protein digestion	De Schrijver and Ollevier (2000)
In vitro	Autochthonous turbot and dab (<i>Limanda limanda</i>) isolates	In vitro	Multiple strains showed adhesion to intestinal mucus, growth within intestinal mucus and antagonism against <i>V. anguillarum</i>	Olsson <i>et al.</i> (1992)

Genera abbreviations: A. = *Aeromonas*, C. = *Carnobacterium*, Lb. = *Lactobacillus*, P. = *Pediococcus*, Str. = *Streptococcus*, V. = *Vibrio*.

Abbreviations: AUT = autochthonous strain; ALL = allchthonous strain; RW = rearing water.

resulted in the control of larval gut microbiota by decreasing Gram-negative populations, as well as increased larval survival and growth (Daga *et al.* 2013). Live prey is an appropriate vector for probiotic delivery to fish larvae (Gatesoupe 1994) and bioencapsulation of bacteria in rotifers can be achieved within 20–40 min (Makridis *et al.* 2000). Villamil *et al.* (2010) showed that a 1 h treatment of *P. acidilactici* supplementation to rotifer culture led to better probiotic retention in rotifers, and in the gut of fed turbot larvae, compared to 24 h. Finally, Villamil *et al.* (2002) conducted *in vitro* experiments to determine the effect of a LAB strain on non-specific immune functions. As with live cells, heat-killed *Lc. lactis* stimulated the immune response of turbot macrophages, but a higher concentration was required compared to live cells.

Probiotics may also influence growth and nutrition of turbot juveniles. Increased protein degradation in the distal segments of the turbot (25–30 g) GI tract has been reported after oral intubation with *Vibrio proteolyticus* (10^{10} ml⁻¹) for a 3 week period. The higher nitrogen digestibility, followed by higher ammonia contents, evidenced the involvement of the microbiota in water-soluble nitrogen contents by the contribution of bacterial proteases and aminopeptidases and potentially the stimulation of endogenous enzyme activities which would account for higher protein digestion, as supported by the elevated fraction of smaller soluble proteins (De Schrijver and Ollevier 2000).

Overall to date, increased larval growth was reported in three studies involving allochthonous strains, while increased survival was demonstrated in five studies conducted under normal rearing conditions (Table 9.4).

9.5 PERCIDAE

9.5.1 European perch (*Perca fluviatilis*)

European perch is a valuable candidate for aquaculture diversification in Europe but farming profitability is compromised by high mortality rate during the larval and early juvenile developmental stages as well as the lack of therapeutic strategies to control outbreaks in culture (Mandiki *et al.* 2011). Hence, the development of prophylactic strategies may contribute to the expansion of European perch culture. Evaluating two administration pathways, Mandiki *et al.* (2011) applied a commercial three-strain *Bacillus* mixture to *Artemia* and/or rearing water (5×10^4 CFU ml⁻¹ per day) of European perch larviculture, resulting in higher larval growth rate and Ig levels only via treated live feed. However, a lower dose was applied via the rearing water.

9.6 CONCLUSION

Aquaculture production is vulnerable to adverse impacts of disease and environmental conditions. The demand for environmentally friendly and sustainable aquaculture has led to increasing research on probiotics for aquatic animals. As presented in this chapter, beneficial effects have been demonstrated following probiotic applications to cold water fish, emphasizing the potential of probiotics in aquaculture production. Probiotic supplementation via the rearing water has been successful in cold water larviculture. This delivery vector is relevant since the intestinal tract, skin and gills are generally accepted routes of entry for bacterial infection, such as furunculosis and vibriosis (Groff and LaPatra 2000; Birkbeck and Ringø 2005;

Jutfelt *et al.* 2006; Ringø *et al.* 2007a; 2007b; Engelsen *et al.* 2008). Although a number of factors effect efficacy, generally a minimal dose of 10^{5-7} CFU ml⁻¹ rearing water has been reported to induce positive effects in fish culture (Strøm and Ringø 1993; Ringø *et al.* 1996; Ringø and Vadstein 1998; Ringø 1999; Ottesen and Olafsen 2000; Hjelm *et al.* 2004; Lauzon *et al.* 2010b; 2010c), while a lower dose ($\sim 10^2$ CFU ml⁻¹) has resulted in no beneficial effects (Bjornsdottir *et al.* 2010). Live feed has also been an appropriate vector for probiotic administration to larvae. Some trials have evaluated both pathways of administration simultaneously with promising results.

The use of probiotics can be recommended to enhance the performance of aquaculture systems provided that operational and economic advantages are attained (Iribarren *et al.* 2012). A better knowledge of the larval/fish gut and rearing system microbiology may allow for the development of efficient and synergistic preventive methods. Novel prophylactic methods will hopefully replace or at least reduce the use of chemotherapeutic treatments. Expanding molecular techniques and new knowledge on the contribution of probiotics in larval nutrition, immunomodulation and host gene expression may lead to focused research and a better understanding of the mechanisms of probiotic action. The need for tailor-made probiotic solutions for successful larviculture has been emphasized (Azad and Al-Marzouk 2008). Future studies should focus on practical industrial-scale applications and demonstrate long term effects. Further, the production feasibility and cost–benefit analysis of developed probiotics should be addressed.

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10 Probiotic Applications in Temperate and Warm Water Fish Species

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ABSTRACT

Environmentally friendly aquaculture practices have seen the introduction of beneficial bacteria, such as probiotics, in the last decade. Probiotics are generally defined as live microbial feed supplements that can benefit the host by modulating the gut microbiota. The endogenous gut microbiotas of fish are integral in mediating the development, maintenance and effective functionality of the intestinal mucosa and gut associated lymphoid tissues (GALT). These microbial populations also provide a level of protection against pathogenic visitors to the gastrointestinal (GI) tract and aid host digestive function via the production of exogenous digestive enzymes and vitamins. Applications of probiotics to temperate and warm water teleosts can stimulate immune responses and enhance growth performance, feed utilization, digestive enzyme activities, antioxidant enzyme activities, disease resistance, larval survival and gut morphology, as well as modulate gene expression and the gut microbiota. They have the potential to elevate the innate defence mechanisms of fish and improve survival following exposure to a number of pathogens. In addition, recent studies have shown the probiotic potential to improve welfare and reproductive success. The studies available on probiotic applications for important temperate and warm water teleosts are discussed in the present review.

10.1 INTRODUCTION

The modern aquaculture industry demands strategies which help to maintain a microbiologically healthy environment to enhance production and profitability. In this context the ban on

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antibiotics as growth promoters (AGPs) has been a challenge and has increased pressure to find alternative methods to control and prevent pathogenic bacterial colonization. In fact, farmed fish are often subjected to environmental stressors (handling, overcrowding, challenging diets etc.) which may cause an imbalance in the intestinal ecosystem and could be a risk factor for pathogen infections. Probiotics are live microorganisms added to feed or rearing water that, when administered to fish in adequate amounts, confer increased viability, enhanced immunity and greater disease resistance. In addition, a possible role of probiotics on fish growth performance has been supported by recent studies suggesting that the use of bacterial probiotics in aquaculture is able to enhance growth through a positive modulation of the insulin-like growth factor (IGF) system associated with a general decrease of myostatin gene expression and lower cortisol levels (Carnevali *et al.* 2006; Avella *et al.* 2010a; 2011; 2012). As described by Waché and collaborators (2006), the use of probiotics may improve growth performance not only due to their beneficial effects on the digestive processes but also by improving stress tolerance (Rollo *et al.* 2006). Chronic and acute stress has negative impacts on the immune system, increasing the susceptibility of fish to infectious diseases.

Disease outbreaks resulting in high mortality rates during the rearing of fish larvae and unpredictable production of juveniles constitute major obstacles for further expansion of the aquaculture industry (Toranzo and Barja 1990; Bloch and Larsen 1993; Novoa *et al.* 1993; Zorrilla *et al.* 1999; Hellberg *et al.* 2002; Ucko *et al.* 2004; Johansen *et al.* 2004; Arijo *et al.* 2005; Nishizawa *et al.* 2006). One of the main beneficial characteristics of probiotics is the ability to inhibit pathogenic bacteria both *in vitro* and *in vivo* through several different mechanisms such as competitive exclusion (Moriarty 1997; Gomez-Gil *et al.* 2000; Balcázar *et al.* 2004; Vine *et al.* 2004), antiviral activities (Kamei *et al.* 1988; Girones *et al.* 1989; Direkbusarakom *et al.* 1998) and host immunomodulation (Picchietti *et al.* 2007; 2009). Recent literature demonstrates that probiotics, after sufficient administration, are able to colonize and multiply in the gut of the host and execute numerous beneficial effects by modulating various biological systems in the host (Nayak 2010; Merrifield *et al.* 2010a; Dimitroglou *et al.* 2011). In particular the main effects of these feed additives are the improved resistance to pathogenic bacteria colonization and enhanced host mucosal immunity, thus resulting in a reduced pathogen load, an improved health status of the animals and a reduced risk of pathogen translocation via the gastrointestinal (GI) tract. In particular, concerning their potency to modulate the immune system, it is widely known that different probiotics, applied as either monospecies or multi-species, can elevate phagocytic, lysozyme, complement and respiratory burst activity as well as the expression of various cytokines in fish (Nayak 2010; Merrifield *et al.* 2010a; Dimitroglou *et al.* 2011). Likewise, probiotics can stimulate the GI immune system of fish with a marked increase in the number of intestinal Ig⁺ cells, acidophilic granulocytes and T cells (Picchietti *et al.* 2007; 2009) and stimulation of intestinal pro-inflammatory cytokine gene expression (Liu *et al.* 2013; Standen *et al.* 2013).

These aspects will be reported for important temperate and warm water species in this chapter. Ichthyologists often group fish species into cold, temperate and warm water groups. This tends to be a rather arbitrary classification because seasonality, habitat range and migration patterns dictate that many fish species fall into more than one of these categories. Indeed, warm water and temperate water fish can tolerate a wide temperature range but they usually have a minimum temperature requirement for reproduction (ca. 20°C for many species) and optimal growth is typically above 20°C, with poor growth or arrested growth below 10–15°C (FAO 1989). In order to provide a systematic overview of probiotic

applications within this book, fish species have been grouped in this manner. In this context this chapter reviews the literature available on important temperate and warm water aquacultured fish species: cyprinids (Cyprinidae), tilapia (Cichlidae), soles (*Solea* spp.), groupers (Epinephelinae), catfishes (Siluriformes), European sea bass (*Dicentrarchus labrax*), gilthead sea bream (*Sparus aurata*), and finally the zebrafish (*Danio rerio*) as an important model organism.

Beyond these warm water species, probiotic benefits have also been reported in cobia (*Rachycentron canadum*) (Geng *et al.* 2012; 2013; Xing *et al.* 2013), oscar (*Astronotus ocellatus*) (Firouzbakhsh *et al.* 2011), spinefoot rabbitfish (*Siganus rivulatus*) (El-Dakar *et al.* 2007), sturgeons (*Acipenser persicus*) (Askarian *et al.* 2011), barramundi (*Lates calcarifer*) (Rengpipat *et al.* 2008), California halibut (*Paralichthys californicus*) (Zacarias-Soto *et al.* 2011), killifish (*Fundulus heteroclitus*) (Lombardo *et al.* 2011), Japanese pufferfish (*Takifugu rubripes*) (Biswas *et al.* 2013), clownfish (*Amphiprion ocellaris*) (Avella *et al.* 2010a), goldfish (*Carassius auratus*) (Sugita *et al.* 2009) and live-bearing ornamental fishes such as guppy (*Poecilia reticulata*), molly (*Poecilia sphenops*), green swordtail (*Xiphophorus helleri*), Southern platyfish (*Xiphophorus maculatus*) (Ghosh *et al.* 2007; 2008) and porthole livebearer (*Poeciliopsis gracilis*) (Hernandez *et al.* 2010).

Numerous investigations have evaluated the feasibility and efficacy of feeding warm water fish with potentially probiotic bacteria; the most documented are the lactic acid bacteria (LAB), *Bacillus* spp. and yeasts. More recently, however, the selection, characterization and application of autochthonous strains (bacteria isolated from the fish or their immediate rearing environment) have also stimulated much interest (Dimitroglou *et al.* 2011; Tapia-Panigua *et al.* 2012). To date, most of these studies have been performed on larvae and juveniles and it has been shown that during the initial feeding period it is possible to establish selected probiotic strains in the fish intestine. The current research suggests that probiotic applications may confer benefits on a number of important warm water fish species.

10.2 EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX* L.)

The European sea bass is the most important commercial fish widely cultured in the Mediterranean; annual production exceeded 120,000 tonnes in 2011 (FAO FIGIS 2013). The major contributors to this production volume are Greece, Turkey, Italy, Spain, Croatia and Egypt. Given the economical relevance of this species, several studies have investigated the gut microbiota and have evaluated the effects of probiotics applications. It has been demonstrated that the gut of European sea bass contains a minor LAB population. LAB isolated from the GI tract of sea bass include: *Lactobacillus fructivorans*, *Lactobacillus brevis*, *Lactobacillus curvatus*, *Lactobacillus acidophilus*, *Lactobacillus viridescens*, *Lactobacillus delbrueckii* subsp. *delbrueckii*, *Lactobacillus lindneri*, *Lactobacillus* spp., *Aerococcus viridans*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Enterococcus faecium*, *Enterococcus faecalis*, *Enterococcus sanguinicola*, *Enterococcus mundtii* and *Enterococcus pseudoavium* (Carnevali *et al.* 2006; Silvi *et al.* 2008; Bouriouni *et al.* 2012). Studies on the application of probiotics for sea bass have utilized *Lactobacillus* spp. (some of which are autochthonous strains), *Bacillus subtilis*, *Brevibacillus brevis*, *Vagococcus fluvialis* and yeasts (Table 10.1) and some of these studies have reported improved growth performance, stress tolerance and elevated larval survivability.

Table 10.1 Summary of *in vivo* sea bass probiotic studies.

Probiot	Parameters investigated	References
<i>Lb. delbrueckii</i> subsp. <i>delbrueckii</i>	GM, GP, STR	Carnevali <i>et al.</i> (2006)
<i>Lb. delbrueckii</i> subsp. <i>delbrueckii</i>	GM, SR, STR	Silvi <i>et al.</i> (2008)
<i>Lb. delbrueckii</i> subsp. <i>delbrueckii</i>	GH, IR	Picchietti <i>et al.</i> (2009)
<i>Lb. farciminis</i> and <i>Lb. rhamnosus</i>	D-EA, GH, GM, GP, M, SR	Frouël <i>et al.</i> (2008)
<i>D. hansenii</i> , <i>S. cerevisiae</i>	GM, D-EA, GP, M, SR	Tovar <i>et al.</i> (2002)
<i>Lb. delbrueckii</i> subsp. <i>delbrueckii</i> , <i>Lb. fructivorans</i> and <i>Lb. plantarum</i>	GP, IR	Abelli <i>et al.</i> (2009)
<i>Lb. plantarum</i> and <i>B. subtilis</i>	DR, PA	Touraki <i>et al.</i> (2012)
<i>Vagococcus fluvialis</i>	DR, PA	Sorroza <i>et al.</i> (2012)
<i>Vagococcus fluvialis</i>	IR	Romàn <i>et al.</i> (2012)
<i>Brevibacillus brevis</i>	GP, PA, SR	Mahdhi <i>et al.</i> (2012)
<i>D. hansenii</i>	D-EA, GP, M, SR	Tovar-Ramírez <i>et al.</i> (2004)
<i>D. hansenii</i>	AO-EA, GP	Tovar-Ramírez <i>et al.</i> (2010)

Source: Adapted from Dimitroglou *et al.* 2011.

Genera abbreviations: *B.* = *Bacillus*, *D.* = *Debaryomyces*, *Lb.* = *Lactobacillus*, *S.* = *Saccharomyces*.

Parameters investigated: AO-EA = antioxidant enzymes, DR = disease resistance, D-EA = digestive/intestinal enzymes, GH = gut histology/immunohistochemistry, GM = gut microbiota (inclusive of GI probiont recovery), GP = growth performance, IR = immunological/haematological response, M = malformations, PA = pathogen antagonism, SR = survival rate, STR = stress tolerance/response/biomarkers.

10.2.1 Growth and stress tolerance

The administration of *Lb. delbrueckii* subsp. *delbrueckii* to sea bass larvae at a final concentration of 10^5 bacteria ml^{-1} via rotifers from 11 to 29 days post hatching (dph) and via *Artemia* nauplii from 30 to 70 dph (treatment group A) or with *Artemia* solely as the carrier from 30 to 70 dph (group B) has been reported (Carnevali *et al.* 2006). In the control group no bacteria were added. Measures of standard length and body weight performed to assess juvenile growth at 70 dph evidenced a significant increase of body weight with respect to control larvae in both groups fed the probiotic. The highest growth was observed by group A (85 ± 5.36 mg) but larvae from group B were also significantly heavier (60 ± 6.7 mg) than the control (47 ± 4.69 mg). In addition, an increase of IGF-I transcription was observed in fish treated with LAB: IGF-I mRNA levels were six times higher in both probiotic treated groups with respect to the control. In contrast, myostatin mRNA transcription was significantly inhibited in probiotic treated groups. These results were in accordance with the increase of body weight reported in this study: fish fed on LAB showed 81% higher body weight in group A and 28% in group B, with respect to the control group. *Lb. delbrueckii* subsp. *delbrueckii* also decreased the cortisol levels.

Among probiotics, yeasts have been commonly isolated in the GI tract of fish and further used as functional feed additives (Gatesoupe 2007). Dietary administration of live *Debaryomyces hansenii*, at ca. 10^6 CFU g^{-1} , to sea bass larvae accelerated maturation of the digestive tract (50 mg versus 25 mg) (Tovar *et al.* 2002) and induced a significant increase in body weight, twice that of the control group, at 48 dph (Tovar-Ramírez *et al.* 2004). In addition, *D. hansenii* administration also reduced the antioxidant status of sea bass larvae as demonstrated by the gene expression and the enzymatic activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX) (Tovar-Ramírez *et al.* 2010). These analyses performed at 23 and 48 dph on the pancreatic segment of the digestive tract (including pancreas,

liver, vertebral axis, muscles, and part of the intestine) evidenced differences between controls and larvae fed yeast. At 23 dph, a peak in the expression of SOD and GPX genes and increased activity of the SOD enzyme were observed in the control group, indicating the presence of oxidative stress; this was not observed in the group fed with the yeast. Changes in enzyme and gene expression patterns were attributed to the presence of yeast, assuming a possible involvement of superoxide anion retention in fish larvae, which could represent importance to the host in increasing cell or tissue responsiveness to growth- and/or differentiation-enhancing factors. No changes of heat shock protein 70 (HSP70) were observed in sea bass larvae fed with *D. hansenii* at 23 and 48 dph.

Illustrating the importance of utilizing the correct dosage, the administration of *D. hansenii* at 7×10^5 CFU g⁻¹ led to a significantly lower growth rate with respect to the control group (Tovar *et al.* 2002), 1×10^6 CFU g⁻¹ led to significant improvements (Tovar- Ramírez *et al.* 2004) and 6×10^6 CFU g⁻¹ led to growth equal to that of the control (Tovar- Ramírez *et al.* 2004).

10.2.2 Effects of probiotics on European sea bass immune system

Manipulation of the larval digestive system in aquaculture, by the addition of probiotics through either culture water or live food, is an important tool to manage fish bacterial communities and pathogen–host interactions (Verschuere *et al.* 1999; 2000a; 2000b). However, the mechanisms of probiotic action in fish are not fully understood, although it is widely accepted that selected probiotic bacteria are able to stimulate the host immune system (Nayak *et al.* 2010; Dimitroglou *et al.* 2011). The first work that assessed the interaction between probiotics and the mucosal immune system of sea bass larvae and post-larvae tested a strain of *Lb. delbrueckii* subsp. *delbrueckii* (AS13B), isolated from the intestinal microbiota of adult sea bass (Picchiatti *et al.* 2009). *Lb. delbrueckii* subsp. *delbrueckii* was administered live to developing sea bass (during gut metamorphosis), using *Brachionus plicatilis* and *Artemia salina* as carriers. The results evidenced that in treated fish gut integrity was unaffected, while the density of acidophilic granulocytes in the intestinal mucosa was significantly higher than in controls. The probiotic induced T cell infiltration into the sea bass intestinal mucosa, which, concomitant with increased total body TcR- β transcripts, was indicative of a stimulatory effect of beneficial bacteria on the sea bass immune system. Furthermore, probiotics induced significantly lower IL-1 β transcripts and a trend towards lower IL-10, Cox-2 and TGF- β transcription in the treated groups compared with the control; these events were interpreted as the direct or indirect effect of beneficial bacteria on fish physiology. It is clear that future studies of the genomic analysis of cytokines and chemokines would help to elucidate the pathways which are involved in mediating the host immunostimulatory effects in order to find suitable probiotic treatments for sustainable aquaculture to reduce the use of antibiotics and guarantee a healthy environment for fish.

For this reason, recent studies have investigated some probiotic strains for use in aquaculture as preventive measures against vibriosis in the sea bass larvae (Touraki *et al.* 2012) and adults (Sorroza *et al.* 2012). In particular, an experimental challenge performed to evaluate the protection offered by a *B. subtilis* strain against infection by *Vibrio* (*Listonella*) *anguillarum* (Touraki *et al.* 2012) demonstrated that the mortality of fish challenged with the pathogen was reduced in the sea bass larvae treated with *B. subtilis* enriched *Artemia* nauplii compared with the untreated group. In addition, the administration of the probiotic *Vagococcus fluvialis*

to adult sea bass (Sorroza *et al.* 2012) induced protection against *V. anguillarum*, reaching a relative percentage survival (RPS) of 42.3% higher than the control group. *In vitro* assays validated the effects of *Vagococcus fluvialis* on the sea bass immune system, showing high values of phagocytic activity and peroxidase content in macrophages and head kidney (HK) leukocytes after incubation with the live or inactivated probiotic cells (Romàn *et al.* 2012).

10.3 GILTHEAD SEA BREEM (*SPARUS AURATA* L.)

The gilthead sea bream is a major fish species farmed in the Mediterranean. In 2011, Mediterranean farms produced 97,000 tonnes (FAO FIGIS 2013). Greece was by far the largest producer (accounting for 49% of production volume), followed by Turkey (15%), Spain (14%) and Italy (6%). In addition, considerable production occurs in Croatia, Cyprus, Egypt, France, Malta, Morocco, Portugal and Tunisia. Gilthead sea bream production also takes place in the Red Sea, the Persian Gulf, and the Arabian Sea. Studies on the application of probiotics for gilthead sea bream have utilized *Lactobacillus* spp., *Bacillus* spp., a diverse range of autochthonous bacterial strains and yeasts (Table 10.2). Such studies have shown benefits for survival, growth, stress tolerance, immunity and disease resistance.

10.3.1 Survival, growth and stress tolerance

The administration of *Lactobacillus fructivorans* (AS17B), isolated from adult gilthead sea bream gut, and *Lactobacillus plantarum* (906), isolated from human faeces, during sea bream development was performed using *Brachionus plicatilis* and/or *Artemia salina* nauplii and dry feed as vectors (Carnevali *et al.* 2004). The *Lb. fructivorans* and *Lb. plantarum* strains were mixed at a proportion of 80% and 20% (w/w), respectively, and added to live food (either rotifers or *Artemia*) at a final concentration of 10^5 bacteria ml^{-1} . One group received a mixture of both bacterial strains via rotifers from 5 to 26 dph and via *Artemia salina* from 27 to 47 dph. In treated groups, probiotic administration significantly reduced larvae and fry mortality but did not improve the body growth or the standard length. In the same specimens the probiotic influence on stress responsiveness was analysed; for this reason sea bream fry (47 dph) were subjected to pH stress (from 6.3 to 8.6). After one hour exposure the cumulative mortalities were recorded. The use of pH as a stressor induced a significantly higher cumulative mortality in the control group than in both probiotic treated groups. Since dead larvae were first observed after 30 min, cortisol levels and HSP70 gene expression were analysed before and after 25 min exposure to pH stress. Interestingly, pre-stress levels of HSP70 gene expression were significantly lower in both probiotic treated groups. Subsequently the pH stress induced a significant increase in HSP70 gene expression in all stressed groups (Rollo *et al.* 2006). The results obtained indicated that the administration of probiotics to sea bream fry induced higher HSP70 levels, indicating a greater potentiality to respond to the harmful conditions possibly present in fish farms. This hypothesis is supported by the fact that the levels of cortisol were significantly lower in both groups under probiotic treatment. These results suggested an improvement in tolerance to acute stress of fry fed with probiotics, by higher repairing processes at the cellular level.

Improvement of growth rate was also observed when a mixture of *B. subtilis*, *Bacillus licheniformis* and *Bacillus pumilus* was provided by rotifers and *Artemia* nauplii and added to the water (group 1) or supplied exclusively via live prey (group 2) (Avella *et al.* 2010b).

Table 10.2 Summary of *in vivo* gilthead sea bream probiotic studies.

Probiotic	Parameters investigated	References
<i>Lb. delbrueckii</i> subsp. <i>lactis</i> , <i>B. subtilis</i>	IR	Salinas <i>et al.</i> (2005)
<i>Lb. delbrueckii</i> subsp. <i>lactis</i> , <i>B. subtilis</i>	IR	Salinas <i>et al.</i> (2008)
<i>Lb. fructivorans</i> and <i>Lb. plantarum</i>	GM, SR	Carnevali <i>et al.</i> (2004)
<i>Lb. fructivorans</i> , <i>Lb. plantarum</i>	GM, SR, STR	Rollo <i>et al.</i> (2006)
<i>Lb. fructivorans</i> , <i>Lb. plantarum</i>	GH, IR	Picchietti <i>et al.</i> (2007)
<i>Lb. delbrueckii</i> subsp. <i>delbrueckii</i> , <i>Lb. fructivorans</i> and <i>Lb. plantarum</i>	GP, IR	Abelli <i>et al.</i> (2009)
<i>Vagococcus fluvialis</i>	IR	Romàn <i>et al.</i> (2012)
Commercial product containing: <i>Lb. plantarum</i> , <i>Lb. bulgaricus</i> , <i>Lb. acidophilus</i> , <i>Lb. rhamnosus</i> , <i>Str. thermophilus</i> , <i>Bi. bifidum</i> , <i>E. faecium</i>	D-EA, GM, GP, SR	Suzer <i>et al.</i> (2008)
<i>Sh. putrefaciens</i> (Pdp11), 51M6 (<i>Shewanella</i> sp.), <i>L. delbrueckii</i> , <i>B. subtilis</i>	IR	Salinas <i>et al.</i> (2006)
<i>Cytophaga</i> spp., <i>Roseobacter</i> spp., <i>Ruegeria</i> spp., <i>Paracoccus</i> spp., <i>Aeromonas</i> spp., <i>Shewanella</i> spp.	SR	Makridis <i>et al.</i> (2005)
<i>Sh. putrefaciens</i> (Pdp11), 51M6 (<i>Shewanella</i> sp.), and <i>Micrococcus</i> sp. (Pdp5)	MA, DR	Chabrilón <i>et al.</i> (2006)
<i>Sh. putrefaciens</i> (Pdp11) and 51M6 (<i>Shewanella</i> spp.)	IR	Díaz-Rosales <i>et al.</i> (2006)
<i>Sh. putrefaciens</i>	BC, GH, GP, IR, STR	Varela <i>et al.</i> (2010)
<i>B. subtilis</i> , <i>Tetraselmis chuii</i> and <i>Phaeodactylum tricornutum</i>	DR, IR	Cerezuela <i>et al.</i> (2012a)
<i>B. subtilis</i> , <i>Tetraselmis chuii</i> and <i>Phaeodactylum tricornutum</i>	GH, GM	Cerezuela <i>et al.</i> (2012b)
<i>D. hansenii</i>	AO-EA, IR, MA	Reyes-Becerril <i>et al.</i> (2008a)
<i>D. hansenii</i>	GM, IR	Tapia-Paniagua <i>et al.</i> (2011)
<i>D. hansenii</i>	GM, IR	Reyes-Becerril <i>et al.</i> (2012)

Source: Adapted from Dimitroglou *et al.* 2011.

Genera abbreviations: *B.* = *Bacillus*, *Bi* = *Bifidobacterium*, *D.* = *Debaryomyces*, *E.* = *Enterococcus*, *Lb.* = *Lactobacillus*, *S.* = *Saccharomyces*, *Sh.* = *Shewanella*, *Str.* = *Streptococcus*.

Parameters investigated: AO-EA = antioxidant enzymes, BC = body composition, D-EA = digestive/intestinal enzymes, DR = disease resistance, GH = gut histology/immunohistochemistry, GM = gut microbiota (inclusive of GI probiont recovery), GP = growth performance, IR = immunological/haematological response, MA = mucus adhesion, SR = survival rate, STR = stress tolerance/response/biomarkers.

Growth (standard length and body weight) significantly increased in both experimental groups, at both larval (47 dph) and juvenile (75 dph) stages. The increases in body weight and total length were in agreement with the expression of genes involved in growth metabolism (IGFI and myostatin) and in animal welfare (HSP70). The higher expression of IGFI and the lower levels of myostatin in groups fed probiotics were concomitant with the lower expression of HSP70 and glucocorticoid receptor (GR), evidencing the beneficial role of the supplied *Bacillus* mixture on fish welfare; however, no differences in survival rate were found among groups (Avella *et al.* 2010b).

Similar effects were observed in the dietary administration of a *Sh. putrefaciens* Pdp11 to sea bream larvae (Varela *et al.* 2010). Under high stocking densities, an improvement in growth performance was evident from 64 dph until the end of the experiment. In addition, most of the metabolic parameters tested in the blood plasma, liver and muscle were comparable between both groups, except for the muscle total α -amino acid values and the hepatic glycogen content which were both higher in specimens fed with the probiotic (Varela *et al.* 2010). To test the beneficial effects of the *Sh. putrefaciens* Pdp11 on stress tolerance, control specimens and fish fed with probiotic were kept under either low (3 kg per m³) or high density (30 kg per m³). The administration of probiotic *Sh. putrefaciens* Pdp11 in the diet promoted growth and improved stress tolerance under high stocking density.

Similar results were previously observed by Suzer and coworkers (2008) with the use of photosynthetic bacteria and *Lactobacillus* spp. (used singularly or in combination) as probiotic supplements for gilthead sea bream. These probiotics were tested in larvae until 35 dph. The administration during larval development was performed with live food (rotifer and *Artemia*; group 1) or with live food and water (group 2) or directly to the water (group 3). The control group received no probiotic. Total bacterial counts among probiotic supplemented groups were significantly different from the total bacterial counts in the controls. In addition, mean digestive enzyme activities of all probiotic treatment groups were significantly different to that of the control. In all probiotic treatments, the specific activities of pancreatic and intestinal enzymes were significantly higher in larvae provided with the probiotic by live food and live food with water but not in the group where probiotic was supplemented only by water. A significant increase (13–105% higher) in survival was observed when the larvae were provided with the probiotic by live food and via water. Similarly a significant increase in SGR (2–9% higher) was observed in groups 1 and 2 where the probiotic was provided via live food. The supplementation of *Lactobacillus* spp. directly to the tank water did not affect growth parameters and digestive enzyme activities, and therefore administration of probiotics by this method would not be effective in terms of husbandry parameters and nutritional condition (Suzer *et al.* 2008).

10.3.2 Effects on the sea bream immune system

Recent studies have evidenced that probiotic bacteria play an important role in enhancing the immune system of adult sea bream and have direct or indirect effects on the intestinal physiology. Such studies can be schematically subdivided into those involving the use of monospecies and multispecies preparations or live bacteria and inactivated bacteria (Salinas *et al.* 2005; 2006; 2008; Díaz-Rosales *et al.* 2006). In this context, Salinas *et al.* (2005) investigated in adult sea bream, at the systemic level, whether or not the stimulation of the innate immune parameters prompted by the oral administration of two live bacteria in combination, *Lb. delbrueckii* subsp. *lactis* and *B. subtilis*, differs from that elicited by monospecies preparations of the same probiotic candidates. The authors observed that the immunostimulatory effect of the two different bacteria administered together can be more effective and more consistent than that of the monostrain probiotic application; it was hypothesized that mixed cultures are complementary by differently modulating host immune responses. In addition, to evaluate the effects of heat-inactivated whole bacteria on sea bream innate immune responses, Diaz-Rosales *et al.* (2006) investigated the effects of dietary administration of two heat-inactivated bacteria from the *Shewanella* genus (*Sh. putrefaciens* Pdp11 and *Shewanella* sp. 51M6, previously isolated from gilthead sea bream skin), singularly or in combination for 1, 2, 3 and 4 weeks. The results showed that the two bacteria had no synergistic

immunostimulatory effects after combined oral administration. This was possibly due to the close phylogenetic relationship between the two bacteria assayed and thus no complementary effect was attained. However, both heat-inactivated bacterial species were capable of modulating the sea bream innate immune system, showing their potential to induce immune responses probably with the recognition by mucosal cells of bacterial antigens or other surface bound structures rather than a host response against probiotic metabolic products or active participation of the probiont in the interactions with the epithelial surface. In fact in a following study, Salinas *et al.* (2008) demonstrated that heat-inactivated *Lb. delbrueckii* subsp. *lactis* and *B. subtilis* applied via the diet to gilthead sea bream induced effects on the systemic humoral and cellular innate immune system as well as on local cellular defences in the gut, confirming that an active participation by the probiont is not always necessary. To assess whether or not the bacterial strains or their origin were related to the immunomodulatory properties of probiotics, Salinas and colleagues (2006) conducted a further study assaying the *in vitro* immunostimulatory properties of four heat-inactivated bacterial species: *Sh. putrefaciens* Pdp11, *Shewanella* sp. 51M6, *Lb. delbrueckii* subsp. *lactis* and *B. subtilis*. The analysis of the individual bacterial species revealed a similar *in vitro* immunostimulatory effect for all the studied strains, which was strongly related to the bacterial concentration, confirming that the use of appropriate bacterial concentrations and administration times is critical to achieve the desired effect on the immune system in aquacultured fish. In addition to the studies on the juvenile and adult sea bream immune system, Picchiatti *et al.* (2007) assessed the potential immunomodulatory properties of lactobacilli (*Lb. fructivorans* AS17B and *Lb. plantarum*) on the developing sea bream immune system to evaluate the beneficial effects of microbial products on larval intestinal immunity. In particular, the work demonstrated that probiotic applications could increase the density of Ig⁺ cells (Figure 10.1A), total acidophilic granulocytes (Figure 10.1B–D) and in particular a subpopulation of acidophilic granulocytes (shown to be phagocytic against *V. anguillarum*) (Chaves-Pozo *et al.* 2005) in the gut of sea bream. However, these effects were dependent on the larval age and/or the duration of the treatment.

The modulation of the intestinal microbiota and immune system of gilthead sea bream was also evaluated following the probiotic administration of the yeast *Debaromyces hansenii* L2 both singly (Reyes-Becerril *et al.* 2012) or in conjunction with inulin (Tapia-Paniagua *et al.* 2011). In these studies, changes in the intestinal microbiota in fish receiving the supplemented diet were detected. These changes coincided with an up-regulation of the expression of immunological genes in the skin and HK at 2 and 4 weeks, respectively (Tapia-Paniagua *et al.* 2011; Reyes-Becerril *et al.* 2012). In addition, Romàn and colleagues (2012) evaluated the *in vitro* effects of the probiotic *Vagococcus fluvialis* on sea bream leukocytes. In particular *Vagococcus fluvialis*, both live and inactivated, produced dose-dependent increments of respiratory burst activity in sea bream leukocytes as previously described by Salinas *et al.* (2006) after treatment with four different probiotic strains. In addition, the complement activity, serum IgM level, respiratory burst, phagocytic activity and expression of seven selected immune-related genes were also evaluated in sea bream HK samples following combined or individual dietary administration with microalgae (*Phaeodactylum tricornutum* and *Tetraselmis chuii*) and *B. subtilis* (Cerezuela *et al.* 2012a). The dietary applications of *B. subtilis*, *Tetraselmis chuii* and *Phaeodactylum tricornutum*, singly or in combination, exhibit up-regulating effects on gilthead sea bream immune parameters, although *Phaeodactylum tricornutum* demonstrated the highest immunomodulatory activity. Despite this no protection was afforded when sea bream were challenged with *Photobacterium damsela* subsp.

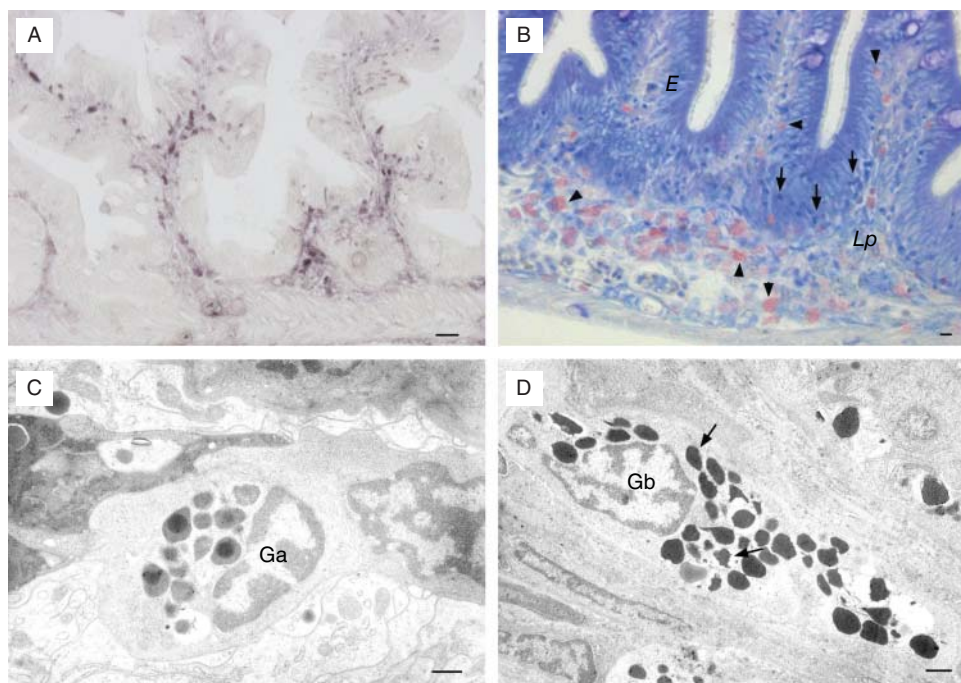


Fig. 10.1 (A) Immunohistochemistry of Ig⁺ intestinal lymphocytes in 99 dph sea bream fry. The polyclonal antiserum ORa immunostained Ig⁺ cells seed both the mucosal epithelium and the lamina propria (probiotic group A). (B) May-Grünwald-Giemsa staining of posterior intestine in 99 dph sea bream fry showing lymphoid cells (arrows) and acidophilic granulocytes (arrowheads) in the mucosal epithelium (E) and lamina propria (Lp) (probiotic group A). (C) Electron microscopy of sea bream granulocytes housed in the intestinal mucosa. This subpopulation (Ga) is characterized by a rounded shape and cytoplasmic granules. The granules have electron dense paracrystalline cores. (D) Electron microscopy of intestinal sea bream granulocytes housed in the lamina propria (Gb). Gb granulocytes have an elongated shape, a distinct granule type and autolysosomes (arrows). Scale bars: A = 20 μm; B = 5 μm; C = 500 nm; D = 1 μm. [Source: Simona Picchiatti *et al.* 2007.] For colour detail see Plate 11.

piscicida (Cerezuela *et al.* 2012a). Curiously, fish fed the experimental diets showed important alterations in the intestinal microbiota and signs of oedema and inflammation that could compromise intestinal barrier function and homeostasis (Cerezuela *et al.* 2012b). Similar changes in the intestinal morphology and microbiota were caused by dietary administration of inulin and *B. subtilis* singly or in combination with gilthead sea bream (Cerezuela *et al.* 2013).

10.4 PROBIOTIC APPLICATIONS IN SOLE SPP.

Probiotics have been studied for use in the rearing of two sole species: Senegalese sole (*Solea senegalensis*), which has become of great interest to fish producers in the last decade due to its high value and the increased market demand (Howell 1997; Imsland *et al.* 2003; Overton *et al.*

Table 10.3 Summary of *in vivo* probiotic applications on sole species.

Probiotic	Parameters investigated	References
Senegalese sole		
<i>Vibrio</i> sp. (2J18), Gram-positive sp. (J84), <i>Shewanella</i> sp. (2J27)	GM, SR	Makridis <i>et al.</i> (2008)
<i>Sh. putrefaciens</i> (Pdp11) and <i>Sh. baltica</i> (Pdp13)	BC, D-EA, GH, GM, GP, SR	Sáenz de Rodríguez <i>et al.</i> (2009)
<i>Sh. putrefaciens</i> (Pdp11) and <i>Sh. baltica</i> (Pdp13)	DR, GP, IR	Díaz-Rosales <i>et al.</i> (2009)
<i>Sh. putrefaciens</i> (Pdp11) and <i>Sh. baltica</i> (Pdp13)	BC, DR, GH, GM, GP, SR	de La Banda <i>et al.</i> (2010)
<i>Sh. putrefaciens</i> (Pdp11)	BC, DR, GP	de La Banda <i>et al.</i> (2012)
<i>Sh. putrefaciens</i> (Pdp11)	GM	Tapia-Paniagua <i>et al.</i> (2010)
<i>Sh. putrefaciens</i> (Pdp11)	BC, GM, GP, SR	Lobo <i>et al.</i> (2013)
Common sole		
<i>E. faecium</i> IMC 511	GP, GM, SR, STR	Avella <i>et al.</i> (2011)
<i>E. faecium</i> IMC 511	GP, STR	Palermo <i>et al.</i> (2011)

Source: Adapted from Dimitroglou *et al.* 2011.

Genera abbreviations: *E.* = *Enterococcus*, *Sh.* = *Shewanella*.

Parameters investigated: BC = body composition, D-EA = digestive/intestinal enzymes, DR = disease resistance, GH = gut histology/immunohistochemistry, GM = gut microbiota (inclusive of GI probiont recovery), GP = growth performance, IR = immunological/haematological response, SR = survival rate, STR = stress tolerance/response/biomarkers.

2010); and common or Dover sole (*Solea solea*). A number of probiotics have been isolated and tested on sole and several strains isolated from different teleost species have also been applied (Dimitroglou *et al.* 2011; Table 10.3). In particular, the probiotic strains Pdp11 and Pdp13, from the Alteromonadaceae family, are among the most well researched Gram-negative bacterial probiotics in fish (Díaz-Rosales *et al.* 2006; 2009; Sáenz de Rodríguez *et al.* 2009; de La Banda *et al.* 2010; Tapia-Paniagua *et al.* 2010; 2012). These strains were originally isolated from gilthead sea bream skin and later identified as *Shewanella putrefaciens* (Pdp11) and *Shewanella baltica* (Pdp13) (Díaz-Rosales *et al.* 2009). These probiotic species have been extensively studied in Senegalese sole and varying benefits have been documented for growth performance, immunomodulation and disease resistance (for a review see Tapia-Paniagua *et al.* 2012). These strains have been reported to modulate the gut microbiota of sole by changing operational taxonomic units (OTU) community profiles, elevating inter-fish community similarities and elevating the abundance of species related to the *Vibrio* genus; readers with a specific interest on their abilities to modulate the gut microbiota are referred to *Chapter 8*.

10.4.1 Effects on growth

Sáenz de Rodríguez and collaborators (2009) administered dietary supplementation of *Sh. putrefaciens* (Pdp11) and *Sh. baltica* (Pdp13) to juvenile Senegalese sole for 60 days. Experimental diets were made in the laboratory by supplementation of the diet with lyophilized cells of Pdp11 and Pdp13 (at 10^9 CFU g⁻¹) in an alginate based top coat. The dose was the same as previous studies which evidenced health benefits in *S. senegalensis* (Díaz-Rosales *et al.* 2009). The controls were fed only with a probiotic-free alginate top coated diet. Growth and nutrient

utilization were significantly higher in fish receiving probiotics than in those fed the control diet. No differences were observed in proximal composition, though significantly higher lipid muscle content was present in fish receiving Pdp13. Those fish also exhibited higher activities of alkaline phosphatase when compared to Pdp11 and control groups. Recently, similar results were obtained by de La Banda *et al.* (2012) by the administration of *Sh. putrefaciens* (Pdp11). This latter study aimed to elucidate the effects of fresh and lyophilized probiotic cells incorporated into fish feed at 10^9 cells g^{-1} : both fresh and lyophilized Pdp11 were provided to juvenile Senegalese sole for 60 days. Growth rates were significantly higher in fish fed the fresh probiotic compared with fish fed the control diet. The growth of fish receiving lyophilized bacteria was similar to that of the control. Body fatty acids profile, total lipids and protein levels were not different among the dietary treatments. These findings suggest that the alteration of microbial composition and activity, as likely to occur more rapidly with fresh cells, may be partly responsible for improved digestive function.

Beneficial effects of probiotic administration were also observed in *Solea solea* by Avella *et al.* (2011). The administration of the *E. faecium* IMC 511 isolated from common sole brood stock as a probiotic in the farming of *Solea solea* larvae and juveniles evidenced the high potentiality of this strain. *E. faecium* IMC 511 was administered twice daily to sole larvae through live feeds, at the final concentration of 10^3 or 10^5 CFU ml^{-1} . Five groups were established: group 1 was the control under a standard feeding regime; group 2 was fed *E. faecium* IMC 511 supplied from the opening of the mouth (2 dph) at the final concentration of 10^3 CFU ml^{-1} ; group 3 was supplied *E. faecium* IMC 511 from the first day of cofeeding (11 dph) at the final concentration of 10^3 CFU ml^{-1} ; group 4 was supplied *E. faecium* IMC 511 from the opening of the mouth (2 dph) at the final concentration of 10^5 CFU ml^{-1} ; and group 5 was supplied *E. faecium* IMC 511 from the first day of co-feeding (11 dph) at the final concentration of 10^5 CFU ml^{-1} . After 50 days administration, probiotic treated sole juveniles had significantly higher body weight and total length. In particular, all probiotic treated groups had significantly higher total length than the control, but no significant differences were observed among the probiotic groups. The body weights of all probiotic treated groups were significantly higher than the control and the higher concentration of probiotic (10^5 CFU ml^{-1}) induced the highest body weight increase. No significant differences were found among the early treated groups (groups 2 and 4) and those administered at the first cofeeding (groups 3 and 5). These results were supported by the molecular data showing a significant decrease of myostatin gene expression in probiotic fed larvae with respect to the control group. Survival was not affected but HSP70 mRNA levels were significantly reduced in probiotic fed larvae with respect to the control group, suggesting a better tolerance of the farm conditions in fish fed the probiotic. This hypothesis was in contrast with cortisol levels which were found to be significantly higher after 10 days of treatment in probiotic fed larvae (Palermo *et al.* 2011), while on days 30 and 50 the levels were similar among all experimental groups. The study suggests that 50 days of probiotic treatment may improve common sole larval growth but not survival.

10.4.2 Effects on survival and the immune system

In order to prevent bacterial diseases which usually hamper intensive sole farming, some studies have sought to isolate bacterial strains with antagonistic activity to sole pathogenic bacteria (Chabrilón *et al.* 2005a; 2005b). In particular, Chabrilón *et al.* (2005b) demonstrated the efficacy of dietary supplemented *Sh. putrefaciens* (strain Pdp11) to reduce *Vibrio harveyi*

induced mortalities in Senegalese sole during an *in vivo* challenge study. In this investigation Senegalese sole, with an average weight of 15–20 g, received the feed supplemented with the potential probiotic Pdp11 for 15 days while the control received a non-supplemented feed. After the *V. harveyi* challenge experiment the mortality in fish receiving the diet supplemented with Pdp11 (10%) was significantly lower than that in fish which received a normal diet without the probiotic (60%). The relative percentage survival (RPS) for the challenge assay with *V. harveyi* was 83.33%. The demonstrated ability of the bacterial strain to interfere with *V. harveyi* adhesion to mucus, as well as the adhesion to host surfaces, were suggested to be partial mechanisms in which Pdp11 reduced sole mortalities during the challenge. In addition, *Sh. putrefaciens* (Pdp11) and *Sh. baltica* (Pdp13) have been reported to reduce mortalities in Senegalese sole after challenges with *Photobacterium damsela* subsp. *piscicida* (Díaz-Rosales *et al.* 2009; de La Banda *et al.* 2010). In particular the results reported by Díaz-Rosales *et al.* (2009) showed that despite their affiliation within the same genus, and the same origin of both microorganisms assayed, their effects on mortality and respiratory burst activity were different. In fact, the cumulative percentage of mortality after the *Photobacterium damsela* subsp. *piscicida* challenge was 100% in the groups fed with the control diet, whereas mortality rates observed in the groups fed with Pdp11 and Pdp13 supplemented diets ranged from 75% to 100% and 65% to 80%, respectively. In all cases the mortality of fish receiving the diet supplemented with Pdp13 was significantly lower than in fish receiving the diet supplemented with Pdp11; however, only Pdp11 significantly enhanced superoxide anion production by sole HK phagocytes. The authors suggested that the increase of the respiratory burst activity of the sole phagocytes could be strain dependent; however, this stimulatory effect was not essential to enhance survival after the challenge with *Photobacterium damsela* subsp. *piscicida*, and thus Pdp13 could exert its effects on immunological abilities differently from respiratory burst activity. More recently de La Banda *et al.* (2012) further investigated the health protection induced by the strain Pdp11 on Senegalese sole juveniles. In this study Pdp11, both fresh and lyophilized cells, were incorporated into fish feed at a concentration of 10^9 cells g⁻¹, and then given to juvenile sole for 2 months. At the end of the study, fish were challenged by intraperitoneal (IP) inoculation with *Photobacterium damsela* subsp. *piscicida*. In this case the RPS obtained ranged between 25% and 44% for fish receiving both probiotic supplemented diets, although only fresh Pdp11 enhanced Senegalese sole growth when compared with control. Such positive effects of *Shewanella* species reported on Senegalese sole juveniles are in agreement with previous data obtained on developing sole (Makridis *et al.* 2008) where early phase larval survival was improved with the application of various bacteria, including *Shewanella*. The authors set up the feeding experiment of sole larvae and post larvae comprising three treatments: cultures of a *Vibrio* sp. (strain 2J18) were added to treatment A; a Gram-positive strain J84 (unidentified Gram-positive, oxidase-negative and catalase-positive) and *Shewanella* sp. (strain 2J27) were added to treatment B; and no bacteria were added to the control, treatment C. The results evidenced that the addition of bacteria in treatment B increased survival of larvae in the first phase of the experiment (0–20 dph) while no differences were observed in survival during the second phase (20–60 dph). Although the current knowledge demonstrates that the use of beneficial microbes at the larval stages can improve sole survival and disease resistance, further studies are necessary to clarify if these benefits are due to microbial control by various mechanisms, such as competition for nutrients, production of bacteriocins and competition for attachment sites, and/or if they are due to immunomodulatory effects.

10.5 GROUPERS

Groupers are fish typically having a stout body and a large mouth from a number of genera in the subfamily Epinephelinae of the family Serranidae and order Perciformes. The common name ‘grouper’ is usually given to fish in one of two large genera: *Epinephelus* and *Mycteroperca*. Groupers are widely distributed in tropical and warm waters worldwide. Due to high consumer demand, and their fast growth, efficient feed conversion, desirable taste and high economic value (Pierre *et al.* 2008; Harikrishnan *et al.* 2011), a grouper farming industry has become established over the last two decades and continues to be an important value added industry in the Far East and South East Asia (Pierre *et al.* 2008). However, the rapid development of intensive grouper culture leads to incidences of infectious diseases caused by bacteria, viruses and parasites that have become more severe and result in serious economic losses in farms and hatcheries (Harikrishnan *et al.* 2010a; 2011). Therefore, large volumes of traditional antibiotics and chemical disinfectants are often used for disease prevention and control. However, the use of antibiotics and chemical disinfectants as a preventive measure has been questioned because they can alter the gut microbiota and induce resistant bacterial populations, with unpredictable long-term effects on public health (Merrifield *et al.* 2010a; Harikrishnan *et al.* 2011). From a scientific point of view, the use of probiotics has been suggested to be an alternative method for the prevention and control of various diseases in aquaculture and a number of studies have been undertaken with groupers (Wang *et al.* 2008a; Qi *et al.* 2009; Nayak 2010; Merrifield *et al.* 2010a; Harikrishnan *et al.* 2011; Pan *et al.* 2013; Table 10.4).

10.5.1 Effects of probiotics on orange-spotted grouper (*Epinephelus coioides*)

Orange-spotted grouper is an economically important marine fish cultured in China and South East Asian countries. A number of allochthonous probiotics have been tested on orange-spotted grouper growth, immune status and disease resistance. A diet containing *Lb. plantarum*, isolated from homemade Korean-style pickled cabbage, at 10^6 , 10^8 and 10^{10} CFU g⁻¹, significantly improved the weight gain and feed efficiency of orange-spotted grouper (Son *et al.* 2009). In addition, grouper fed the probiont at 10^8 CFU kg⁻¹ suffered minimum mortality when challenged with *Streptococcus* sp. for 5 days and an iridovirus for 7 days; the probiotic groups also showed significantly higher complement, lysozyme and glutathione activities after 4 weeks (Son *et al.* 2009). Grouper fed a diet containing *Saccharomyces cerevisiae* at levels of 10^3 , 10^5 and 10^7 CFU kg⁻¹ for four weeks demonstrated significantly increased weight gain and feed efficiency, and induced up-regulation of innate cellular and humoral immune responses together with increased resistance to *Streptococcus* sp. and iridovirus challenges, especially in the 10^7 CFU kg⁻¹ group (Chiu *et al.* 2010). Dietary administration of *B. subtilis* E20 isolated from fermented boiled soybeans fed to *E. coioides* at levels of 10^4 , 10^6 and 10^8 CFU g⁻¹ could improve the feed efficiency and growth rate, and induce up-regulation of innate cellular and humoral immune responses together with increasing the resistance against *Streptococcus* sp. and an iridovirus (Liu *et al.* 2012).

Five potential beneficial bacteria have been isolated from the gut of juvenile grouper *E. coioides* and identified as *B. pumilus* SE5, *Bacillus clausii* DE5, *Psychrobacter* sp. SE6, *Lc. lactis* MM1 and *E. faecium* MM4 (Sun *et al.* 2009). *In vitro* studies showed that the five strains exhibited antagonistic activity against fish pathogens and had good tolerance to artificial gastric

Table 10.4 Summary of *in vivo* probiotic applications with groupers.

Probiotic	Parameters investigated	References
<i>Epinephelus coioides</i>		
<i>Lb. plantarum</i>	DR, GM, GP, IR	Son <i>et al.</i> (2009)
<i>Lc. lactis</i> , <i>E. faecium</i>	D-EA, GP, IR	Sun <i>et al.</i> (2012b)
<i>Lc. lactis</i>	GM	Sun <i>et al.</i> (2012c)
<i>E. faecium</i>	GM	Sun <i>et al.</i> (2012a)
<i>B. pumilus</i>	GM	Sun <i>et al.</i> (2011a)
<i>B. pumilus</i> , <i>B. clausii</i>	AO-EA, D-EA, GM, GP, IR, SR	Sun <i>et al.</i> (2013)
<i>B. pumilus</i> , <i>B. clausii</i>	GP, IR, SR	Sun <i>et al.</i> (2010a)
<i>B. clausii</i>	GM	Yang <i>et al.</i> (2012)
<i>B. subtilis</i>	IR, GM, GP, SR	Purwandari and Chen (2013)
<i>B. subtilis</i>	AO-EA, DR, GM, GP, IR	Liu <i>et al.</i> (2012)
<i>B. coagulans</i>	DR, IR, PA	Pan <i>et al.</i> (2013)
<i>Psychrobacter</i> sp.	GM	Yang <i>et al.</i> (2011)
<i>Psychrobacter</i> sp.	AO-EA, D-EA, GP, IR, SR	Sun <i>et al.</i> (2011b)
<i>S. cerevisiae</i>	DR, GM, GP, IR, SR	Chiu <i>et al.</i> 2010
<i>Epinephelus bruneus</i>		
<i>Lb. sakei</i>	DR, IR	Harikrishnan <i>et al.</i> (2010b)
<i>Mycteroperca rosacea</i>		
<i>D. hansenii</i>	AO-EA, DR, IR, STR	Reyes-Becerril <i>et al.</i> (2008b)
<i>D. hansenii</i>	AO-EA, DR, GP, IR	Reyes-Becerril <i>et al.</i> (2011)

Genera abbreviations: *B.* = *Bacillus*, *D.* = *Debaryomyces*, *E.* = *Enterococcus*, *Lb.* = *Lactobacillus*, *Lc.* = *Lactococcus*, *S.* = *Saccharomyces*.

Parameters investigated: AO-EA = antioxidant enzymes, D-EA = digestive/intestinal enzymes, DR = disease resistance, GM = gut microbiota (inclusive of GI probiont recovery), GP = growth performance, IR = immunological/haematological response, PA = pathogen antagonism, SR = survival rate, STR = stress tolerance/response/biomarkers.

and intestinal fluids (Sun *et al.* 2009; 2010b). Therefore, it was speculated that these strains may be positively related to the growth and health of grouper, and may be potential probiotic strains. Subsequent *in vivo* studies revealed that individual dietary administration of the five potential probiotics at a dose of 10^8 CFU g⁻¹ for 60 days could significantly improve the feed utilization of *E. coioides* (Sun *et al.* 2010a; 2011b; 2012b). Interestingly, enhanced specific activities of digestive enzymes were observed in the probiotic groups, while the five probiotic strains did not secrete any digestive enzymes (protease, amylase and lipase) under *in vitro* conditions. Therefore, the authors speculated that the probiotics may induce the secretion of digestive enzymes and this might have led to improvement in the whole digestive process, enhancing the digestibility of feed and thereby the nutritive utilization of feed (Sun *et al.* 2011b; 2012b). The increase in digestive enzyme activities and therefore the digestibility of feed through the use of probiotics have also been demonstrated in common carp by *Bacillus* sp. (Yanbo and Zirong 2006) and in gilthead sea bream larvae by *Lactobacillus* spp. (Suzer *et al.* 2008).

The effect of these five probiotic strains on the immune response of *E. coioides* has been investigated. *B. pumilus* SE5 and *B. clausii* DE5 enhanced the serum complement C3 (C3) and IgM levels at day 30 and serum leukocyte phagocytic activity and lysozyme activity at day 60 (Sun *et al.* 2010a); *Psychrobacter* sp. SE6 significantly improved the complement C4 (C4) level at day 60 (Sun *et al.* 2011b); and *Lc. lactis* and *E. faecium* significantly enhanced serum C3 levels and *Lc. lactis* also improved serum lysozyme activity (Sun *et al.* 2012b).

In conclusion, probiotics could affect the general immune responses in *E. coioides*. So far, however, no information is available about the effect of probiotics on the mucosal immune response of *E. coioides*. Therefore, as proposed by Merrifield *et al.* (2010a), future works should aim to enhance our understanding of these interactions by assessing the expression of immune activatory or immunoregulatory cytokines, pattern recognition receptors (PRRs) and anti-microbial proteins using molecular techniques such as transcriptomics and proteomics. Such studies will elucidate the cellular and molecular mechanisms involved in probiotic modulation of the mucosal immune system, informing future approaches in aquaculture in the maintenance of an efficient immune system which is reflected in fish quality and productivity.

In general, probiotic applications have demonstrated a range of growth and health benefits in *E. coioides* (Son *et al.* 2009; Chiu *et al.* 2010; Sun *et al.* 2010a; 2011b; 2012b), but the mechanisms involved in mediating these responses are largely unknown. It is suggested that a clear understanding of the effect of probiotics on the autochthonous gut microbiota is integral to illustrate the underlying mechanisms which result in host benefits (Ferguson *et al.* 2010; Merrifield *et al.* 2010a). It has been reported that *B. pumilus* SE5 (Sun *et al.* 2011a) and *B. clausii* DE5 (Yang *et al.* 2012) exerted no significant impact on the autochthonous gut microbiota, while *Psychrobacter* sp. SE6 (Yang *et al.* 2011), *Lc. lactis* MM1 (Sun *et al.* 2012c) and *E. faecium* MM4 (Sun *et al.* 2012a) elevated the microbial diversity along the GI tract of *E. coioides*. Interestingly, various potentially beneficial bacteria were selectively stimulated, while some potential harmful species were suppressed by probiotic *B. pumilus* SE5, *B. clausii* DE5, *Psychrobacter* sp. SE6 and *Lc. lactis* MM1 (Yang *et al.* 2011; 2012; Sun *et al.* 2011a; 2012c; 2013). This might suggest that the autochthonous gut microbiota of *E. coioides* was modulated to some degree by the probiotics (refer to Chapter 8).

10.5.2 Effects of probiotic on kelp grouper (*Epinephelus bruneus*)

Recently, culture of kelp grouper *E. bruneus* started in Korea and Japan with controlled larval production. The effect of *Lb. sakei* BK19 (10^8 cells g^{-1}) on *E. bruneus* streptococcosis resistance (caused by *Streptococcus iniae* and *Streptococcus parauberis*) and the innate immune response was evaluated at 1, 2 and 4 weeks by Harikrishnan *et al.* (2010b). Maximum reduction in mortalities was observed in fish fed the probiotic diet for two weeks after challenge with the pathogens when compared to the infected group fed with the basal diet; similarly the cellular and humoral immune responses such as HK macrophage phagocytic and peroxidase activities, serum lysozyme activity, and total protein levels increased significantly. The results reveal that, in streptococcosis infected kelp grouper, feeding on an *Lb. sakei* BK19 enriched diet affords a higher level of disease resistance due to stimulation of the immune system.

10.5.3 Effects of probiotic on leopard grouper (*Mycteroperca rosacea*)

The leopard grouper, *M. rosacea*, is found along the Atlantic coast from the Gulf of California to the state of Jalisco in Mexico, and has potential for aquaculture because it shares similar characteristics with other commercial grouper species (Pierre *et al.* 2008). In the juvenile *M. rosacea*, dietary administration of live yeast *D. hansenii* strain CBS 8339 at 10^6 CFU g^{-1} for 4 weeks promoted a high survival rate and significantly increased IgM as well as antioxidant enzymes such as SOD and CAT (Reyes-Becerril *et al.* 2008b). A subsequent study reported that

D. hansenii strain CBS 8339 enhanced leopard grouper growth performance but did not significantly affect the assayed immunological parameters after 4 weeks of feeding (Reyes-Becerril *et al.* 2011). However, post infection with *A. hydrophila*, fish fed the yeast-supplemented diet displayed a significant increase in the levels of plasmatic IgM, SOD and CAT activities. CAT and HSP70 gene expression in liver were up-regulated before and after infection of *A. hydrophila*.

10.6 TILAPIA

After carps, tilapias are the second most abundantly produced fish group with total global production approaching 4 million tonnes in 2011 (FAO FIGIS 2013). The Nile tilapia (*Oreochromis niloticus*) accounts for the largest volume (2.8 million tonnes), followed by Nile-blue hybrid (*Oreochromis niloticus* × *Oreochromis aureus*; 360,000 tonnes), the Mozambique tilapia (*Oreochromis mossambicus*; 35,000 tonnes) and various other species and hybrids.

Relative to other fish species, the application of probiotics to tilapia is extensive (Table 10.5). *Bacillus* spp. (*B. coagulans*, *B. subtilis*, *B. licheniformis*, *B. pumilus* and *B. firmus*), LAB (*Lb. plantarum*, *Lb. rhamnosus*, *Lb. acidophilus*, *Lb. brevis*, *E. faecium*, *P. acidilactici* and *Lc. lactis*), other Gram-positive species (*Micrococcus luteus* and *Clostridium butyricum*), Gram-negative species (*Pseudomonas* spp., *Rhodopseudomonas palustris* and *Citrobacter freundii*) and yeast (*S. cerevisiae*) have been tested in tilapia trials. The majority of these studies have focused on Nile tilapia and applications have demonstrated a range of growth benefits (Lara-Flores *et al.* 2003; Aly *et al.* 2008b; 2008c; Wang *et al.* 2008b; Essa *et al.* 2010; Zhou *et al.* 2010a; 2010b; Jatoba *et al.* 2011), stimulated some aspects of the non-specific immune response (Aly *et al.* 2008b; Wang *et al.* 2008b; Ferguson *et al.* 2010; Zhou *et al.* 2010a; 2010b; Goncalves *et al.* 2011; Jatoba *et al.* 2011; Pirarat *et al.* 2011; Villamil *et al.* 2012; Liu *et al.* 2013; Standen *et al.* 2013) and improved resistance to various bacterial pathogens (Aly *et al.* 2008a; 2008b; Ngamkala *et al.* 2010; Villamil *et al.* 2012; Liu *et al.* 2013). A comprehensive review of probiotic applications in tilapia is presented by Welker and Lim (2011).

10.6.1 Effects of probiotics on tilapia growth performance

Improved tilapia growth performance has been reported with the application of *M. luteus* (Abd El-Rhman *et al.* 2009), *E. faecium* (Lara-flores *et al.* 2003; Wang *et al.* 2008b), *Lb. acidophilus* (Lara-flores *et al.* 2003; Aly *et al.* 2008b), *Lb. plantarum* (Essa *et al.* 2010; Jatoba *et al.* 2011), *Lc. lactis* (Zhou *et al.* 2010b), *B. coagulans* (Zhou *et al.* 2010a), *B. pumilus* (Aly *et al.* 2008c), *B. subtilis* (El-Haroun *et al.* 2006; Aly *et al.* 2008b; Essa *et al.* 2010; Salem 2010), *B. licheniformis* (El-Haroun *et al.* 2006), *Rhodopseudomonas palustris* (Zhou *et al.* 2010a) and *S. cerevisiae* (Lara-flores *et al.* 2003; Abdel-Tawwab *et al.* 2010; Essa *et al.* 2010). Such improvements can lead to considerable economic gains (El-Haroun *et al.* 2006).

The mechanisms behind these improvements in growth performance are not fully elucidated, but a number of studies have reported elevated GI digestive enzyme activities in fish fed probiotics, which is likely to be a contributory factor. For example, concomitantly with elevated growth performance, elevated amylase, protease and lipase activities have been observed in tilapia fed *B. subtilis* and/or *Lb. plantarum* and elevated protease activity in tilapia fed

Table 10.5 Summary of *in vivo* probiotic applications with tilapia.

Potential probiont	Parameters investigated	References
Nile tilapia (<i>Oreochromis niloticus</i>)		
<i>B. pumilus</i>	DR, IR, GP, SR	Aly <i>et al.</i> (2008c)
Commercial product containing <i>B. subtilis</i>	BC, GP, IR, SR	Salem (2010)
<i>B. subtilis</i>	DR	Mohamed and Refat (2011)
Commercial product containing <i>B. subtilis</i>	BC, GP	El-Haroun <i>et al.</i> (2006)
<i>B. coagulans</i> , <i>B. subtilis</i> and <i>Rhodopseudomonas palustris</i>	AO-EA, GP, IR, SR	Zhou <i>et al.</i> (2010a)
<i>B. subtilis</i> , <i>Lb. plantarum</i> and <i>S. cerevisiae</i>	BC, D-EA, GP, SR	Essa <i>et al.</i> (2010)
<i>B. subtilis</i> , <i>Lb. acidophilus</i> , <i>Clostridium butyricum</i> and <i>S. cerevisiae</i>	DR, IR, STR	Taoka <i>et al.</i> (2006)
<i>B. subtilis</i> and <i>Lb. acidophilus</i>	DR, GP, IR, SR	Aly <i>et al.</i> (2008b)
<i>B. pumilus</i> , <i>B. firmus</i> and <i>Citrobacter freundii</i>	DR, PA	Aly <i>et al.</i> (2008a)
<i>B. subtilis</i> , <i>B. licheniformis</i> , <i>E. faecium</i> , <i>P. acidilactici</i> and <i>S. cerevisiae</i>	DR, GM, GP, IR, SR	Shelby <i>et al.</i> (2006)
<i>B. amyloliquefaciens</i> and <i>Lactobacillus</i> sp.	GM, GP, IR, SR	Ridha and Azad (2012)
<i>Bacillus</i> sp. and presumptive LAB	GP, PA, SR	Apún-Molina <i>et al.</i> (2009)
<i>E. faecium</i>	GP, IR, SR	Wang <i>et al.</i> (2008b)
<i>Enterococcus</i> sp.	GM, PA	Del'Duca <i>et al.</i> (2013)
<i>E. faecium</i> ^a and <i>Lb. acidophilus</i> , <i>S. cerevisiae</i>	BC, GP, SR	Lara-Flores <i>et al.</i> (2003)
<i>Lc. lactis</i>	GP, IR, PA, SR	Zhou <i>et al.</i> (2010b)
<i>Lb. acidophilus</i>	DR, IR, SR	Villamil <i>et al.</i> (2012)
<i>Lb. rhamnosus</i>	GH, GP, IR	Pirarat <i>et al.</i> (2011)
<i>Lb. rhamnosus</i>	DR, GH, IR	Pirarat <i>et al.</i> (2006)
<i>Lb. rhamnosus</i>	DR, GH, IR	Ngamkala <i>et al.</i> (2010)
<i>Lb. rhamnosus</i>	GP, STR	Goncalves <i>et al.</i> (2011)
<i>Lb. plantarum</i>	GM	Bucio Galindo <i>et al.</i> (2009)
<i>Lb. plantarum</i>	DR, GP, IR, PA, SR	Abumourad <i>et al.</i> (2013)
<i>Lb. plantarum</i>	GP, GM, IR, SR	Jatoba <i>et al.</i> (2011)
<i>P. acidilactici</i>	GH, GM, GP, IR, SR	Ferguson <i>et al.</i> (2010)
<i>P. acidilactici</i>	BC, GH, GM, GP, IR, SR	Standen <i>et al.</i> (2013)
<i>M. luteus</i> and <i>Pseudomonas</i> sp.	BC, DR, GP, IR, PA, SR	Abd El-Rhman <i>et al.</i> (2009)
<i>S. cerevisiae</i>	BC, DR, GP, IR, SR	Abdel-Tawwab <i>et al.</i> (2008)
<i>S. cerevisiae</i>	BC, DR, GP, IR, SR	Abdel-Tawwab (2012)
Hybrid tilapia (<i>Oreochromis niloticus</i> × <i>Oreochromis aureus</i>)		
<i>Lb. brevis</i> and <i>Lb. acidophilus</i>	DR, GM, GP, IR, PA, SR	Liu <i>et al.</i> (2013)
Galilee tilapia (<i>Sarotherodon galilaeus</i> L.)		
<i>S. cerevisiae</i>	BC, GP, SR, STR	Abdel-Tawwab <i>et al.</i> (2010)

^aPreviously *Streptococcus faecium* (Schleifer and Kilpper-Balz 1984).

Genera abbreviations: *B.* = *Bacillus*, *E.* = *Enterococcus*, *Lb.* = *Lactobacillus*, *Lc.* = *Lactococcus*, *M.* = *Micrococcus*, *P.* = *Pediococcus*, *S.* = *Saccharomyces*.

Parameters investigated: AO-EA = antioxidant enzymes, BC = body composition, DR = disease resistance, GH = gut histology/immunohistochemistry, GM = gut microbiota (inclusive of GI probiont recovery), GP = growth performance, IR = immunological/haematological response, PA = pathogen antagonism, SR = survival rate, STR = stress tolerance/response/biomarkers.

S. cerevisiae (Essa *et al.* 2010). Other potential mechanisms include improved microbiota composition or activity and may potentially include elevated intestinal endocytic activity or improved intestinal morphology as reported with probiotic applications in other fish species (Sáenz de Rodríguez *et al.* 2009; Merrifield *et al.* 2010b; Pirarat *et al.* 2011).

On the other hand, a number of studies have reported no difference in tilapia growth performance with probiotic applications (Shelby *et al.* 2006; Ferguson *et al.* 2010; Liu *et al.* 2013; Standen *et al.* 2013) or even decreased growth (Abd El-Rhman *et al.* 2009). These studies highlight the importance of selecting the appropriate probiont and optimum dosage. It should be noted that growth performance in line with that of the control group, that is, no improvements in growth performance, should be viewed positively if other benefits such as elevated immune responses and disease resistance are achieved.

10.6.2 Effects of probiotics on tilapia immunity and disease resistance

An investigation of the efficacy of live and dead probiotic cells, supplemented via the feed or rearing water, revealed that the oral administration of live cells seemed to be most effective at stimulating the non-specific immune responses of Nile tilapia (Taoka *et al.* 2006). Subsequently, most research therefore on probiotics has been conducted with the supplementation of live probiotics to feed. The effects of probiotics on the immune response of tilapia are quite comprehensive. Most studies have focused on the innate response, owing to its greater role in fish as opposed to homeotherms, and the effect at both the systemic and the localized (i.e. intestinal) levels have been observed.

At the systemic level it has been commonly reported that various probiotics can improve tilapia serum or plasma lysozyme activity (Taoka *et al.* 2006; Aly *et al.* 2008b; Wang *et al.* 2008b; Ferguson *et al.* 2010; Zhou *et al.* 2010a; 2010b; Ridha and Azad 2012), serum complement activity/concentration (Wang *et al.* 2008b; Pirarat *et al.* 2006; 2011), serum bactericidal activity (Taoka *et al.* 2006; Abdel-Tawwab *et al.* 2008; Aly *et al.* 2008b; Pirarat *et al.* 2011) and serum total Ig levels (Ridha and Azad 2012); elevate peripheral leukocyte levels (Aly *et al.* 2008c; Ferguson *et al.* 2010); modulate the proportions or abundance of peripheral leukocyte subpopulations (Aly *et al.* 2008c; Standen *et al.* 2013); enhance HK chemiluminescence activity (Pirarat *et al.* 2011) and respiratory burst activity (Taoka *et al.* 2006; Aly *et al.* 2008b; 2008c; Wang *et al.* 2008b; Zhou *et al.* 2010a; 2010b); and modulate immunoregulatory gene expression profiles in immunologically relevant organs (e.g. kidney and spleen) (Pirarat *et al.* 2011; Villamil *et al.* 2012; Liu *et al.* 2013). In addition, in some studies haematological profiles and serum or plasma biochemistry parameters have also been affected by probiotic treatment (Taoka *et al.* 2006; Wang *et al.* 2008b; Abdel-Tawwab *et al.* 2008; Aly *et al.* 2008b; Abd El-Rhman *et al.* 2009; Salem 2010; Abdel-Tawwab *et al.* 2010; Ferguson *et al.* 2010; Zhou *et al.* 2010a; 2010b; Jatoba *et al.* 2011; Ridha and Azad 2012; Abumourad *et al.* 2013).

At the localized level, the supplementation of *Lb. rhamnosus* GG (at 10^{10} CFU g⁻¹) has been reported to promote tilapia intestinal structure and mucosal immunity (Pirarat *et al.* 2011). Tilapia fed the probiotic for 30 days displayed greater villous height in all parts of the intestine and, significantly, in the proximal and distal regions. The number of intraepithelial leukocytes (IELs) and the abundance of a subpopulation of acidic granulocytes was significantly higher in the proximal intestine of the probiotic group than in the control group. In addition, the abundance of goblet cells was significantly higher in the distal intestine. In addition, the same probiotic (*Lb. rhamnosus* GG) and same dose was applied by Ngamkala *et al.* (2010)

who reported reduced intestinal damage following *Aeromonas* challenges. Similarly, Standen *et al.* (2013) reported that *P. acidilactici* treatment may potentiate the immuno-responsiveness of the tilapia intestine as demonstrated by up-regulation of intestinal TNF α gene expression and elevated IEL and goblet cell levels. Liu *et al.* (2013) fed various levels of *Lb. brevis* or *Lb. acidophilus* to hybrid tilapia for 35 days and assessed gene expression profiles (HSP70, TGF- β , IL-1 β and TNF α) in the intestine, spleen and kidney at several time points. The findings revealed a complex pattern for each probiotic strain, dose, time point and organ for each gene assessed. In the intestine, elevations in the expression of all the genes tested were observed at some time points with some doses but the responses were not consistent. Feeding the highest dose (1×10^9 cells g $^{-1}$) of *Lb. brevis* produced the most consistent effect with elevated HSP70 (at days 10 and 35), TGF- β (day 35), IL-1 β (days 10 and 35) and TNF α (day 35) mRNA levels compared to the control. The expression profiles were also inconsistent to some extent in the spleen and kidney but the fish fed the highest dose of *Lb. brevis* commonly exhibited higher HSP70, TGF- β , IL-1 β and TNF α gene expression in both organs. Subsequently, the mortality rate of tilapia after an *A. hydrophila* immersion challenge was significantly lower in this treatment group than in the control.

A number of other probiotic studies have also reported improved disease resistance of probiotic treated tilapia. Elevated survival against *A. hydrophila* challenge has been reported with the application of *Micrococcus luteus* (Abd El-Rhman *et al.* 2009), *S. cerevisiae* (Abdel-Tawwab *et al.* 2008), *Lb. brevis* (Liu *et al.* 2013), *Lb. acidophilus* (Aly *et al.* 2008b; Villamil *et al.* 2012; Liu *et al.* 2013) and *B. subtilis* (Aly *et al.* 2008b). In addition, elevated resistance has been observed against *Edwardsiella tarda* with *Lb. rhamnosus* (Pirarat *et al.* 2006), against *Str. iniae* with *B. subtilis* (Aly *et al.* 2008b) and *Lb. acidophilus* (Aly *et al.* 2008b), and against *Pseudomonas fluorescens* with *B. subtilis* (Aly *et al.* 2008b), *Lb. acidophilus* (Aly *et al.* 2008b) and *Lb. plantarum* (Abumourad *et al.* 2013).

10.7 CARPS

By volume, carps account for the largest component of fish culture production globally. Total production in 2011 exceeded 23 million tonnes, with production predominantly in China (16 million tonnes) and India (4 million tonnes) (FAO FIGIS 2013). Important species include silver carp (*Hypophthalmichthys molitrix*, 5.3 million tonnes), grass carp (*Ctenopharyngodon idellus*, 4.5 million tonnes), common carp (*Cyprinus carpio*, 3.7 million tonnes), Crucian carp (*Carassius carassius*, 2.9 million tonnes), catla (*Catla catla*, 2.4 million tonnes), Indian major carp/rohu (*Labeo rohita*, 1.4 million tonnes) and mrigal (*Cirrhinus mrigala*, 340,000 tonnes). Probiotic applications have been reported in Indian major carp, catla, common carp, grass carp, koi carp (*Cyprinus carpio koi*) and gibel carp (*Carassius auratus gibelio*) (refer to Table 10.6).

10.7.1 Effects of probiotics on carp growth

Improved growth performance has been reported in Indian major carp (*Labeo rohita*) fed *B. subtilis* (Kumar *et al.* 2006), *B. circulans* (Ghosh *et al.* 2002; 2003), *Lb. plantarum* (Giri *et al.* 2013a), combinations of *B. subtilis* + *Lc. lactis* + *S. cerevisiae* (Mohapatra *et al.* 2012a; 2012b) and combinations of *B. subtilis* + *Pseudomonas aeruginosa* + *Lb. plantarum* (Giri *et al.* 2013b); common carp fed *Bacillus* sp., *Lb. acidophilus*, *E. faecium*, photosynthetic bacteria and *S. cerevisiae* (Bogut *et al.* 1998; Yanbo and Zirong 2006; Ramakrishnan *et al.* 2008);

Table 10.6 Summary of *in vivo* probiotic applications with carps.

Potential probiont	Parameters investigated	References
Indian major carp/rohu (<i>Labeo rohita</i>)		
<i>B. circulans</i>	GP, SR	Ghosh <i>et al.</i> (2002)
<i>B. circulans</i>	DE-A, GP, SR	Ghosh <i>et al.</i> (2003)
<i>B. subtilis</i>	DR, IR, GP	Kumar <i>et al.</i> (2006)
<i>B. subtilis</i>	DR, IR	Kumar <i>et al.</i> (2008)
<i>B. subtilis</i>	IR	Nayak <i>et al.</i> (2007)
<i>B. subtilis</i> , <i>Lc. lactis</i> and <i>S. cerevisiae</i>	BC, DE-A, GM, GP	Mohapatra <i>et al.</i> (2012a)
<i>B. subtilis</i> , <i>Lc. lactis</i> and <i>S. cerevisiae</i>	GP, IR, STR	Mohapatra <i>et al.</i> (2012b)
<i>B. subtilis</i> , <i>Ps. aeruginosa</i> and <i>Lb. plantarum</i>	DR, GP, IR	Giri <i>et al.</i> (2013b)
<i>Lb. plantarum</i>	GP, IR, DR, SR	Giri <i>et al.</i> (2013a)
<i>Ps. aeruginosa</i>	DR, IR	Giri <i>et al.</i> (2012)
<i>Catla</i> (<i>Catla catla</i>)		
<i>Lb. plantarum</i> and <i>B. megaterium</i>	BC, DR, GM, GP, IR, PA, SR	Parthasarathy and Ravi (2011)
Common carp (<i>Cyprinus carpio</i>)		
<i>E. faecium</i> , <i>Carnobacterium divergens</i>	DR, GM, IR, PA	Gopalakannan and Arul (2011)
<i>E. faecium</i> ^a	GM, GP	Bogut <i>et al.</i> (1998)
<i>Lb. acidophilus</i> and <i>S. cerevisiae</i>	BC, GM, GP, SR	Ramakrishnan <i>et al.</i> (2008)
Photosynthetic bacteria and <i>Bacillus</i> sp.	DE-A, GP, SR	Yanbo and Zirong (2006)
Grass carp (<i>Ctenopharyngodon idellus</i>)		
<i>B. coagulans</i> , <i>Lb. acidophilus</i> and <i>Rhodopseudomonas palustris</i>	DE-A, GP, SR	Wang (2011)
Koi carp (<i>Cyprinus carpio koi</i>)		
<i>B. subtilis</i> C-3102	DR, GM, GP, IR, SR	He <i>et al.</i> (2011a)
Gibel carp (<i>Carassius auratus gibelio</i>)		
<i>S. cerevisiae</i>	GM, GP, IR	He <i>et al.</i> (2011b)

^aPreviously *Streptococcus faecium* (Schleifer and Kilpper-Balz 1984).

Genera abbreviations: *B.* = *Bacillus*, *C.* = *Carnobacterium*, *E.* = *Enterococcus*, *Lb.* = *Lactobacillus*, *Lc.* = *Lactococcus*, *Ps.* = *Pseudomonas*, *S.* = *Saccharomyces*.

Parameters investigated: BC = body composition, DR = disease resistance, DE-A = digestive/intestinal enzymes, GM = gut microbiota (inclusive of GI probiont recovery), GP = growth performance, IR = immunological/haematological response, PA = pathogen antagonism, SR = survival rate, STR = stress tolerance/response/biomarkers.

koi carp fed *B. subtilis* (He *et al.* 2011a); gibel carp fed a fermented yeast product (*Saccharoculture*) (He *et al.* 2011b); and grass carp fed *B. coagulans*, *Rhodopseudomonas palustris* and *Lb. acidophilus* (Wang 2011).

The mechanisms which underpin the observed improvements in growth performance are partly described and in many cases include modulated gut microbiota and elevated gastric digestive enzyme activities. For example, concomitantly with improved weight gain, grass carp fed *B. coagulans* displayed improved intestinal protease, amylase and cellulase activities, and grass carp fed *Rhodopseudomonas palustris* and *Lb. acidophilus* displayed improved intestinal amylase activities (Wang 2011). The application of *Bacillus* sp. and photosynthetic

bacteria has been reported to elevate lipase, protease and amylase activities in the intestine of common carp (Yanbo and Zirong 2006). The application of live *B. subtilis*, *Lb. lactis* and *S. cerevisiae* (at 10^8 CFU g⁻¹) significantly improved Indian major carp weight gain and specific growth rate (SGR), as a result of improved feed conversion ratio (FCR), protein efficiency ratio, apparent digestibility of dietary dry matter and protein, and elevated intestinal protease and lipase activities (Mohapatra *et al.* 2012a). With the exception of dry matter digestibility, these improvements were not observed in carps fed a diet supplemented with dead *B. subtilis*, *Lb. lactis* and *S. cerevisiae* cells, which indicates that the probiotics actively contributed to the digestive process, either directly via contributions of digestive enzymes and vitamins, and/or indirectly in that their metabolic activities induced elevations in the enzyme secretions or activities of the host.

10.7.2 Carp immunity and disease resistance

A number of studies have provided information on the immune responses of Indian major carp/rohu fed *B. subtilis*, *Lb. plantarum* and *Ps. aeruginosa*, catla fed *Lb. plantarum* and *B. megaterium*, common carp fed *E. faecium*, koi carp fed *B. subtilis*, and gibel carp fed *S. cerevisiae* (Table 10.6). In addition, the resistance to pathogen challenge has also been studied in Indian major carp/rohu fed *B. subtilis*, *Lb. plantarum* and *Ps. aeruginosa*, catla fed *Lb. plantarum* and *B. megaterium*, common carp fed *E. faecium*, and koi carp fed *B. subtilis* (Table 10.6).

The dietary provision of *B. subtilis* has commonly been reported to improve the immune response and disease resistance of carps. Improved survival of Indian major carp against *A. hydrophila* (IP challenged) has been reported and a dose response was observed (Kumar *et al.* 2006). The mean survival level increased significantly from 18.75% in the control (no probiotic) fed carp to 68.75%, 81.25% and 87.50% in the carp fed *B. subtilis* at 0.5, 1.0 and 1.5×10^7 CFU g⁻¹ feed, respectively. In keeping with these elevated survival rates, Kumar *et al.* (2008) reported increased proportions of peripheral granulocytes and monocytes and neutrophil respiratory burst activity in a dose-dependent manner pre- and post-IP *A. hydrophila* challenge. In addition, increased serum bactericidal activity against *A. hydrophila* was also demonstrated, with the improvements elevated in a dose-dependent manner. Nayak *et al.* (2007) fed Indian major carp *B. subtilis* at 10^8 CFU g⁻¹ for 60 days and reported elevated peripheral leukocyte levels and serum protein in probiotic fed carp as well as elevated antibody titres post challenge with *E. tarda* bacterin.

Improved resistance of Indian major carp to *A. hydrophila* has also been reported with the application of dietary *Lb. plantarum* (Giri *et al.* 2013a) and *Ps. aeruginosa* (Giri *et al.* 2012). Compared to the control group (11%), significantly higher survival rates were observed in the fish groups fed diets containing *Ps. aeruginosa* at 10^7 CFU g⁻¹ (66.66%) and 10^9 CFU g⁻¹ (55.55%) (Giri *et al.* 2012). *Lb. plantarum* provided at 10^6 , 10^8 and 10^{10} CFU g⁻¹ significantly improved survival against *A. hydrophila* but probiotic provision at 10^8 CFU g⁻¹ provided significantly better survival rates than all other treatments. Dose-dependent responses were also apparent in the immunological responses assayed. At day 60, Indian major carp fed all *Lb. plantarum* supplemented diets displayed elevated serum lysozyme activity, alternative complement activity and head kidney (HK) respiratory burst activity, and carp fed the probiont at 10^8 CFU g⁻¹ displayed the highest levels. Elevated HK macrophage phagocytosis activity was observed in all groups but SOD activity was only elevated in carp fed the probiont at 10^8 CFU g⁻¹ (Giri *et al.* 2013a). In similar fashion, after 60 days of feeding on *Ps. aeruginosa* elevated lysozyme activity, alternative complement activity, HK phagocytosis

and HK respiratory activities were significantly elevated at all levels tested, but the improvements were greatest in all cases in carp fed *Ps. aeruginosa* at 10^7 CFU g⁻¹. Many of these immune responses, and improved resistance to *A. hydrophila*, were also reported when using simultaneous combinations of *B. subtilis*, *Ps. aeruginosa* and *Lb. plantarum* (Giri *et al.* 2013b). Gopalakannan and Arul (2011) demonstrated that dietary provision of *E. faecium* (RPS = 75 and 78 on days 30 and 60, respectively) and *C. divergens* (RPS = 50 and 61 on days 30 and 60, respectively) improved common carp resistance to *A. hydrophila*. Both probiotics also elevated neutrophil NBT activity. Improved resistance of catla to *A. hydrophila* has also been reported with *Lb. plantarum* and *B. megaterium* applications (Parthasarathy and Ravi 2011), and koi carp fed *B. subtilis* displayed reduced, albeit not significantly, mortalities when challenged with *A. hydrophila* (He *et al.* 2011a). In addition, elevated hepatic expression of TNF α , HSP70, TGF- β and IL-10 and intestinal expression of IL-1 β , TNF α , TGF- β and IL-10 genes were observed in *B. subtilis* fed koi (He *et al.* 2011a).

Although somewhat limited in their scope, these studies provide clear evidence that probiotics can improve a number of immunological responses of carp species and that these responses confer protection against *A. hydrophila* infections.

10.8 ZEBRAFISH (*DANIO RERIO*)

The zebrafish and human genomes share extensive conserved syntenic fragments and zebrafish is therefore increasingly seen as a powerful and highly amenable model system. The zebrafish has been used to elucidate the molecular mechanisms involved in several different physiological processes, including host–microbiome responses, and as such the model has been used extensively to study probiotics (Table 10.7).

10.8.1 Effects of probiotic on zebrafish reproduction, development and survival

A number of recent studies have evidenced the positive role of *Lb. rhamnosus* IMC 501 on zebrafish gamete quality, spawning rates, oocyte growth and maturation, larval development, fecundity, backbone calcification and the expression of genes which regulate growth, development and immunity (Table 10.7). The roles of the probiotics in zebrafish development, reproduction and nutrient metabolism have been reviewed by Carnevali *et al.* (2013) and again here in *Chapter 12*. Readers with a specific interest in this topic are directed to these reviews. Further information is available with respect to modulation of the gastric microbiota, growth performance, immunity and disease resistance.

10.8.2 Zebrafish immunity and disease resistance studies

The antimicrobial activities of *Lb. plantarum* JCM 1149^T (observed to have positive adhesive capabilities to the zebrafish gut) and *Lb. acidophilus* JCM 1132^T (observed to be less capable of adhering to the zebrafish gut) were assessed against *A. hydrophila* *in vitro* and *in vivo* by Zhou *et al.* (2012a). Both probiotic strains showed strong, and similar, *in vitro* antimicrobial activities against *A. hydrophila*. In the 2 week *in vivo* challenge study dietary *Lb. acidophilus* JCM 1132 improved the survival rate by 3%, 7% and 37%, compared to the control, when provided at 10^6 cell g⁻¹, 10^7 cell g⁻¹ and 10^8 cell g⁻¹, respectively. However,

Table 10.7 Summary of *in vivo* probiotic applications with zebrafish.

Potential probiont	Parameters investigated	References
<i>Lb. rhamnosus</i> IMC 501	F	Gioacchini <i>et al.</i> (2010a)
<i>Lb. rhamnosus</i> IMC 501	F	Gioacchini <i>et al.</i> (2010b)
<i>Lb. rhamnosus</i> IMC 501	F	Gioacchini <i>et al.</i> (2010c)
<i>Lb. rhamnosus</i> IMC 501	GM, F	Gioacchini <i>et al.</i> (2011)
<i>Lb. rhamnosus</i> IMC 501	GM, F	Gioacchini <i>et al.</i> (2012)
<i>Lb. rhamnosus</i> IMC 501	F	Giorgini <i>et al.</i> (2010)
<i>Lb. rhamnosus</i> IMC 501	F	Gioacchini <i>et al.</i> (2013)
<i>Lb. rhamnosus</i> IMC 501	GP, LD	Avella <i>et al.</i> (2012)
<i>Lb. rhamnosus</i> and <i>Lb. casei</i>	F, GH, GM, IR	Qin <i>et al.</i> (2013)
37 commensal or probiotic Gram-positive and Gram-negative bacteria often used as probiotic strains in the food industry and/or aquaculture	DR, IR	Rendueles <i>et al.</i> (2012)
Lactobacilli (multiple species)	DR, GM, PA	Zhou <i>et al.</i> (2012a)
<i>B. coagulans</i>	DR, IR, PA	Pan <i>et al.</i> (2013)

Genera abbreviations: *B.* = *Bacillus*, *Lb.* = *Lactobacillus*.

Parameters investigated: DR = disease resistance, GM = gut microbiota (inclusive of GI probiont recovery), F = fecundity/gonadal development/spawning rates etc., GP = growth performance, IR = immunological/haematological response, PA = pathogen antagonism.

dietary provision of *Lb. plantarum* JCM 1149 was more effective and enhanced the survival rate by 33% (when provided at 10^6 cell g^{-1}), 60% (10^7 cell g^{-1}) and 50% (10^8 cell g^{-1}). These findings imply that the capacity of the probiotic to adhere to and populate the GI tract is an important factor in determining their efficacy in promoting disease resistance. In addition, the dosage was clearly an important factor also in this study. Similarly, *B. coagulans* fed zebrafish displayed improved survivability, in a dose-dependent manner, and reduced pathogen translocation/infection levels when challenged with *Vibrio vulnificus* (Pan *et al.* 2013). Although not always consistently, the following genes were up-regulated in zebrafish fed fodder containing *B. coagulans* at $\geq 10^6$ CFU (per 50 ml): TLR4 (at 96 h post *V. vulnificus* challenge), TRAM-1 (at 24, 72 and 96 h), NF- κ B (at 24, 48, 72 and 96 h), IL-1 β (at 24, 48 and 72 h) and TNF α (at 24, 72 and 96 h) compared to control (non-probiotic fed) zebrafish. TLR4 plays an important function in detecting LPS, present in Gram-negative bacterial cell walls including pathogens such as *V. vulnificus*, and initiates a downstream signalling pathway which includes the signal adaptor molecule TRAM-1 and the primary transcription factor NF- κ B, which regulates the transcription of pro-inflammatory cytokines such as IL-1 β and TNF α . In turn, NF- κ B can also be up-regulated in a positive feedback loop by pro-inflammatory cytokines such as IL-1 β and TNF α . This is the first study in fish to have assessed the efficacy of probiotics to modulate the expression of intermediary genes in the signalling pathway from pathogen detection (e.g. TLR4) to cytokine gene responses (i.e. elevated IL-1 β and TNF α) and demonstrates the ability of *B. coagulans* to prime the immune response which leads to an elevated ability to respond to *V. vulnificus* challenge (Pan *et al.* 2013). However, considering that the expression of downstream signalling molecules and the cytokines were up-regulated (from 24 h) before TLR4 (96 h) it is likely that other signalling PRRs, such as TLR5, may have been involved (and potentially earlier) in the detection of the pathogen. Indeed, *V. vulnificus* major flagellin

(FlaB) has been shown to interact with TLR5 expressed on Caco-2 cells and that the binding of FlaB with TLR5 subsequently activated downstream signalling (including elevated NF- κ B) to produce the chemokine IL-8 (Lee *et al.* 2006).

10.9 CATFISHES

Globally nearly 3 million tonnes of farmed catfish is produced annually (FAO FIGIS 2013). *Pangassius* spp. (1.4 million tonnes), Amur catfish *Silurus asotus* (397,000 tonnes), channel catfish *Ictalurus punctatus* (376,000 tonnes), yellow catfish *Tachysurus fulvidraco* (217,000 tonnes), African catfish *Clarias gariepinus* (194,000 tonnes), wels catfish/sheat fish *Silurus glanis* (1500 tonnes), walking catfish/Indian Magur *Clarias batrachus* and silver catfish *Rhamdia quelen* are amongst the most important species. Information on the gut microbiota compositions of catfish species, and the factors which influence them, is quite limited (Chapter 5 and Chapter 6). However, a review of the literature available on the channel catfish gut microbiota, and the potential to modulate it, is provided by Burr *et al.* (2012).

The selection of probiotic candidates for, and the application of probiotics to, catfish species is in its infancy compared to many other farmed fish, but some information is available for channel catfish, wels catfish, African catfish, walking catfish/Indian Magur, silver catfish and striped catfish (*Pangasianodon hypophthalmus*) (refer to Table 10.8). Some studies have sought to characterize and assess the potential efficacy of autochthonous catfish bacterial strains in *in vitro* tests and these studies have demonstrated the ability of some bacterial strains to antagonize pathogens (Hamid *et al.* 2012; Ran *et al.* 2012; Zhou *et al.* 2012b). *Lc. lactis* subsp. *lactis* strains (isolated from the intestine of African catfish) were able to inhibit the growth of *Salmonella typhimurium* and *Escherichia coli* (Hamid *et al.* 2012), and *B. subtilis* strains (isolated from the intestine of yellow catfish) and their metabolites could antagonize and reduce the growth of *Flavobacterium columnare*, *Edwardsiella tarda*, *A. hydrophila*, *Aeromonas sobria* and *E. coli* (Zhou *et al.* 2012b). Ran *et al.* (2012) also demonstrated that the channel catfish intestinal tract contained a number of *Bacillus* spp. which could inhibit the growth of fish pathogens. *B. subtilis*, *B. pumilus*, *B. cereus*, *B. methylotrophicus* and *B. amyloliquifaciens* strains showed antimicrobial activity against *A. hydrophila*, *Edwardsiella ictaluri*, *Ew. tarda*, *Flavobacterium columnare*, *Str. iniae*, *Yersinia ruckeri* and *Saprolegnia ferax* *in vitro*.

Some of the *Bacillus* strains isolated by Ran *et al.* (2012) were also tested *in vivo*, and in a static rearing system with daily water changes (20–30 min) some degree of protection was afforded to juvenile channel catfish when challenged with *Edwardsiella ictaluri* in a 45 min immersion exposure; 18 days post challenge the strains improved survivability rates by up to 15%. Perhaps surprisingly, a clear degree of protection was also apparent for juvenile striped catfish when challenged with a 30 min immersion exposure to *Ew. ictaluri*; mean mortality rates 18 days post challenge were reduced from 71% in the control (non-probiotic fed fish) to 10–57% in the *Bacillus* fed fish (Ran *et al.* 2012). Dietary provision of *B. thuringiensis* has been reported to improve the survival rate of African catfish IP challenged with *A. hydrophila* (Renshaw *et al.* 2011). Although mortality rates were not presented, the effects of dietary provision of *Lb. acidophilus* on the haematological and immunological responses and histopathology of pathogen challenged African catfish have been investigated (Al-Dohail *et al.* 2011). Al-Dohail *et al.* (2011) reported that infected catfish

Table 10.8 Summary of *in vivo* probiotic applications with catfishes.

Potential probiont	Parameters investigated	References
Channel catfish (<i>Ictalurus punctatus</i>)		
Multiple <i>Bacillus</i> strains	DR, GM, PA	Ran <i>et al.</i> (2012)
<i>B. subtilis</i> , <i>B. megaterium</i> , <i>B. polymyxa</i> and <i>B. licheniformis</i>	GP, SR	Queiroz and Boyd (1998)
<i>E. faecium</i> , <i>P. acidilactici</i> , <i>Lactobacillus</i> spp., <i>B. subtilis</i> , <i>B. licheniformis</i> and multiple commercial mixtures	DR, GP, IR	Shelby <i>et al.</i> (2007)
African catfish (<i>Clarias gariepinus</i>)		
Commercial product containing <i>B. subtilis</i>	BC, GP, SR	El-Haroun <i>et al.</i> (2007)
<i>B. thuringiensis</i>	DR, IR	Renshawry <i>et al.</i> (2011)
<i>Lb. acidophilus</i>	GP, IR, SR	Al-Dohail <i>et al.</i> (2009)
<i>Lb. acidophilus</i>	DR, IR	Al-Dohail <i>et al.</i> (2011)
Striped catfish (<i>Pangasianodon hypophthalmus</i>)		
Multiple <i>Bacillus</i> strains	DR	Ran <i>et al.</i> (2012)
Walking catfish/Indian Magur (<i>Clarias batrachus</i>)		
<i>Lb. sporogenes</i> , <i>S. boulardii</i> , and multiple strains	DR, IR	Dahiya <i>et al.</i> (2012)
Silver catfish (<i>Rhamdia quelen</i>)		
<i>B. cereus</i> var. <i>toyoi</i> and <i>S. boulardii</i>	GP, PA	de Souza <i>et al.</i> (2012)
Wels catfish/sheat fish (<i>Silurus glanis</i>)		
<i>E. faecium</i>	GM, GP	Bogut <i>et al.</i> (2000)

Bacterial genera abbreviations: *B.* = *Bacillus*, *E.* = *Enterococcus*, *Lb.* = *Lactobacillus*, *P.* = *Pediococcus*, *S.* = *Saccharomyces*. Parameters investigated: BC = body composition, DR = disease resistance, GM = gut microbiota (inclusive of GI probiont recovery), GP = growth performance, IR = immunological/haematological response, PA = pathogen antagonism, SR = survival rate.

(IP challenged with *Staphylococcus xylosus*, *A. hydrophila* or *Str. agalactiae*) fed on probiotic diets modulated haematological parameters, and histopathology revealed a reduction in the extent of necrosis, degeneration and lesions in the liver and kidney. Other studies have shown elevated immunological responses in non-challenged catfish; *Lb. acidophilus* fed African catfish displayed elevated peripheral leukocyte levels, serum total protein levels and serum total Ig levels (Al-Dohail *et al.* 2009), and *P. acidilactici* fed channel catfish displayed elevated serum total protein levels, serum total Ig levels and complement activity (Shelby *et al.* 2007). Besides immunological responses and disease resistance, probiotic applications have also been reported to improve catfish growth performance (Queiroz and Boyd 1998; Bogut *et al.* 2000; El-Haroun *et al.* 2007; Al-Dohail *et al.* 2009) and catfish pond culture production (Queiroz and Boyd 1998; El-Haroun *et al.* 2007).

Very little attention has been focused on the impacts of probiotic applications on the gut microbiota in catfish studies. It has however been reported that dietary supplementation of *E. faecium* could reduce wels catfish intestinal levels of *Staphylococcus*, *E. coli*, Enterobacteriaceae and *Clostridium* spp. (Bogut *et al.* 2000). The *Bacillus* strains characterized by Ran

et al. (2012) were also tested for their persistence in the intestine of channel catfish. Catfish were fed *Bacillus* spore-supplemented feeds (ca. 10^9 CFU g⁻¹) for 7 days followed by a normal non-supplemented feed for 3 days. High ($>\log 5$ CFU g⁻¹) *Bacillus* levels were observed in all of the probiotic fed fish 3 days after the cessation of dietary provision, and no probiotic *Bacillus* were detected in the control fed fish. Both of these studies used a culture based approach which looked at a limited number of bacterial groups and therefore future studies should also utilize culture-independent methods to provide a more complete and accurate representation of the impact of probiotics on the catfish microbiota, and to determine the efficacy of probiotics to colonize and persist in the GI tract of catfish species.

10.10 GENERAL CONCLUSIONS

The literature available provides clear evidence that probiotics modulate GI communities and improve the welfare, stress tolerance, growth, metabolism, reproduction, localized and systemic immune responses and disease resistance of temperate and warm water fish species. These observations were achieved through multidisciplinary approaches based on microbiological, biomolecular, bio-histochemical and proteomical methods developed by several laboratories focusing on these species, but further research into the mechanisms of action, and the endogenous host microbiota, is necessary to enable informed decisions on which probiotics are applicable for each species and for each specific set of culture conditions. Further, studies should expand to cover emerging warm water aquaculture species such as cobia and tuna species.

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11 Probiotic Applications in Crustaceans

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ABSTRACT

Crustacean aquaculture, and more specifically shrimp culture, faces several challenges in order to increase production yield while maintaining sustainability. The various disease outbreaks that have been continuously affecting the sector for almost two decades, together with the increasing demand for environmentally friendly aquaculture and the pressure from customers for safe and traceable products, are fundamentally modifying the culture practices of shrimp and other crustacean species. It is today well accepted that these challenges can only be faced through the development of better management practices together with technical innovations. Among the solutions currently considered, those relying on nutrition and intestinal health management can play a critical role. The application of probiotics in aquaculture has been developed in this context and is now widely applied in crustacean aquaculture as a complementary tool for management of disease. This development is mostly driven by practical and empirical approaches in shrimp farming; however, specific in-depth studies related to the biological effects and mechanisms of action of probiotics in crustaceans are gaining more importance. This chapter presents a review of the research conducted on probiotic applications in crustacean species with special emphasis on the modes of action investigated so far and the main benefits derived from it. The limits and future perspectives are also addressed.

11.1 INTRODUCTION

Today crustacean aquaculture represents a significant source of world aquatic products, reaching a market value estimated at US\$22.7 billion (FAO 2011). Among farmed crustaceans, marine shrimps of the Penaeidae family represent the largest component.

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The diminishment of natural stocks, the increasing cost of fishing wild shrimps and the current increase in demand for aquatic products directly favours the development of shrimp aquaculture. As an example, the farming of white shrimp (*Litopenaeus vannamei*) originating from the Pacific east coast is rapidly growing worldwide; from 2002 to 2010, the production of this species rose from 200,000 to 600,000 tonnes in Thailand (Wyban 2007; FAO 2011). The remarkable growth achieved in the shrimp farming industry over the last 30 years has been made possible thanks to the intensification and globalization of the seafood trade. However, the increase in productivity has been accompanied by increased risk of exposure to stressful conditions together with the emergence of several diseases. In fact, disease was rated the most important issue (coming before production cost of feed and fishmeal availability) for the shrimp aquaculture industry in the survey by the Global Outlook for Aquaculture Leadership in 2011 (Valderrama and Anderson 2011). Indeed viral and bacterial diseases have posed a serious challenge for the shrimp farming industry for almost two decades (Bondad-Resantoso *et al.* 2005). As an example, the recently identified infectious myonecrosis virus (IMNV) in *Penaeus vannamei* has caused direct economic losses of US\$0.2 billion to \$1 billion to the shrimp industry in Indonesia since its first appearance in East Java in 2006 (Sunarto and Sutanto 2011). More generally, according to the FAO, monetary losses could range from national-level estimates of US\$17.5 million to \$650 million depending on the outbreak episode, and the global estimate is US\$3.2 billion (FAO 2011). Disease not only affects the sector in socio-economic terms (losses in income, employment, market access or market share, investment and consumer confidence, food shortages and closure of businesses) but also limits its development and sustainability (with impacts on costs, trade and biodiversity).

The control and prevention of diseases, especially those of bacterial origin, have been historically achieved through the use of chemicals such as antimicrobial agents. Antibiotic products were also used for their growth promoting effect. However, due to the lack of sustainability and health concerns over the use of such chemicals, their use is today becoming increasingly restricted by governmental policies, certification programmes and farmers' organizations. It seems obvious today that the emergence of new pathogens and bacterial resistance was the result of hazardous management practices. Improving farming practices as well as increasing product quality to meet consumers' preferences constitute some of the main challenges that the industry is facing. This will become possible thanks to the development of better management practices (BMPs) in aquaculture in order to help farmers manage health and food safety. Such practices are now being implemented in several countries with success and are seen as the basis for application of GAP and HACCP schemes in aquaculture (Subasinghe and Ababouch 2009).

Among these practices, the management of microbial communities within aquaculture systems is gaining momentum and is likely to become one of the most promising areas of progress, especially since it is now understood that the delicate microbial balance within the digestive tract and the surrounding environment have a huge effect on the immune system, nutrition and other physiological aspects of aquatic animals. The use of probiotics as 'biofriendly agents' to better manage the health of the host and its resistance to diseases and/or to favour growth and feed utilization through a variety of mechanisms is now a tool increasingly implemented in farmers' BMPs, specifically in crustacean aquaculture.

For the purpose of the current review it is important to define what we mean here by using the broad term 'probiotic'. As proposed elsewhere (Merrifield *et al.* 2010), we will suppose here that a probiotic is any microbial cell provided via the diet or rearing water that benefits

the host and therefore the farmer and the final consumer. We will then only consider direct probiotic effects on the host, which are achieved, in part at least, by improving the microbial balance of the animal. Nevertheless, as extensive applications exist in shrimp farming, several references will also be made to microorganisms used as bioremediation agents.

In the past two decades research has been conducted to select probiotic strains and evaluate their effects on crustaceans, specifically on marine shrimps. Commercial probiotic preparations, already available for terrestrial animals, have also been evaluated. The constantly increasing number of studies on this topic has made it possible to achieve first reviews detailing the various developments made in the use of probiotics in aquatic cultured species, including crustaceans (Gatesoupe 1999; Verschuere *et al.* 2000a; Gomez-Gil *et al.* 2000; Farzanfar 2006; Balcázar *et al.* 2006; Wang *et al.* 2008; Castex 2009; Qi *et al.* 2009). However, in contrast to the broad application of probiotics in commercial shrimp farms (hatcheries and grow-out farms), relatively few in-depth studies on their mechanism of action have been published so far. Therefore many questions about how probiotics actually work in crustaceans still remain unanswered. According to the probiotic definition (Fuller 1989; Gatesoupe 1999; Merrifield *et al.* 2010), the primary effect of probiotics relies on the modulation of the intestinal microbiota of the host. Then, a first question to consider is whether probiotics are actually able to modify the associated microbiotas of crustaceans. This review will address this fundamental question and will update the main information presently available on the mechanisms of action of probiotics in crustaceans.

Even though information on mechanisms is scarce, this chapter will summarize the various benefits of probiotics in crustacean species reported in many scientific studies and field reports which have led to probiotics becoming accepted among the scientific communities as well as the professionals in this sector (e.g. farmers, feed producers, veterinarians). Although the probiotic concept is gaining momentum, statistical data about the efficacy of probiotics in commercial farms are scarce and more pilot scale studies are needed to assess the efficacy of a particular probiotic under farming conditions. Nevertheless, beyond these considerations, probiotic applications have been a practical and commercial reality in shrimp farming for more than 10 years, especially in Asia. For instance, Qi *et al.* (2009) report that more than 100 companies in China are producing various types of probiotics for applications in aquaculture. The authors estimated that around 50,000 tonnes of commercial probiotic products were sold annually in China with a market value estimated at €50 million. To a lower extent, and even if figures are lacking, similar situations can be encountered in other Asiatic countries such as India and Thailand (Nimrat and Vuthiphandchai 2011), or in South and Central America as reported for instance in Ecuador (Cedeño 2007; Rodríguez *et al.* 2007). A survey conducted by the research Institute CENAIM in Ecuador of over 56 farms showed that 89% of farms were using probiotics, more than 20 different products were being applied, and in 57% of the cases the bacterial strains used were of local origin. This scale of commercial development of probiotics leads therefore to regulatory and industrial issues (refer to *Chapter 13*). For instance, product quality and consistency are very critical issues when it comes to the safety and efficacy of the products being used by farmers (Wang *et al.* 2008). A recent report from Thailand highlighted these particular issues and argued that more attention must be paid to evaluation of the quality of the commercial probiotics used in commercial shrimp farms (Nimrat and Vuthiphandchai 2011).

11.2 MAIN MICROORGANISMS EVALUATED AND USED AS PROBIOTICS IN CRUSTACEAN AQUACULTURE

The probiotic bacteria used in aquaculture are from many phylogenetic lineages; however, most of the probiotics studied belong to two bacterial divisions, the Firmicutes (e.g. *Bacillus* spp., *Lactobacillus* spp., *Lactococcus* spp., *Carnobacterium* spp. etc.) and the Gammaproteobacteria (e.g. *Vibrio* spp., *Pseudomonas* spp., *Shewanella* spp. etc.), while yeasts remain rarely studied (Gatesoupe 2007). In the case of crustaceans, the diversity of microorganisms assessed as probiotics is important. The range of microorganisms examined has encompassed Gram-positive bacteria (*Bacillus* spp., *Micrococcus* sp., *Lactobacillus* spp., *Lactococcus* spp., *Pediococcus* sp. and *Arthrobacter* sp.), Gram-negative bacteria (*Vibrio* spp., *Pseudomonas* spp., *Bdellovibrio* spp., *Aeromonas* spp., *Pseudomonas* spp., *Halomonas* sp. and *Alteromonas* spp.), yeasts (*Saccharomyces* spp., *Phaffia* sp.) and microalgae (*Tetraselmis* sp.).

The main probiotic bacteria documented and used in crustacean farming belong to the *Bacillus* genus (Moriarty 1998; Rengpipat *et al.* 1998; 2000; 2003; 2008; Vaseeharan and Ramasamy 2003; Ziaei-Nejad *et al.* 2006; Decamp *et al.* 2008; Boonthai *et al.* 2011). Other Gram-positive bacteria such as *Lactobacillus* spp. (Villamil *et al.* 2003; Venkat *et al.* 2004; Chiu *et al.* 2007; Viera *et al.* 2008), *Lactococcus lactis* (Harzevili *et al.* 1998), *Pediococcus acidilactici* (Castex *et al.* 2008; 2010), *Micrococcus* sp. (Antony *et al.* 2011) and *Arthrobacter* sp. (Li *et al.* 2006; 2008; Pai *et al.* 2010) have received some attention and are used in some commercial products (Nimrat and Vuthiphandchai 2011). Autochthonous Gram-negative bacteria strains such as *Vibrio* spp. (Garriques and Arevalo 1995; Moriarty 1998; Verschuere *et al.* 2000a; Alavandi *et al.* 2004; Rodríguez *et al.* 2007; Krupesha Sharma *et al.* 2010), *Bdellovibrio* spp. (Qi *et al.* 2009), *Aeromonas* sp. (Verschuere *et al.* 2000b), *Pseudomonas* spp. (Chythanya *et al.* 2002; Alavandi *et al.* 2004; Vijayan *et al.* 2006; Hai *et al.* 2007; 2009; Pai *et al.* 2010), *Halomonas* sp. (Zhang *et al.* 2009) and *Alteromonas* sp. (Abraham 2001) have also been documented and used in some parts of the world, although the development of commercial products containing such microorganisms would be very limited. Furthermore, even though the scientific literature available on the use of photosynthetic bacteria is scarce, bacteria such as *Thalassobacter utilis* (Nogami *et al.* 1997), *Rubrivivax gelatinosa*, *Rhodobacter capsulata*, *Rhodobacter spheroides* and *Phaeospirillum fulvum* are extensively used in Chinese aquaculture (Qi *et al.* 2009), probably being the earliest and most widely used probiotics in China since the 1980s. Finally, yeast probiotic strains belonging to the species *Saccharomyces cerevisiae* or marine yeasts are also considered, though results to date show that such microorganisms can be efficient probiotics to optimize shrimp cultivation (Scholz *et al.* 1999; Burgents *et al.* 2004; Yang *et al.* 2011).

11.2.1 *Bacillus* spp.

The most well studied probiotics for crustacean applications belong to the genus *Bacillus* (Farzanfar *et al.* 2006). Generally, *Bacillus* species tested in shrimp culture have been selected according to their antimicrobial activities toward pathogenic *Vibrio* spp. based on *in vitro* antagonism assays (Rengpipat *et al.* 1998; Decamp *et al.* 2008). *Bacillus* spp. are saprophytic Gram-positive spore forming bacteria naturally present in a wide range of environmental

conditions (in air, water, dust, soil and sediment). It is reported that *Bacillus* species are ubiquitous and even dominate the marine microbiota (Priest 1993). Present in the water column as well as the sediment, *Bacillus* spp., especially *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus coagulans*, are considered autochthonous members of the crustacean's environment and a component of the intestinal microbiota entering the gut via the food or water. However, it is interesting to highlight that the most recent studies, which used molecular techniques to characterize the gut microbiota of crustaceans, rarely report the presence of *Bacillus* species within the gastrointestinal (GI) tract (Li P. *et al.* 2007; Liu H. *et al.* 2011a), suggesting either a minor component of the microbiota belongs to this genus or it constitutes a transient population.

Moriarty (1998) emphasized several reasons for the use of *Bacillus*, rather than antibiotics, to improve shrimp culture success: *Bacillus* strains naturally produce many different antibiotic compounds, secrete many enzymes, compete for nutrients and surface adhesion sites, and hence are able to inhibit other bacteria. Furthermore, the robustness of these spore forming bacteria is often viewed as a great advantage as it may enable passage across the gastric barrier and offer better stability during feed processing and shelf life/storage. The large interest in the *Bacillus* genus in shrimp aquaculture can indeed be explained by these characteristics, but it is also for historical reasons as the first successful studies of probiotic in shrimps were conducted with *Bacillus* species (Rengpipat *et al.* 1998; Moriarty 1998).

Among *Bacillus* spp., *B. subtilis* is the most recurrent species being tested and used in crustaceans either alone (Rengpipat *et al.* 1998; 2000; 2003; Moriarty 1998; Dalmin *et al.* 2001; Meunpol *et al.* 2003; Vaseeharan and Ramasamy 2003; Marques *et al.* 2006a; Balcázar *et al.* 2007; Yu *et al.* 2008; Liu *et al.* 2009; Tseng *et al.* 2009; Liu K.-F. *et al.* 2011b; Shen *et al.* 2010) or in combination with other *Bacillus* spp. (Decamp *et al.* 2008; Boonthai *et al.* 2011), other probiotic species (Wang *et al.* 2005; Wang 2007) or prebiotic supplements (Li *et al.* 2009; Daniels *et al.* 2010; Zhang *et al.* 2011). The other *Bacillus* species documented so far as probiotics for crustacean are *B. licheniformis* (Li K. *et al.* 2007), *B. coagulans* (Zhou *et al.* 2009) and *B. pumilus* (Hill *et al.* 2009).

Interestingly *B. subtilis* is also widely used in terrestrial animals and in humans, with claims of bacteriotherapy and bacterioprophyllaxis of GI disorders (Cutting 2011). In shrimp, Vaseeharan and Ramasamy (2003) reported the beneficial effect of *B. subtilis*, isolated from shrimp culture ponds, on the survival of black tiger shrimp (*Penaeus monodon*) juveniles challenged with *Vibrio harveyi*, when the probiotic was delivered in the culture water at a density of $10^6 - 10^8$ CFU ml⁻¹. Furthermore, these authors demonstrated under *in vitro* and *in vivo* conditions that the probiotic was able to control the growth of *V. harveyi*. Balcázar and Rojas-Lunas (2007) also evaluated the dietary supplementation of a *B. subtilis* strain (UTM 126) on the survival of juvenile Pacific white shrimp *Litopenaeus vannamei* following an immersion challenge with *Vibrio parahaemolyticus*. The strain was initially isolated from the digestive tract of adult *L. vannamei* according to its antimicrobial activity against *V. harveyi*, *Vibrio vulnificus*, and *Vibrio fluvialis* (Balcázar *et al.* 2007). The results indicated that the probiotic strain exhibited *in vitro* antagonism toward *V. parahaemolyticus* and could induce a significant increase in the survival rate and performance of the host (weight gain and feed conversion ratio, FCR) when fed for 28 days to juveniles at 10^5 CFU g⁻¹ of diet. Rengpipat *et al.* (1998) also isolated a *Bacillus* strain (strain S11) from the gut of healthy *Penaeus monodon* with antimicrobial properties against *V. harveyi* and *V. parahaemolyticus*. In several *P. monodon* studies, and under various experimental conditions (Rengpipat *et al.* 1998; 2000; 2003; 2008), it has been reported that this probiotic strain, when administered as fresh cells at a concentration of 10^9

to 10^{10} CFU g⁻¹ of diet, could (1) increase growth and survival in pond and tank culture, (2) increase resistance to pathogenic *V. harveyi* strains, (3) activate both cellular and humoral immune defences, and (4) provide competitive exclusion in the shrimp gut. Meunpol *et al.* (2003) confirmed the efficiency of this strain and reported, in association with an ozone treatment, the beneficial effect conferred by *Bacillus* S11 on the survival of *P. monodon* post larvae, concomitantly with a reduction in *V. harveyi* concentration in the culture water. More recently, evaluations of *Bacillus* spp. probiotics were conducted on other crustacean species. Daniels *et al.* (2010) investigated the effect of a combination of dietary *Bacillus* sp. and yeast mannan oligosaccharides (MOS) on European lobster (*Homarus gammarus* L.) larvae. Beyond the classic effects on performance and survival, this study highlights for the first time the effect of a *Bacillus* spp. mixture on various parameters of lobster larvae such as modification in the gut microbial communities and improvements in intestinal morphology.

As described later in this chapter, one of the mechanisms of action by which *Bacillus* spp., especially *B. subtilis*, provide host benefits is by modulation of the immune response of the host. Such effects have been well documented in humans, terrestrial animals (Green *et al.* 1999; Cutting *et al.* 2011), fish (Avella *et al.* 2010) and also in crustaceans (Tseng *et al.* 2009; Liu *et al.* 2011b). The effect of *Bacillus* spp. on digestion and their putative contribution to nutritional processes have also been evaluated in crustaceans.

It is important to add here that *Bacillus* strains are also being studied, and extensively used, as bioremediation agents in shrimp farming (Moriarty 1999). The potential role and benefits of such microorganisms on the nutrient cycle and microbial processes in aquaculture ponds have been described by Moriarty (1997) and readers with a specific interest in this topic are referred to that review article. For instance, the author compared results from different farms in the Philippines and concluded that the addition of several *Bacillus* spp. to the pond water, at a concentration of 10^4 to 10^5 CFU ml⁻¹, allowed the culturing of shrimps over 160 days without an outbreak of luminescent *Vibrio* disease, while control ponds were affected by luminescent *Vibrio* disease before 80 days of culture (Moriarty 1998; 1999). The author demonstrated that the probiotic treatment modified the bacterial species composition of the pond water and sediment with a particular reduction in the number of luminous *Vibrio* spp. Apart from pathogenic bacteria, another main concern in intensive shrimp farming is organic enrichment and nitrogenous waste, including ammonium and ammonia in the culture pond. In relation to this, probiotics such as selected *Bacillus* spp. (Moriarty 1998; Song *et al.* 2011), eventually coupled with other bacterial strains (Devaraja *et al.* 2002; Matias *et al.* 2002; Wang and He 2011), have been added to pond water with the aim of boosting mineralization of the organic matter and increasing water quality. Thus, bacterial species belonging to the *Bacillus*, *Pseudomonas*, *Acinetobacter*, *Cellulomonas*, *Rhodopseudomonas*, *Aerobacter*, *Nitrosomonas* and *Nitrobacter* genera are known to promote mineralization of organic wastes (Thomas *et al.* 1992). For instance, *Nitrosomonas* spp. will help in the oxidation of ammonia while *Nitrobacter* spp. will oxidize nitrites and this process can help to prevent the build-up of toxic ammonia. Recently, a *B. coagulans* strain isolated from highly intensive shrimp ponds in China was also reported to show interesting aerobic denitrification characteristics for nitrite removal in shrimp ponds (Song *et al.* 2011). Some of the benefits of using such bacterial products include the reduction of blue-green algal populations and of nitrate, nitrite, ammonia and phosphate levels, the increase of dissolved oxygen concentrations and the promotion of organic matter decomposition (Boyd 1995). Unfortunately well designed studies on the benefits of applying such bacterial products in aquaculture rearing systems are still scarce (Shariff *et al.* 2001; Devaraja *et al.* 2002).

11.2.2 Lactic acid bacteria (LAB)

Another approach to the development of new probiotic applications in crustacean aquaculture consists in testing proven human or terrestrial animal probiotics such as strains of lactic acid bacterial (LAB) species. Because LAB are the most commonly applied probiotics in terrestrial animal nutrition, their use as probiotics has been proposed for aquatic species (Ringø and Gatesoupe 1998). Moreover since regulations in many countries make it mandatory to get authorization for the use of probiotics in animal feeds, the use of already identified and documented bacterial species, such as LAB or those belonging to the genus *Bacillus*, will tend to be increasingly extended to aquaculture (Gatesoupe 2002) as the licensing of new species is relatively expensive (Hong *et al.* 2005).

LAB potentially have several interesting probiotic properties: they are generally recognized as safe (Leuschner *et al.* 2010), they have the ability to adhere to cells, they may stimulate the growth of beneficial microorganisms and outcompete harmful bacteria, and they reinforce the organism's natural defence mechanisms (Reid 1999; Vázquez *et al.* 2005). For instance, as reviewed in the previous chapters a large number of probiotic studies report the beneficial effects of LAB probiotics on immunological and haematological parameters in fish, although the immunomodulatory mechanisms are only partly described.

Contrary to what is recorded in fish, in crustaceans most of the studies on intestinal microbiota do not report the presence of LAB in their digestive tract (for a review see *Chapter 6*). However, the current knowledge on the intestinal microbiota of crustaceans is still very limited and the majority of studies conducted so far have used conventional culture based microbiological methods, therefore providing incomplete information (Dempsey *et al.* 1989; Harris 1993; Moss *et al.* 2000; Oxley *et al.* 2002). Only a limited number of studies have used molecular methods to describe the microbiota of shrimps (Li P. *et al.* 2007; Johnson *et al.* 2008; Castex 2009; Liu *et al.* 2011a) or other crustaceans (Battison *et al.* 2008; Daniels *et al.* 2010). Further work in this direction is essential to better understand the presence, diversity and role of the intestinal microbial populations in crustaceans, and manipulate them in a way which promotes a better host health status. Even if the presence of LAB as normal inhabitants of the digestive tract of crustacea still needs to be addressed, the continual application of LAB species to crustaceans may lead to high GI levels of LAB and modulated microbial populations. Castex *et al.* (2008) reported that *Ped. acidilactici* MA18/5M administered continuously at a concentration close to 10^7 CFU g⁻¹ of feed was retrieved at levels between 10^4 and 10^5 CFU g⁻¹ in the gut of *Litopenaeus stylirostris*. This result was associated with a significant decrease in the *Vibrio* spp. levels in the intestine. Similar observations, *Vibrio* inhibition and probiotic recovery were reported in the intestine of *L. vannamei* fed a diet supplemented with an autochthonous *Lactobacillus plantarum* at 2×10^7 CFU⁻¹ (Viera *et al.* 2008). Unfortunately, studies with LAB probiotics have rarely reported the probiotic count achieved in the digestive tract of the host, but interestingly levels of recovered probiotic similar to those reported in these two studies on penaeid shrimps were also reported with *Bacillus* spp. probiotics on *P. monodon* and *Fenneropenaeus indicus* (Rengpipat *et al.* 2000; Ziaei-Nejad *et al.* 2006), suggesting an optimal probiotic bacteria level between log 4 and 6 CFU g⁻¹ in the intestine of penaeid shrimps.

As with fish, the effect of LAB on the immunological response of crustaceans has also been addressed, especially in penaeid shrimps. For instance, Chiu *et al.* (2007) investigated the effect of an *Lb. plantarum* strain on several immune parameters of *L. vannamei*. The author's report that administering this strain of *Lb. plantarum* can enhance the non-specific immunity (phenoloxidase (PO) activity, as well as prophenoloxidase (proPO) and peroxinectin (PE) mRNA

transcription) and the antioxidant enzyme superoxide dismutase activity (SOD) of the white shrimp. They also showed that this effect was associated with increased pathogen clearance efficiency and resistance to a pathogenic *V. alginolyticus* strain, suggesting that the probiotic was able to induce immune modulation and a subsequent increase in resistance to bacterial infection. Additional studies suggested a similar effect of LAB on the immune and antioxidant defences of shrimp resulting in higher resistance to bacterial infections (Castex *et al.* 2009; 2010; Viera *et al.* 2007; 2008).

Finally, the effect of LAB on some nutritional aspects in different marine shrimp species has also received some attention (Uma *et al.* 1999; Venkat *et al.* 2004; Castex 2009). Nevertheless, future studies should highlight the potential and the precise mode of action of LAB as probiotics for crustaceans.

11.2.3 Other Gram-positive bacteria

A number of other Gram-positive bacteria have also received attention as potential probiotics for crustacean culture. Li *et al.* (2006) compared an *Arthrobacter nicotianae* strain to an antibiotic treatment (Chloramphenicol) to protect Chinese shrimp post larvae from pathogenic *Vibrio* spp. To the authors' knowledge this study is the first evaluation of *Arthrobacter* sp. as a possible probiotic bacterium in aquaculture. While bath infection with a combination of pathogenic *Vibrio* spp. (*V. parahaemolyticus*, *Vibrio anguillarum* and *Vibrio nereis*), isolated from sick 'red-leg' shrimps, led to 43% mortality of the post larvae at the end of the 14 day experimental period, the shrimps receiving the probiotic bath treatment showed an 82.6% survival rate which was a level of protection similar to that obtained with the antibiotic treatment. The authors isolated the strain from *Fenneropenaeus chinensis* culture water samples but did not clearly report why this particular microorganism was selected. Nevertheless, they evoked several interesting properties of microorganisms from the genus *Arthrobacter* such as the production of antimicrobial compounds, the ability to nitrify and to use a wide range of organic substances, or again their ability to produce amino acids, vitamins and polysaccharides. Recently, Pai *et al.* (2010) also concluded that an *Arthrobacter* sp. strain (MCCB 104) functioned as probiotic in *P. monodon* larvae but suggested immunostimulatory and digestive enzyme-supporting modes of action in the absence of *in vitro* antagonistic effects toward *Vibrio* spp.

A recent study from Antony *et al.* (2011) showed the effect of three probiotic preparations containing a *Bacillus* strain and a *Micrococcus* strain alone, or in combination, on sub-adult black tiger shrimps. Even though *Micrococcus* sp. has been rarely identified as a normal inhabitant of the GI tract of penaeid shrimps, these authors focused their investigation on the putative effect of one strain on the immunity and the subsequent ability of the shrimp to resist a white spot syndrome virus (WSSV) challenge. The study revealed that the probiont administered individually or in combination with a *Bacillus* sp. was found to modulate the level of expression of antimicrobial peptide genes in *P. monodon*, leading to higher survival rate of the shrimps following a WSSV infection. However, the *Micrococcus* strain was found to be less effective than the *Bacillus* strain when tested alone.

11.2.4 Gram-negative bacteria

Gram-negative bacteria, especially Gammaproteobacteria, are also being used extensively as probiotics for crustaceans. Indeed members of the Gammaproteobacteria phylum are amongst the most commonly reported, and often most abundant, species present in the

intestinal microbial communities, or larval associated microbiota, of crustaceans according to numerous studies using conventional culture based or molecular culture-independent techniques (Oxley *et al.* 2002; Payne *et al.* 2006; Johnson *et al.* 2008; Daniels *et al.* 2010; Liu *et al.* 2011a). Autochthonous probiotic strains are often locally isolated, produced and used, most of the time based on an empirical and field led approach. Although this practice is widely applied, only a limited number of published reports exist. An interesting example is the development of probiotic products achieved in South American shrimp hatcheries and initiated by farmers with the aim of improving the levels of beneficial *Vibrio* spp., the sucrose fermenting bacteria, in their systems (Garriques and Wyban 1993). The first successful application was reported in Ecuadorian hatcheries by Griffith (1995). Further, Garriques and Arevalo (1995) isolated a *V. alginolyticus* strain from seawater and used it to treat larval mortality when challenged with *V. parahaemolyticus*. Other trials by the same authors confirmed the improvement in survival and also showed a growth promoting effect, though statistical analysis of their results carried out later by Gomez-Gil *et al.* (2000) indicated no statistical difference in survival and weight gain between treated and control groups. Nevertheless, since 1995 broad applications of probiotic *V. alginolyticus* (Ili strain) have taken place in commercial penaeid hatcheries in Ecuador. The main application of this probiotic was to control larval diseases and the applications therefore resulted in a large reduction in antimicrobial products being used in hatcheries. The work reported by these authors suggested that *V. alginolyticus* may confer some degree of protection against disease even if a lack of scientific explanation was evident. According to Rodríguez *et al.* (2007), the Ili strain of *V. alginolyticus* isolated from healthy shrimp larviculture is currently used in CENAIM's larvae production protocols. The strain is administered at the nauplii 5 stage and successfully mitigates what is commonly referred to as 'bolitas' syndrome or Zoea 2 syndrome (Vandenbergh *et al.* 1999).

In contrast to the broad application of Gram-negative bacterial probiotics in the field, relatively few in-depth studies have been published on this subject. Alavandi *et al.* (2004) isolated a *Pseudomonas* sp. and *V. fluvialis* strains from the gut of farmed sub-adult *P. monodon*. Selection was based on several properties such as: predominance in the gut microenvironment, ability to secrete extracellular macromolecules and digestive enzymes, and production of iron sequestering compounds like siderophores. However, the authors concluded that the selection criteria used were not satisfactory as they did not measure any effect on the immune indicators (such as haemocyte counts, PO activity and antibacterial activity in the haemolymph) of *P. monodon* shrimp. Other attempts were more successful, as demonstrated by the works of Chythanya *et al.* (2002), Gullian (2004), Balcázar (2003) and Balcázar *et al.* (2006; 2007). The first of these selected a *Pseudomonas* strain I-2 (tentatively identified as *Ps. aeruginosa*), isolated from seawater, and demonstrated its inhibitory activity towards several pathogenic Vibrionaceae such as *V. parahaemolyticus*, *V. harveyi*, *V. fluvialis*, *V. vulnificus* and *Photobacterium damsela*. They concluded that *Pseudomonas* I-2 strain could be used as a biocontrol agent in shrimp hatcheries and farms. More recently, Hai *et al.* (2007) also *Pseudomonas synxantha* and *Ps. aeruginosa* strains for use in the culture of western king prawns (*Penaeus latisulcatus*). The selection was based on the results of five inhibition tests conducted with various pathogens. Interestingly these authors looked at the *in vivo* effect of these probiotics at different levels (Hai *et al.* 2009; Hai and Fotadar 2009). They first report that the probiotics were able to improve the health (determined by assessing haemocyte numbers and bacterial load in the haemolymph) of the shrimp, and that their combination was more efficient than the

standalone strains. The authors report some levels of protection of the probiotic-fed prawns when challenged with *V. harveyi*, even if no data were provided. They finally reported that when supplemented to western king prawn diet (at 2×10^6 CFU g⁻¹) over 84 days of culture, the combination of these two strains led to improved growth, survival and FCR – this last benefit being partially associated with changes in the morphology of the intestine. Balcázar *et al.* (2007) compared the efficacy of different probiotic species to limit the mortality of *L. vannamei* following immersion challenge with *V. parahaemolyticus*. Interestingly the study demonstrated a similar level of protection with Gram-positive and Gram-negative bacteria compared with the control group, and recommended *V. alginolyticus* UTM102 and *Ps. aestumarina* SLV22 as promising dietary probiotics to prevent vibriosis in shrimps. Pai *et al.* (2010) recently showed that two strains of *Pseudomonas* sp. (MCCB 102 and MCCB 103) were also able to improve disease resistance of *P. monodon* larvae through active antagonism toward *Vibrio* spp. and a particular *V. harveyi* strain. Interestingly they also selected two other probiotic species from different genera (*Bacillus* and *Arthrobacter*) able to provide similar benefits to the larvae but with a non-antagonistic supporting mode of action.

Other authors have recently reported alternative approaches to using *Vibrio* sp. in shrimp diets (Krupesha Sharma *et al.* 2010). These authors assessed *Vibrio* cells as potential immunostimulants for *P. monodon* by testing the effect of heat inactivated *V. alginolyticus* cells and heat inactivated *V. alginolyticus* biofilm. The use of *V. alginolyticus* biofilm cells added to the feed at a dose of 10^9 CFU g⁻¹ of shrimp per day for 14 days resulted in an immunostimulation and an effective enhancement of *P. monodon* resistance to *V. alginolyticus* infection and WSSV. Despite these encouraging results, the use of *Vibrio* spp. (or other autochthonous Gram-negative bacteria) isolated locally remains controversial today since some strains have been described to be pathogenic for crustaceans, as is the case for *V. alginolyticus* (Lightner 1993). Clearly safety concerns must be considered in the use of taxa considered pathogenic for aquatic animals before allowing wider applications. Indeed, bacteria that are harmless for some species might acquire virulence or be pathogenic for other species (Irianto and Austin 2002). Adapted regulations on feed additives must then be set up locally in order to control these applications.

11.2.5 Yeast

Although various inactive yeast fractions (MOS, β -1,3 glucans, or yeast extract fractions) have received some attention as feed ingredients for marine and freshwater shrimp species (Sahoo *et al.* 2008; Ringø *et al.* 2010; Smith *et al.* 2003) as well as other crustaceans (Daniels *et al.* 2010; Sang *et al.* 2010) and are today widely used in formulated aquafeeds, the use of live yeasts as probiotics has received little attention in crustacea.

One of the first preliminary reports on the use of live yeast and fungi in crustaceans is the study of Intriago *et al.* (1998) which reported that a red pigmented yeast and a chitin-degrading fungus were able to improve the growth performance of *L. vannamei* larvae. A more detailed report from Scholz *et al.* (1999) compared different yeasts (a baker's yeast *S. cerevisiae*, a *Saccharomyces exiguus* and *Phaffia rhodozyma*) to improve performance of juvenile *L. vannamei* over a 7 week tank culture experiment. Their results revealed that even though no clear immunostimulatory effect was observed for the parameters assessed, all three yeasts, and especially the *Phaffia*, had a positive effect on the animals, leading for instance to a better survival. However, the probiotic dosage used in this study was not clearly reported and would merit further attention to evaluate the feasibility of such application. In a more recent study, Immanuel

et al. (2007) fed *P. monodon* post larvae with *Artemia* nauplii enriched with/without a commercial probiotic preparation containing a live strain of *S. cerevisiae*. They showed that shrimp fed enriched *Artemia* exhibited slightly higher weight gain and higher specific growth rate (SGR). More interestingly, in the probiotic group, increased survival of post larvae subjected to *V. harveyi* infection was also observed and associated with a reduced pathogen load in the muscle and the digestive gland. A recent study conducted in a commercial farm in Mexico assessed the effect of a particular strain of *S. cerevisiae* (*S. cerevisiae* var. *boulardii*) fed either alive or inactivated (at a 0.04% inclusion rate) to *L. vannamei* juveniles (experiment using floating cages), and revealed that only the 'live' form was able to significantly improve survival and feed utilization over the 60 day experiment (Lallemand unpublished results).

Patra and Mohamed (2003) were the first to report the application of the same yeast strain (*S. cerevisiae* var. *boulardii*) in an aquatic live feed organism. The aim of this study was to enrich *Artemia* nauplii with the probiotic and then to look at resistance to a *V. harveyi* infection. Indeed, *Artemia* nauplii, which are recognized as the best natural live feed available for larval culture, are often used as a model organism to study host–microbe interactions, and to evaluate probiotic bacteria before testing on target organisms (Marques *et al.* 2006b). In this study, the authors reported enhanced survival of nauplii receiving the yeast compared to the control larvae. Interestingly a dose effect could be detected with best results obtained when *S. boulardii* was administered at 10^4 CFU ml⁻¹ (90% survival rate after 48 h compared to 40% in the control group). A study by Soltanian and colleagues (2007) considered the effects of various yeast strains administered to gnotobiotic *Artemia* culture. They revealed a protective effect of the different yeast supplements toward *Vibrio* pathogens; the intensity of the protection was dependent on the yeast strain tested and was likely linked to the structure and the composition of the yeast cell wall.

Marine yeasts have also been studied for their probiotic potential. The yeast *Candida sake* was assessed for its immunostimulatory properties in *F. indicus* sub-adults (Sajeevan *et al.* 2006). The authors reported that *C. sake* S165 supported an optimal immune response and increased the survival of shrimps after a WSSV challenge. However, significant improvements were only reported with a 10% inclusion rate which is impractical at the industrial scale. More recently, Yang *et al.* (2011) selected a red marine yeast (*Rhodospiridium paludigenum*) based on its high content of carotenoids and its ability to grow in *L. vannamei* intestinal mucus. Subsequently it was demonstrated that this yeast could enhance growth, survival and antioxidant competence of *L. vannamei* when fed at 10^8 CFU g⁻¹ of feed (fresh yeast) or at a 1% inclusion rate (dry yeast). However, once again the high dosage levels considered can be questionable in terms of both practical implication for feed formulations and cost. Dose–response studies should therefore be addressed in order to further assess the efficacy of live yeast as probiotics for crustaceans.

11.3 PROBIOTIC MODES OF ACTION

Several studies on probiotics in crustaceans have been published over the last two decades. However, in the past the authors have mainly focused their evaluations on the benefits in terms of survival, resistance to disease or growth performance in order to demonstrate the concept. Even though more in-depth studies have been published over the last five years, little information is available today on the precise mode of action of probiotics in crustaceans. Besides,

as suggested by many authors, the specific mechanisms of probiotics resulting in the observed host benefits are probably of a very diverse nature and unlikely to arise from independent mechanisms (Gatesoupe 1999; Merrifield *et al.* 2010). This complicated synergistic multi-factorial relationship makes the demonstration of probiotic modes of action complicated, especially under *in vivo* conditions and especially when multi-strain probiotics are considered. Furthermore for crustaceans, the diversity of the culture systems (e.g. extensive, intensive, super intensive, closed recirculation, static biofloc, flow through etc.) must also be considered as they may influence the probiotic effects.

Today our understanding in crustaceans is mostly derived from humans, terrestrial animals, or more recently studies in fish. This section will focus on the proposed mechanisms of action of probiotics (direct effects on the host) for shrimps and other crustacean species, with specific focus on the localized mechanisms and effects on the digestive tract. Proposed, and investigated, modes of action in crustaceans include the production of inhibitory compounds, competition for chemicals or available energy, competition for adhesion sites, interference with quorum sensing, modulation of the host immune response, provision of nutrients and contributions to digestive enzyme activity.

11.3.1 Modulation of the gut microbiota

It is today accepted that the effects of probiotics in aquatic species arise from an initial modification of the GI microbiota. This microbial shift leads to changes in intestinal conditions, resulting in effects at different levels such as immune modulation or modulation of digestive functions. This initial modification, seen as the primary effect of probiotics on the host, can go from a simple introduction of a new microorganism species in the microbial ecosystem of the digestive tract to an entire shift of the microbial communities shown in fish. This shift is the result of specific or non-specific antagonisms toward autochthonous microbes. Unfortunately, this primary effect of probiotics has often been overlooked in crustacean studies and many assumptions are made through our experiences in other animals. Indeed most of the papers reporting the effect of probiotics on the physiology and performance of crustaceans do not investigate what is actually happening within the digestive tract. Some investigators do however enumerate or track the probiont in the gut microbiota, but rarely have studies looked at the probiotic effect on the composition of the indigenous microbiota. A number of studies have reported the effect of probiotics on the total bacterial count and/or cultivable *Vibrio* sp. in the digestive tract of crustaceans (Rengpipat *et al.* 2003; Venkat *et al.* 2004; Castex *et al.* 2008; Li *et al.* 2009; Zhang *et al.* 2009; Boonthai *et al.* 2011; Zhang *et al.* 2011); however, to our knowledge only a limited number of documents report the effect of probiotics, or even prebiotics, on the microbiota of crustaceans with application of molecular techniques (Daniels *et al.* 2010; Thompson *et al.* 2010; Liu *et al.* 2011a). Thompson and colleagues considered the use of molecular techniques to investigate the effect of Gram-negative probionts in Pacific white shrimp, *L. vannamei*. In this study, terminal restriction fragment length polymorphism (T-RFLP) was used to analyse bacteria within faecal and mid/hindgut samples. A limited bacterial diversity and distinct changes in microbial communities were observed when a *Vibrio gazogenes* was used as dietary probiotic over an 8 week period. PCR-DGGE of the 16S rRNA V3 region has been used to study the effects of *Bacillus* spp., and prebiotic MOS, on the GI microbial communities of *H. gammarus* larvae (Daniels *et al.* 2010). Microbial community profiles were observed to shift away from control lobsters in those fed *Bacillus* spp., in combination with prebiotics, with a reduction in species richness and diversity

highlighting a stabilization in bacterial communities in the probiotic fed *H. gammarus*. Both studies used traditional culture-dependent and more advance culture-independent methods of microbial analysis to provide a more holistic picture of changes in bacterial communities. However the lack of knowledge about the presence, role and diversity of the intestinal microbial communities in crustaceans limit interpretation of these findings. Additional studies on the crustacean microbiota using modern culture-independent techniques (PCR-DGGE, qRT-PCR, FISH and next-generation sequencing) are required to address this issue and to better identify and understand the basic probiotic effect in crustaceans. Furthermore, studies looking at the microbial communities in different sections of the GI are also required. Indeed the digestive tract of crustaceans is composed of three major sections – foregut, midgut and hindgut – with different morphological characteristics and functions. These differences are likely to have an effect on the associated microbial communities, as was demonstrated by Johnson *et al.* (2008) in their study on the microbial communities in the foreguts and hindguts of *L. vannamei* juveniles raised in a closed recirculating system. 16S rDNA sequences indicated that the microbial communities in the rearing water were more diverse than those in shrimp gut, and that the foregut and the hindgut harbour distinct communities. Indeed, DNA sequence of selected DGGE bands indicated that the foregut contained sequences belonging to *Mycobacterium* spp., *Propionibacterium* spp., *Desulfocapsa* spp., and chloroplast genes while the hindgut showed a lower diversity with sequences exclusively related to *Vibrio* spp. Such studies provide useful information to better understand the sensitivity of crustaceans to enteric bacterial infections and to potential methods of microbial manipulation. For instance, crayfish, having a much shorter midgut compared to penaeid shrimps, are less susceptible to oral *Aeromonas hydrophila* infections, which could be related to preliminary reports which suggest that the midgut is a preferential infection site for this bacterium (Jiravanichpaisal 2011).

11.3.2 Production of inhibitory substances

In a study of the probiotic *Bacillus* S11, Rengpipat *et al.* (2000) assumed competitive exclusion in the shrimp's gut as they observed a reduction in *V. harveyi* concentration in the gut of *P. monodon* fed the probiotic when challenged with the pathogenic bacteria. However, no information was provided by the authors as to how the probiotic might compete with the pathogen, though the production of inhibitory substances may be assumed since the bacteria was selected for its *in vitro* antimicrobial activity against *Vibrio* spp. The antagonism against pathogens, especially by the production of inhibitory substances, is probably the mode of action most frequently suggested in current literature. This can be explained by the fact that the preliminary screening processes of probiotics often rely on antagonism assays towards pathogenic bacteria (Vershuere *et al.* 2000a). The methods used in such studies are based on the capacity of the bacteria to produce some extracellular substances that inhibit other bacterial strains, as recently detailed by Hai *et al.* (2007). Rengpipat *et al.* (1998) selected a *Bacillus* S11 strain, from over 350 isolates from samples of mud, water and black tiger shrimp, based on its high antimicrobial performance against pathogenic *V. harveyi* and *V. parahaemolyticus*. Decamp *et al.* (2008) used a similar approach in order to select several *Bacillus* strains included in a commercial probiotic. Antimicrobial effects of bacteria generally come from the following substances released alone or in combination: antibiotics, bacteriocins, siderophores, lysozymes, proteases, hydrogen peroxide or organic acids reducing the pH of the medium, and/or ammonia (Verschuere *et al.* 2000a). Gildberg *et al.* (1997) stated that among the extracellular substances produced by probiotics, bacteriocins would be the most important. For instance, antimicrobial activities of

Bacillus spp. have been linked to polymyxin, bacitracin and gramicidin antibiotic production (Gullian *et al.* 2004). More specifically, the mechanisms by which *Bacillus* spp. control *Vibrio* spp. and other pathogens in crustacean intestines are associated with their capacity to produce antibiotic agents and to compete for nutrients and habitat with other bacteria (Moriarty 1998). In the case of LAB, it is commonly accepted that the primary causative effect is the reduction of pH via organic acid production and the removal of carbohydrates (Vázquez *et al.* 2005). Nevertheless, it is also accepted that the probiotic effects of many LAB are also based on the production of diverse antibacterial metabolites (bacteriocins in particular).

However, as emphasized by Kesarcodi-Watson *et al.* (2008), the *in vitro* screening for production of inhibitory substances presents two major limitations: (1) it excludes other potential probiotics which possess different modes of action that are undetected by agar plate methods; and (2) there is potentially no direct link between *in vitro* and *in vivo* assays as already reported (Gram *et al.* 2001). Moreover, bacteria, such as *Vibrio* spp., might develop resistance if the production of growth inhibitory compounds is the only mode of action. Based on this statement, the European Union and the European Food Safety Association (EFSA) stated that microorganisms intended for use as probiotics should not be able to produce any antimicrobial substances used as antibiotics in humans or animals (Anadón 2006). Therefore, *in vivo* validations are absolutely necessary. A plethora of *in vivo* trials has been carried out with shrimp species in order to (1) validate that *in vitro* properties of probiotics can be achieved *in vivo* (Vaseeharan and Ramasamy 2003; Castex *et al.* 2008; Boonthai *et al.* 2011); (2) assess the possible pathogenicity of a probiotic to the host (Chythanya *et al.* 2002); and (3) evaluate the degree of protection a probiotic may confer during a challenge with a pathogen (Rengpipat *et al.* 1998; Ajitha *et al.* 2004; Gullian *et al.* 2004; Balcázar *et al.* 2007; Castex *et al.* 2010). For instance, Vaseeharan and Ramasamy (2003) demonstrated inhibitory effects of cell-free extracts of *B. subtilis* BT23 against *V. harveyi* and then confirmed *in vivo* that this strain, when administered at 10^6 – 10^8 CFU ml⁻¹ for 6 days, confers a 90% reduction in accumulated mortality of *P. monodon* challenged with *V. harveyi*. In a recent study, Boonthai *et al.* (2011) observed, over a 120 day trial in tanks, that a dietary combination of several *Bacillus* species led to a log ≥ 3 increase in the total *Bacillus* counts in the intestine and digestive gland of *P. monodon* within 30 days, subsequently associated with a significant control of the cultivable *Vibrio* levels after the 30 days. Similar results were also reported in *L. stylirostris* with an LAB probiotic over a 10 week experiment in commercial ponds (Castex *et al.* 2008). Other studies have tried to elucidate the molecules involved in the antagonistic activity. Chythanya *et al.* (2002) reported the inhibitory activity of cell-free extract of *Ps. aeruginosa* I-2 and showed that the extract, when applied at ≥ 20 mg ml⁻¹, was able to reduce *V. harveyi* concentrations in rearing water by over a log unit. This effect was associated with the inhibitory effect of the chloroform extract of *Pseudomonas* I-2 cell-free supernatant and the author suggested that pyocyanine could be the antibacterial agent responsible for this effect.

11.3.3 Interference with quorum sensing

Another putative mode of action recently studied concerns the inhibition of virulence of pathogenic bacteria via interference of quorum sensing. ‘Quorum sensing’ is the process by which bacteria communicate and coordinate the expression of certain genes in response to signal molecules. These quorum sensing signal molecules were found to be involved in the regulation of virulence factors in many pathogenic bacteria, including the fish and crustacean pathogen *V. harveyi*. Recently, Defoirdt *et al.* (2005) and Tinh *et al.* (2007) demonstrated

that the *V. harveyi* quorum sensing system regulated the virulence of the bacterium towards gnotobiotic *Artemia franciscana* and *Br. plicatilis*, respectively. The use of quorum sensing disrupting compounds has been suggested as a promising strategy in order to inhibit the virulence of pathogenic bacteria in aquaculture (Defoirdt *et al.* 2004). For instance, when added at optimal concentrations, halogenated furanones, produced by a marine red algae and previously shown to disrupt quorum sensing signal molecules, protected *Artemia* from several pathogenic *Vibrio* spp. (Defoirdt *et al.* 2006). Therefore, steering the microbial communities toward microorganisms able to degrade these signalling molecules can be seen as a promising strategy.

Interestingly, quorum sensing disrupting compounds affect virulence of pathogenic *Vibrio* spp. but do not affect their growth, thus limiting the risk of resistance development compared to antibiotics. Hence, the search for new probiotic bacteria which can act as signal molecule inactivating bacteria is a future challenge in aquaculture systems.

11.3.4 Competition for nutrients and adhesion sites

Other studies suggest competitive exclusion by probiotic bacteria competing for resources such as nutrients and adhesion sites. The latter has been often suggested but unfortunately little evidence to demonstrate it under *in vivo* conditions can be found in the literature (Lee *et al.* 2000). Studies on this subject are limited because little is known about the colonization of the crustacean's digestive tract by bacteria and the existence of specific attachment sites (Harris 1993). It is accepted that crustaceans have a less developed GI microbiota than fish and that the microbiota would be predominately transient in the foregut and the hindgut regions. Bacterial concentration and composition are thus likely to be influenced by a variety of parameters, such as the culture system, temperature, bacterial communities of the culture medium, feeding, moulting, intestinal transit and defecation (Moss *et al.* 2000). Also while most research has focused on bacteria that are able to attach to intestinal mucus, transient bacteria have also been demonstrated to exert beneficial effects in crustaceans (Castex *et al.* 2008; 2009; 2010). Therefore, although it may potentially be beneficial, it is not necessary for a probiotic to display 'colonization' to exert a local effect during transient passage through the GI system. In this case, regular administration of the probiotic via the feed can be a means to artificially sustain their presence in the digestive tract (Castex 2009; Merrifield *et al.* 2010).

When, or if, adhesion and colonization of the mucosal surfaces by probiotic bacteria do occur, the attachment of pathogens can be prevented or their removal can be achieved from an infected digestive tract (Vershuere *et al.* 1999; Vine *et al.* 2004). In crustaceans, one of the best examples was provided by Gullian *et al.* (2004) who demonstrated colonization of the digestive gland of *L. vannamei* by several probiotic strains and highlighted competitive interaction of these strains with pathogenic *V. harveyi*. For instance, the probiotic strain *Vibrio* P62 was able to reduce the colonization of *V. harveyi* S2 by 60%, and to displace the indigenous microbiota of the digestive gland. Of course one must be aware that the adhesion of a probiotic to the gut wall does not necessarily imply that competition for adhesion sites is the only mode of action involved.

Several studies also suggest competition for nutrients or energy sources (Rico-Mora *et al.* 1998; Verschuere *et al.* 1999). Rico-Mora *et al.* (1998) showed that a probiotic strain, isolated for its high capacity to grow in organic-poor substrates, was able to prevent the establishment of *V. alginolyticus* in a diatom culture, whereas no inhibitory effect of the strain was detected toward other *Vibrio* spp. Others have also suggested that the competition for iron could be a

possible mechanism (Gram *et al.* 1999). However, reports or suggestions of such mechanisms in crustaceans are presently absent.

11.3.5 Modulation of the host immune response

There are many reports regarding the immunostimulatory properties of microorganism fractions (e.g. lipopolysaccharides from bacteria cell walls, β -glucans from the yeast cell walls etc.) in crustaceans (Smith *et al.* 2003; Sajeevan *et al.* 2009; Bai *et al.* 2010). As extensively detailed in the previous chapters, many studies have also revealed that live microorganisms can stimulate the non-specific immune system of fish; in particular, LAB are often reported as efficient immunomodulators in fish. However despite the large number of studies available, the immunomodulatory effects of probiotics in fish systems are, at present, not fully understood. The same observation can be made for crustaceans with many studies reporting the effect of probiotics on immunity even though relatively little information is available to explain the mechanisms behind these observations (Rengpipat *et al.* 2000; Gullian *et al.* 2004; Alavandi *et al.* 2004; Sajeevan *et al.* 2006; Rodríguez *et al.* 2007; Chiu *et al.* 2007; Li *et al.* 2008; 2009; Hai *et al.* 2009; Hai and Fotadar 2009; Zhang *et al.* 2009; 2011; Peraza-Gomez *et al.* 2011). Balcázar *et al.* (2006) suggested that bacteria that did not colonize the gut would not help in improving the immune system of shrimp. This seems to be in accordance with the results of Gullian *et al.* (2004) who associated the enhancement of the global immunity index conferred by *Vibrio* sp. (P62 and P63) and *Bacillus* probiotic strains in shrimps to their degree of colonization. However, this aspect has not been further documented and more studies are required to identify whether colonization is a prerequisite for immune stimulation or modulation in crustaceans.

In crustaceans this mode of action is mainly investigated with probiotics from the *Bacillus* genus. Rengpipat *et al.* (2000) suggested that *Bacillus* S11 provided protection against vibriosis by activating both cellular and humoral defences in tiger shrimp. This effect was characterized by improved immunity indices such as total haemocyte count, phagocytic activity, PO activity and a higher haemolymph antibacterial activity. They attributed this effect to the peptidoglycan present at the surface of the probiont wall which can stimulate granulocytes and enhance phagocytosis (Itami *et al.* 1998). Other authors have confirmed such effects on PO activity, phagocytosis, pathogen clearance efficiency and increased resistance to pathogenic bacteria with other *Bacillus* strains (Tseng *et al.* 2009). Interestingly these authors pursued their investigation and further explained this effect by an up-regulation of the expression of two proPO isoform genes (Liu *et al.* 2011b).

Balcázar (2003) reported that the immune modulation conferred by a mixture of *Bacillus* spp. and *Vibrio* spp. was associated with a better resistance to WSSV. Interestingly a recent study from Antony *et al.* (2011) showed, for the first time, that dietary probiotics (*Micrococcus* sp. and *Bacillus* sp. alone or in combination) were able to up-regulate the expression of specific antimicrobial peptide (AMP) genes (ALF, penaeidin-3 and -5) in haemocytes and several tissues of the giant tiger shrimp; previous experiments had shown similar priming effects with lipopolysaccharides (Okumura 2007). AMPs are a primitive first-line innate host defence mechanism, displaying activities against bacteria, fungi and viruses. Today, AMPs are well described in crustaceans and are known to be involved in the resistance of shrimps to bacterial and viral infection (Bachère *et al.* 2004). When shrimps were challenged by WSSV, a general down-regulation of the AMP genes was reported (with the exception of ALF); however, probiotic treated shrimps showed a greater level of protection together with enhanced quantities of

all AMP gene transcripts measured in the haemocytes (anti-lipopolysaccharide factor, crustins and penaeidins) compared to the control (Antony *et al.* 2011). The study demonstrated variations in the expression profile of AMP genes resulting in different levels of protection against WSSV dependent on the probiotic strain.

Interestingly new strategies based on probiotics are also currently being developed in order to improve the resistance of crustaceans to WSSV. Even though crustaceans lack a true adaptive immune system, it is now reported that memory responses could be induced by applying inactivated pathogens or recombinant proteins against WSSV (Namikoshi *et al.* 2004). Candidate 'vaccines' based on structural proteins of the virus have been evaluated. For instance VP28, an envelope protein from WSSV, has shown promising protective effects (Witteveldt 2004). However the application of this 'vaccine' would require injection which is not feasible at the industrial level. Therefore the use of live bacteria, such as *B. subtilis*, has been explored as a vehicle for oral delivery of VP28. Successful attempts using a recombinant *B. subtilis* strain able to secrete VP28 have been conducted in shrimp and crayfish (Fu *et al.* 2010). This study reported a lower susceptibility of *F. chinensis* sub-adults to WSSV infection when fed for 20 days a diet coated with the recombinant *B. subtilis* strain (20% mortality compared to 100% in the positive control group) and suggested that the enhancement of the activity of inducible nitric oxide synthase (iNOS) and NO observed could play an important role in the antiviral defence of the shrimp. Interestingly the unmodified *B. subtilis* fed group was able to marginally reduce the mortality compared to the positive control group.

In addition to *Bacillus* spp., a number of investigations have studied the immunomodulatory capacity of other bacterial genera. Specifically, Chiu *et al.* (2007) were among the first to assess the effect of an LAB probiotic on the immune system of shrimps. This study demonstrated that an *Lb. plantarum* strain, administered at 10^7 CFU g⁻¹ of feed, enhanced the immune response of *L. vannamei* from 48 hours after its introduction to the diet, and subsequently increased shrimp resistance to *V. alginolyticus*. They suggested that *Lb. plantarum* may enhance the antioxidant status of the shrimp based on the increased SOD activity and peroxinectin mRNA transcription levels. The antioxidant defence system of the organism acts to maintain the lowest possible levels of reactive oxygen species in the cell, and is recognized as an essential component of an organism's self-maintenance. Recent studies have demonstrated the involvement of the antioxidant system following bacterial infection or virus challenges in marine shrimps (Liu *et al.* 2007; Mathew *et al.* 2007). Castex *et al.* (2009; 2010) showed that the administration of the dietary probiotic *Ped. acidilactici* MA18/5M to *L. stylirostris* was able to support the antioxidant system during an infection by a highly virulent *Vibrio nigripulchritudo* strain. Even if the exact mechanisms behind it remain unknown at this stage, one of the hypotheses formulated to explain this effect was that the probiotic may have contributed to increase the overall physiological status of the shrimp, including antioxidant status and immune response. Other authors also reported recently the effect of a live yeast based probiotic (the red yeast, *Rhodospiridium paludigenum*) on the performance and the antioxidant enzymes activities in the serum, muscle and digestive gland of *L. vannamei*, supporting again the enhancement of the antioxidant status as another possible explanation for the probiotic effect in crustaceans (Yang *et al.* 2011).

In conclusion, the information available to date demonstrates that probiotics are potent immunomodulators in crustaceans. Future studies are required, specifically to identify direct and indirect (through a modulation of the microbiota) interaction of probiotics on the localized immune status of crustaceans. However, with their diverse mechanisms of action (competitive

exclusion and immunomodulation) probiotics have several advantages over the application of pure immunostimulants, precisely for the prevention of bacterial or viral infection.

11.3.6 Direct or indirect nutritional effects

As explained by Verschuere *et al.* (2000a), it is often unclear whether a probiotic effect on growth is attributed to better health (i.e. biocontrol) or if it is a consequence of the nutritional effects of the probiont, acting as food source or contributing to the digestive function. It is obvious that interaction may exist in some cases between the two effects. This is where confusion can arise between zootechnical additives, acting mainly in maintaining an organism's health and favouring growth, and veterinary additives acting strictly as biocontrol agents. A combination of nutritional contribution and disease control will probably yield the best probiotic effect.

Very few studies report that probiotic bacteria are good candidates for improving the digestion of nutrients and the growth of crustaceans, and the mechanisms behind such benefits remain unclear (Venkat *et al.* 2004; Lin *et al.* 2004; Wang 2007). Interestingly, growth improvement is mainly reported when probiotics are administered in the feed (Castex 2009). Even if little is known about the role of the intestinal microbiota on nutritional processes in marine invertebrates (Harris 1993), the microbiota may serve as a supplementary source of food and nutrients such as vitamins, exogenous enzymes or essential amino acids (Dall and Moriarty 1983). Thus the manipulation of the intestinal microbial communities could be a way to improve the energetic pool provided by the microbial biomass through a direct production of nutrients in the mucosa or a better feed digestibility. For instance, Erasmus *et al.* (1997) demonstrated that enteric bacteria play an integral role in abalone nutrition by hydrolysing complex polysaccharide components of macroalgae to simple polymers and smaller units which are rapidly assimilated by the host. Indeed it was reported that abalone enteric bacteria produced enzymes capable of degrading agar, carrageenan, laminarin and alginate and that 70–90% of the enzyme activities were extracellular, suggesting that bacterial enzymes were secreted into the lumen of the gut.

It is accepted that probiotics may improve digestive activity by synthesis of vitamins, by cofactors or by improving enzymatic activities (Fuller 1989; Gatesoupe 1999). For instance, Gorospe *et al.* (1996) explained the nutritional contribution of *Pseudomonas* sp. in *Artemia* culture as the bacteria being an additional source of protein, and Yu *et al.* (1998) demonstrated that vitamin B12 producing bacteria can be used as a nutritive complement for rotifers *Brachionus plicatilis*. More globally, probiotics may favour growth by a number of mechanisms, either alone or in interaction, by: (1) increasing the pool of digestive enzymes in the GI tract or stimulation of digestive enzyme production by the host, (2) increasing nutrient availability to the organism for absorption in the GI tract (for example by pre-digesting some elements of the feed), and/or (3) providing additional bacterial biomass which the host can use as an additional nutrient source. A nice example is provided by Doeschate and Coyne (2008) who suggested three levels of actions for the probiotic *Pseudoalteromonas* sp. strain C4 on the nutrition of farmed abalone *Haliotis midae*: (1) pre-digestion of alginate in kelp based feed, (2) increased alginate lyase activity in the abalone digestive tract, and (3) utilization of strain C4 as a protein source.

In crustaceans, the information available is restricted to the production of enzymes contributing to digestion and/or the stimulation of digestive enzyme activities of the host by the probiont. Ochoa-Solano and Olmos-Soto (2006) selected three *Bacillus* strains and showed

their high protease, carbohydrase and lipase activities which they suggested could be used as probiotic in order to increase feed utilization by the shrimp. Recently, Liu *et al.* (2009) isolated a protease-producing bacterium (*B. subtilis* E20), and demonstrated that, when administered to shrimp juveniles at 10^8 CFU kg⁻¹ of feed, this probiotic improved growth performance and increased protease activity in the digestive tract of the shrimps. Ziaei-Nejad *et al.* (2006) also associated the growth improvement of the *F. indicus* shrimp, cultured with mixed *Bacillus* probiotics, with an increase in the specific amylase, lipase and protease activities in the digestive tract. However, based on the low colonization rate of the *Bacillus* strain in the digestive tract of the shrimps, the authors suggested that the exogenous enzymes produced by the probiotic strains may account for only a small proportion of total enzyme activity. Therefore, in order to explain the improved growth rate and FCR recorded in the probiotic treatments, the authors assumed that the probiotic might stimulate endogenous enzymes produced by the shrimp. Other studies suggest the same hypothesis (Wang 2007; Castex *et al.* 2008) and Wang (2007) reported that these effects were not improved by increasing concentration, suggesting that such probiotic effect could reach an upper limit in the shrimp intestine.

Additional studies are required to better understand the contribution of probiotics to nutrition, and their potential to address the challenges of the next generation of feeds that would likely use more complex formulations (using the growing range of raw materials available in the market) in order to avoid the dependence on particular raw materials

11.4 RELATED BENEFITS IN CRUSTACEAN AQUACULTURE

Despite a lack of precise understanding of the mechanisms behind the use of probiotics, many studies and field reports provide evidence of the efficacy of the probiotic concept in crustaceans. The following benefits have been extensively reported: improved disease resistance (against bacterial and viral diseases), immune response, health status, growth performance, feed utilization, and modulation of the intestinal microbiota. Tables 11.1 and 11.2 present a summary of some of the most relevant *in vivo* probiotics studies published with crustacean species, for larvae, early post larvae and larger developmental stages (juveniles and sub-adults). Regarding shrimps, although most of the trials on larvae, post larvae, juvenile, sub-adult and breeder shrimps were carried out with the black tiger (*P. monodon*) and white shrimp (*L. vannamei*), a substantial number of studies have also taken place on marine and freshwater shrimp species such as *F. chinensis*, *F. indicus*, *L. stylirostris* and *Macrobrachium rosenbergii*.

Indeed, unlike finfish aquaculture, the use of probiotics in crustacean culture, specifically shrimp culture, is now a common and widely adopted management practice used by farmers. Therefore, some practical considerations and issues regarding large scale experiments are provided in the following sections.

11.4.1 Main benefits reported in larvae and early post larvae stage

11.4.1.1 Overview

Maeda and Liao (1992) were the first to report the beneficial effect of bacterial strains on penaeid shrimp larvae. This study demonstrated that a soil bacterial strain (*Thalassobacter utilis* PM-4), with *in vitro* inhibitory activity against *V. anguillarum*, was able to increase larvae

Table 11.1 Summary of relevant *in vivo* probiotics applications reported in crustacean larviculture.

Animal species	Probiotic strain	Route of administration	Rearing conditions	Measured parameters	Pathogen challenge	Reference
Shrimp <i>Penaeus monodon</i>	<i>Lb. rhamnosus</i> and two commercial probiotics (including <i>Ped. acidilactici</i>)	Water	Experimental tanks	DR, S, GP	<i>V. anguillarum</i>	Panigrahi <i>et al.</i> (2011)
	<i>Pseudomonas</i> sp., <i>Bacillus</i> sp., <i>Arthrobacter</i> sp. independently	Water	Experimental tanks	DR	<i>V. harveyi</i>	Pai <i>et al.</i> (2010)
	Combination of <i>Bacillus</i> spp.	Water	Commercial hatchery	S		Decamp <i>et al.</i> (2008)
	<i>B. cereus</i> , <i>Paenibacillus</i> spp., <i>Paenibacillus polymyxa</i> independently	Water	Experimental tanks	DR	<i>V. harveyi</i> and <i>V. vulnificus</i>	Ravi <i>et al.</i> (2007)
	<i>B. subtilis</i> S11	Water	Experimental tanks	DR, GM	<i>V. harveyi</i>	Meunpol <i>et al.</i> (2003)
	<i>Ps. aeruginosa</i>	Water	1 litre beaker	S		Chythanya <i>et al.</i> (2002)
	<i>B. subtilis</i> S11	Feed	Experimental tanks	DR, IR	<i>V. harveyi</i>	Rengpipat <i>et al.</i> (2000)
	Combination of <i>Lactobacillus</i> sp. and <i>S. cerevisiae</i>	Feed	Experimental tanks	S, GP		Uma <i>et al.</i> (1999)
	<i>Thalassobacter utilis</i> PM-4	Water	Experimental tanks	S, GP		Maeda and Liao (1992)
	<i>B. subtilis</i> E20	Water	Experimental tanks	S, IR, GP, EP, AM, stress tolerance	Salinity, temperature, nitrite	Liu <i>et al.</i> (2011b)
<i>Litopenaeus vannamei</i>	Combination of <i>Bacillus</i> spp.	Water	Commercial hatchery	S		Decamp <i>et al.</i> (2008)
	<i>V. alginolyticus</i>	Water	Experimental tanks	S, DR, IR	WSSV	Rodriguez <i>et al.</i> (2007)
	<i>Lb. plantarum</i> and an unidentified LAB independently	Water	Experimental tanks	S, GP, AM	<i>V. harveyi</i>	Viera <i>et al.</i> (2007)
						(continued overleaf)

Table 11.1 (continued)

Animal species	Probiotic strain	Route of administration	Rearing conditions	Measured parameters	Pathogen challenge	Reference
	Red yeast	Water	Experimental tanks	GP		Intriago <i>et al.</i> (1998)
	<i>V. alginolyticus</i>	Water	Commercial hatchery	DR	<i>V. parahaemolyticus</i>	Garriques and Arevalo (1995)
<i>Fenneropenaeus indicus</i>	Combination of <i>Bacillus</i> spp.	Water or <i>Artemia</i>	Experimental tanks	S, GP, FU, GM		Ziaei-Nejad <i>et al.</i> (2006)
<i>Fenneropenaeus chinensis</i>	<i>Arthrobacter</i> XE-7	Water	Experimental tanks	DR, AM, EP	<i>V. parahaemolyticus</i> , <i>V. nereis</i> , <i>V. anguillarum</i>	Li <i>et al.</i> (2006)
<i>Macrobrachium rosenbergii</i>	<i>B. subtilis</i>	Water	Experimental tanks	S, GP		Keysami <i>et al.</i> (2007)
	<i>Lb. acidophilus</i> and <i>Lb. sporogenes</i> independently	Feed or <i>Artemia</i>	Experimental tanks	S, GP, GM		Venkat <i>et al.</i> (2004)
Crab						
<i>Portunus trituberculatus</i>	<i>Thalassobacter utilis</i> PM-4	Water	Experimental tanks	S		Nogami and Maeda, (1992), Maeda (1994), Nogami <i>et al.</i> (1997)
Lobster						
<i>Homarus gammarus</i>	Combination of <i>Bacillus</i> spp.	Feed	Experimental tanks	GP, GM, GH		Daniels <i>et al.</i> (2010)

Microbial genera: *B.* = *Bacillus*, *Lb.* = *Lactobacillus*, *Ped.* = *Pediciococcus*, *Ps.* = *Pseudomonas*, *S.* = *Saccharomyces*, *V.* = *Vibrio*.

DR = diseased resistance, GM = gastrointestinal microbiota, IR = immune response, S = survival, GP = growth performance, FU = feed utilization, GH = gastrointestinal histology, EP = environmental parameters, AM = associated microbiota.

Table 11.2 Summary of relevant *in vivo* probiotics applications reported in juvenile and sub-adult crustaceans.

Animal species	Probiotic strain	Route of administration	Rearing conditions	Measured parameters	Pathogen challenge	Reference
<i>Penaeus monodon</i>	<i>Bacillus</i> MCCB101, <i>Micrococcus</i> MCCB104 independently and their combination	Addition to diet	Aquarium	DR, IR	WSSV	Antony <i>et al.</i> (2011)
	Combination of <i>B. subtilis</i> , <i>B. polymyxa</i> , <i>B. megaterium</i> , <i>B. licheniformis</i> and <i>B. thuringiensis</i>	Addition to diet	Experimental tanks	GP, FU, GM		Boonthai <i>et al.</i> (2011)
	<i>Lb. acidophilus</i> and <i>S. cerevisiae</i> independently	Addition to <i>Artemia</i>	Experimental tanks	GP, DR, GM, S	<i>V. harveyi</i>	Immanuel <i>et al.</i> (2007)
	<i>Streptomyces</i> sp.	Addition to diet	Experimental tanks	S, GP, AM, EP		Das <i>et al.</i> (2006)
	Not reported	Addition to culture water	Commercial earthen ponds	AM, GM		Vaseeharan <i>et al.</i> (2004)
	<i>Pseudomonas</i> sp. and <i>V. fluvialis</i> independently	Addition to culture water	Experimental tanks	IR		Alavandi <i>et al.</i> (2004)
	<i>B. subtilis</i> BT23	Addition to culture water	Experimental tanks	DR	<i>V. harveyi</i>	Vaseeharan and Ramasamy (2003)
	(A) Combination of <i>Bacillus</i> sp. and <i>Saccharomyces</i> sp. (B)	Addition to culture water	Earthen ponds	S, GP, FU,		Devaraja <i>et al.</i> (2002)
	Combination of <i>Bacillus</i> sp., <i>Nitrosomonas</i> sp. and <i>Nitrobacter</i> sp.	Addition to culture water	Earthen ponds	GP, FU, EP		Matias <i>et al.</i> (2002)
	(A) Combination of <i>Bacillus</i> sp. and <i>Saccharomyces</i> sp. (B)	Addition to culture water	Earthen ponds	GP, FU, EP		Matias <i>et al.</i> (2002)
	Combination of <i>Bacillus</i> sp., <i>Nitrosomonas</i> sp. and <i>Nitrobacter</i> sp.	Addition to culture water	Earthen ponds	GP, FU, EP		Matias <i>et al.</i> (2002)
	<i>Bacillus</i> spp.	Addition to culture water	Earthen pond	S, GP, AM, EP		Dalmin <i>et al.</i> (2001)

(continued overleaf)

Table 11.2 (continued)

Animal species	Probiotic strain	Route of administration	Rearing conditions	Measured parameters	Pathogen challenge	Reference
	Not reported	Addition to culture water	Commercial earthen ponds	S, FU, AM, EP		Shariff <i>et al.</i> (2001)
	<i>Bacillus</i> S11	Addition to diet	Experimental tanks and aquarium	S, GP, GM, AM, DR	<i>V. harveyi</i>	Rengpipat <i>et al.</i> (1998)
	<i>Bacillus</i> S11	Addition to diet	Experimental tanks	S, GP, IR, GM, AM, DR	<i>V. harveyi</i>	Rengpipat <i>et al.</i> (2000)
	<i>Bacillus</i> S11	Addition to diet	Cages in earthen ponds and aquarium	GP, GM, DR	<i>V. harveyi</i>	Rengpipat <i>et al.</i> (2003)
	<i>Bacillus</i> S11	Addition to diet	Experimental tanks	DR, AM, GM	<i>V. harveyi</i>	Meunpol <i>et al.</i> (2003)
	<i>Bacillus</i> S11	Addition to diet	Experimental tanks	G, GM, EP		Rengpipat <i>et al.</i> (2008)
	<i>Bacillus</i> spp.	Addition to culture water	Earthen ponds	S, GP, AM		Moriarty (1998)
<i>Penaeus latissulcatus</i>	<i>Ps. synxantha</i> , <i>Ps. aeruginosa</i> independently and combined	Addition to diet	Experimental tanks	S, DR, GP, IR, FU, GH		Hai <i>et al.</i> (2009), Hai and Fotedar (2009)
<i>Litopenaeus vannamei</i>	Combination of <i>Bacillus</i> sp., <i>Nitrosomonas</i> sp., <i>Nitrobacter</i> sp. and <i>Lactobacillus B. subtilis</i>	Addition to culture water	Commercial ponds	EP		Wang and He (2011)
	<i>Rhodospiridium paludigenum</i>	Addition to diet	Experimental tanks	GP, IR, GM		Shen <i>et al.</i> (2010)
	<i>B. subtilis</i> E20	Addition to diet	Experimental tanks	GP, IR		Yang <i>et al.</i> (2011)
	<i>Bacillus</i> sp.	Addition to diet	Experimental tanks	DR, IR	<i>V. alginolyticus</i>	Tseng <i>et al.</i> (2009)
	<i>Lb. plantarum</i>	Addition to diet	Experimental tanks	S, GP, FU, BP		Yu <i>et al.</i> (2008)
	<i>Arthrobacter</i> XE-7	Addition to diet	Experimental tanks	GM, IR	<i>V. harveyi</i>	Viera <i>et al.</i> (2008)
		Addition to diet	Experimental tanks	DR, IR, GM, EP	<i>V. parahaemolyticus</i>	Li <i>et al.</i> (2008)

<i>V. alginolyticus</i>	Addition to culture water	Experimental tanks and commercial earthen ponds	DR, IR	WSSV	Rodríguez <i>et al.</i> (2007)
<i>V. alginolyticus</i> , <i>B. subtilis</i> , <i>Roseobacter gallaeciensis</i> , <i>Ps. aestumarina</i> independently	Addition to diet	Experimental tanks	DR, GP, FU, IR	<i>V. parahaemolyticus</i>	Balcázar <i>et al.</i> (2007)
<i>B. subtilis</i>	Addition to diet	Experimental tanks	DR	<i>V. harveyi</i>	Balcázar and Rojas-Luna (2007)
Combination of <i>Rhodobacter sphaeroides</i> and <i>B. coagulans</i>	Addition to diet	Aquarium	GP, FU	Wang (2007)	
<i>Lb. plantarum</i>	Addition to diet	Experimental tanks	DR, IR	<i>V. alginolyticus</i>	Chiu <i>et al.</i> (2007)
<i>B. licheniformis</i>	Addition to culture water	Experimental tanks	IR		Li <i>et al.</i> (2007)
Not reported	Addition to culture water	Earthen ponds	EP, production yield		Wang <i>et al.</i> (2005)
Combination of <i>Bacillus</i> spp.	Addition to diet	Experimental tanks	FU		Lin <i>et al.</i> (2004)
<i>Vibrio</i> P62, P63, <i>Bacillus</i> P64 independently	Addition to culture water	Aquaria	GP, IR, GM	<i>V. harveyi</i>	Gullian <i>et al.</i> (2004)
Combination of <i>B. subtilis</i> , <i>B. megaterium</i> , <i>B. polymyxa</i> , <i>B. licheniformis</i>	Addition to culture water	Outdoor tanks	S, GP, FU, EP	McIntosh <i>et al.</i> (2000)	
<i>V. hepatarius</i> , <i>Vibrio</i> sp., <i>Bacillus</i> sp. independently	Addition to diet	Experimental tanks	DR, GP, IR	<i>V. harveyi</i> and WSSV	Balcázar (2003)
<i>S. cerevisiae</i> , <i>Phaffia rhodozyma</i> , <i>S. exiguus</i> independently	Addition to diet	Fiberglass tanks	DR, GP, FU, IR	<i>V. harveyi</i>	Scholz <i>et al.</i> (1999)
<i>Ped. acidilactici</i>	Addition to diet	Cages in earthen ponds	S, GP, FU, GM		Castex <i>et al.</i> (2008)
<i>Litopenaeus stylirostris</i>	Addition to diet	Experimental tanks	DR, GM, IR	<i>V. nigripulchritudo</i>	Castex <i>et al.</i> (2009)
	Addition to diet	Experimental tanks	DR, IR	<i>V. nigripulchritudo</i>	Castex <i>et al.</i> (2010)

(continued overleaf)

Table 11.2 (continued)

Animal species	Probiotic strain	Route of administration	Rearing conditions	Measured parameters	Pathogen challenge	Reference
<i>Fenneropenaeus indicus</i>	Combination of <i>Bacillus subtilis</i> , <i>B. licheniformis</i> , <i>B. polymyxa</i> , <i>B. laterosporus</i> , <i>B. circulans</i>	Addition to diet	Experimental tanks	GP, FU, IR		Castex <i>et al.</i> (2011)
	Marine yeast <i>Candida sake</i> S165	Addition to culture water	Experimental tanks and earthen ponds	S, GP, FU, GM, AM		Ziaei-Nejad <i>et al.</i> (2006)
	<i>Lb. acidophilus</i> , <i>Streptococcus cremoris</i> , and two strains of <i>Lb. bulgaricus</i> independently	Addition to diet	Experimental tanks	DR, IR	WSSV	Sajeevan <i>et al.</i> (2006)
	Combination of <i>Saccharomyces</i> sp., <i>Lb. acidophilus</i> and <i>Streptococcus faecium</i>	Addition to diet	Experimental tanks	DR	<i>V. alginolyticus</i>	Ajitha <i>et al.</i> (2004)
<i>Macrobrachium rosenbergii</i>	<i>Lb. acidophilus</i> and <i>Lb. sporogenes</i> independently	Addition to diet	Experimental tanks	DR, GP, FU, FC	<i>V. alginolyticus</i>	Uma <i>et al.</i> (1999)
			Experimental tanks	S, GP, FU		Venkat <i>et al.</i> (2004)

Microbial genera: *B.* = *Bacillus*, *Lb.* = *Lactobacillus*, *Ped.* = *Pediococcus*, *Ps.* = *Pseudomonas*, *S.* = *Saccharomyces*, *V.* = *Vibrio*.
 DR = diseased resistance, GM = gastrointestinal microbiota, IR = immune response, S = survival, GP = growth performance, FU = feed utilization, FC = feed consumption, GH = gastrointestinal histology, EP = environmental parameters, AM = associated microbiota, BP = blood parameters.

survival and to promote the growth of *P. monodon* nauplii. Later experiments from the same authors showed a similar effect for the swimming crab larvae *Portunus trituberculatus* when the probiotic was added daily to seawater from zoea 1 to zoea 3 (Nogami *et al.* 1997). Interestingly the level of the probiotic bacteria was recorded at 10^5 to 10^6 CFU ml⁻¹ in culture water and a concomitant decrease in *V. anguillarum* concentration was measured in seawater. Large scale experiments in commercial infrastructure in Japan confirmed this protective effect with an average survival rate increased by twofold when the probiotic was added.

However, since then relatively few in-depth studies have been published on this topic and, as previously reported in this chapter, most of the information available today comes from practical and empirical experiences in commercial hatcheries.

Most of the investigations of probiotics in crustacean larviculture were motivated by the need for alternative solutions to replace antibiotics. Also the reports available today generally demonstrate the ability of probiotic strains to improve the resistance of larvae and early post larvae to diseases, especially those of bacterial origin such as vibriosis (Gomez-Gil *et al.* 2000; Garriques and Arevalo 1995; Pai *et al.* 2010; Panigrahi *et al.* 2011). As presented in Table 11.1, mainly Gammaproteobacteria, *Bacillus* sp. and LAB have been assessed as probiotics for crustacean larvae. The Gammaproteobacteria (*Vibrio* sp.) and *Bacillus* sp. probiotics tested were almost exclusively isolated from the local environment while both autochthonous and allochthonous LAB were considered.

An illustrative example is the previously quoted development of the *V. alginolyticus* probiotic (Ili strain) in Ecuadorian shrimp hatcheries. Recent studies have also evaluated the effect of *Bacillus* on shrimp larvae or early post larvae. Ravi *et al.* (2007) isolated three strains from the marine sediment according to their antagonistic activity against specific *Vibrio* spp. These strains were identified as *Paenibacillus* sp., *Bacillus cereus* and *Paenibacillus polymyxa*. The inoculation of *Paenibacillus* sp. and *B. cereus* at two concentrations (10^4 and 10^5 CFU ml⁻¹) separately in the larval rearing tanks resulted in increased survival rates of *P. monodon* post larvae challenged with pathogenic *V. harveyi* and other *Vibrio* spp. Lactic acid bacteria, and specifically *Lactobacilli*, were less frequently tested but showed improvement in both growth performance and resistance to vibriosis of freshwater and marine shrimp larvae; this effect is often attributed to an antagonism toward *Vibrio* spp. or enhancement of the immune response (Viera *et al.* 2007; Panigrahi *et al.* 2011). It is also observed that the intensity of the effect seemed to be dependent on the LAB species. Indeed Panigrahi *et al.* (2011) reported that *Lactobacillus rhamnosus* and *Ped. acidilactici* strains were both able to improve the survival and growth of *P. monodon* from mysis to PL-25 when administered at 10^5 CFU ml⁻¹; however, only the *Ped. acidilactici* strain was able to significantly protect PL-25 when subjected to a *V. anguillarum* infection. Similar findings were obtained by Viera *et al.* (2007), who reported similar efficiency of two autochthonous LAB strains under ‘unchallenged conditions’, while the *Lb. plantarum* strain was more effective at improving the resistance of *L. vannamei* mysis to a pathogenic *V. harveyi* compared to unidentified LAB species.

The increased resistance of shrimps to vibriosis, or even WSSV, has also been linked to an effect of probiotics on the immune response of the animal. However, very few authors have comprehensively investigated this putative mode of action in crustacean larvae. In the study of Liu *et al.* (2011b), the authors reported the effect of *B. subtilis* E20 on the up-regulation of the expression of two proPO isoform genes (Söderhäll and Cerenius 1998). Interestingly these results were coherent with a previous study from the same authors which showed that the same probiotic was able to improve PO activity and phagocytic activity in *L. vannamei* juveniles;

these results were associated with an improved resistance to a *Vibrio* infection. Whether this effect on the immune response of shrimp was also implicated in the improved tolerance of the probiotic fed post larvae to salinity change (freshwater and 60‰ salt water) and nitrite stress (up to 300 mg l⁻¹ of nitrite N) was however not investigated. Other authors also reported modulation of the immune response in juvenile white shrimp fed a *V. alginolyticus* probiotic during larviculture stages under a WSSV challenge but no clear correlation between immune parameters and viral resistance was apparent (Rodriguez *et al.* 2007).

Beyond the resistance to vibriosis, some studies have also investigated the effect of probiotics on other parameters such as larval development or susceptibility to stress. For instance, Liu *et al.* (2011b) recently evaluated the effect of a specific *B. subtilis* strain (E20), isolated from human food, on the development, tolerance to environmental stress and immune response of white shrimp larvae. Interestingly, the results showed that *B. subtilis* E20, administered in the water culture at a high concentration (10⁶ CFU ml⁻¹), was able not only to improve larval survival but also to accelerate development. This probiotic effect on larval development was also reported in other shrimp species such as *M. rosenbergii* larvae, for which *B. subtilis* treated *Artemia* nauplii accelerated the rate of metamorphosis (Keyzami *et al.* 2007). This effect has often been attributed to an ability of the probiotic strain to improve the nutritional status of shrimps through an increase in digestive enzyme activities such as proteases (Ziaei-Nejad *et al.* 2006; Liu *et al.* 2009; Zhou *et al.* 2009).

Based on these findings and the development of empirical approaches, probiotic application in larval production has quickly emerged in the main shrimp producing countries and is now a commonly applied alternative to antibiotics. Decamp *et al.* (2008) reviewed field data concerning the use of a commercial probiotic preparation containing mixed *Bacillus* spp. strains, based on data from Thai and Brazilian hatcheries, with *P. monodon* and *L. vannamei* respectively. The authors report that when directly administered in the larval tank at 1–5 × 10⁴ CFU ml⁻¹, the *Bacillus* strains, which were selected from over 70 strains of *Bacillus* according to their *in vitro* antagonism towards pathogenic *Vibrio* species, yielded a similar performance as prophylactic antibiotic treatments.

11.4.1.2 Vector of administration and administration cycle

In crustacean larvae and early post larvae, the main way to deliver microbial products is through direct application into the water (Table 11.1). In this case, based on the data available today and on unpublished data (Chim, personal communication), the optimal dosage of probiotics used in crustacean larviculture seems to be between 10⁴ and 10⁶ CFU ml⁻¹.

However, other vectors of administration via inert or live feeds have also been investigated. *Artemia* nauplii are widely used in marine crustacean hatcheries around the world. *Artemia* like other live feed can be a source of pathogenic bacteria in hatchery environments (Avila-Villa *et al.* 2011; Chapter 16) and probiotics have been proposed as a solution to reduce the risks of contamination (Meunpol *et al.* 2003; Vershuere *et al.* 2000a). Changes in bacterial assemblages as well as protection against pathogens have often been measured when probiotics are applied to live feeds including *Artemia* and rotifers (Verschuere *et al.* 1999; Marques *et al.* 2006a; Rojas-Garcia *et al.* 2008; Qi *et al.* 2008; Pintado *et al.* 2010).

Artemia have also been proposed as an efficient way to deliver nutrients, antimicrobial agents, vaccines, or probiotics to fish and crustacean larvae. Several studies have been conducted to assess probiotic encapsulated *A. franciscana* nauplii in shrimp larvae and post larvae

(Gomez-Gil *et al.* 1998; Ziaei-Nejad *et al.* 2006; Immanuel *et al.* 2007). Ziaei-Nejad *et al.* (2006) demonstrated that delivering a *Bacillus* probiotic to shrimp larvae through *Artemia* was more efficient than direct administration in the water in order to increase the probiotic level in the GI tract. Venkat and colleagues (2004) assessed the dietary application of two *Lactobacillus* strains in live and formulated feeds fed to post larval *M. rosenbergii*. It was observed that the growth of the probiotic fed groups was greater than the control group and that encapsulation of *Lactobacillus sporogenes* into live feed produced the greatest increase in weight gain, feed efficiency and protein gain compared to the formulated feed. This may indicate that bioencapsulation of probiotics into live feed vectors would be the optimal administration route during the early life stages of crustaceans.

In the same way, several authors have suggested that probiotic treatments might be more efficient if first applied at the larval stages, particularly before the opening of the mouth. Rodríguez *et al.* (2007) assessed the Ili probiotic strain (*V. alginolyticus*) on *L. vannamei* larvae and post larvae challenged with WSSV and showed that when administered during larviculture, the probiotic confers an enhanced level of protection and a subsequent higher survival rate of shrimps under farm conditions. Similarly, in their study, Ziaei-Nejad *et al.* (2006) examined the effects of a commercial *Bacillus* probiotic on digestive enzyme activity, survival and growth of *F. indicus*, from nauplii to post larval stages, and reported that the improvement in growth parameters was higher when larvae received the probiotic from the zoea stage.

11.4.2 Main benefits in juveniles and sub-adults

11.4.2.1 Overview

In the case of advanced post larvae stages or juveniles and sub-adult crustaceans, experiments are usually run in small scale tanks or in aquaria. A summary of the most relevant scientific studies is presented in Table 11.2.

Studies have assessed probiotic administration via the diet or directly into ponds. In many cases, *in vitro* inhibitory assays toward pathogenic bacteria were generally performed (even if not presented) and *in vivo* infection challenges with pathogens were achieved (Castex 2009). Experimental challenges focused on vibriosis even though the effect of probiotics on the susceptibility to WSSV has now received more attention (Antony *et al.* 2011). An important consideration with challenge trials is the duration of the experiment as studies are usually conducted over a quite short period (few weeks) and long-term effects should be addressed.

As previously mentioned, the data available suggest an optimal probiotic bacteria level in the gut between log 4 and log 6 CFU g⁻¹ in the intestine of penaeid shrimps whatever the probiotic species/strain considered. This information is of particular interest from practical perspectives in order to adjust the probiotic concentration in the feed depending on the feeding regime (Castex *et al.* 2011). More studies are required in order to design adaptive probiotic feeding programs depending on the shrimp species, size and feeding regime. Additionally more attention should in future be paid to the evaluation of a selected probiotic strain(s) under different culture systems, specifically closed recirculating system, as such data are presently lacking.

Finally, to our knowledge, the use of probiotics for broodstock has never been documented in crustaceans but this topic does merit some interest. Indeed, it has been demonstrated that broodstock conditioning has a great influence on reproductive performance and larval quality (Racotta *et al.* 2003). Therefore probiotic effects, especially at the nutritional level, could have

beneficial consequences on reproductive performance of broodstock animals and larval quality, as previously reported with other feed supplements (Paibulkichakul *et al.* 2008).

11.4.2.2 Consideration with large scale experiments

It is interesting that very few published studies have been carried out in commercial farms or under pond conditions. It is obvious that the efficiency of probiotics at a commercial scale will require *in situ* validation, ideally in ponds, to highlight their true value for the farmers. People who have worked on the evaluation of probiotics in ponds know that such work is complex because it has to take into account numerous factors, often highly variable (different pond sizes and configurations, zootechnical practices, date and density of seeding, origin and quality of post larvae etc.). To compare treatments and to determine precisely whether a probiotic has an effect or not, it is thus essential to standardize these variables as much as possible for every pond and ideally to have numerous replicates in order to reach an acceptable statistical power (Chim *et al.* 2009). For instance, in their study, Devaraja *et al.* (2002) used three replicate ponds of 0.5 ha in order to evaluate two commercial bioremediation products, but they did not show significant differences among the treatments in the average production at the end of the culture period, even though one treatment seemed to give better results. The authors finally advised the use of larger sample sizes for further studies to show the potential benefits of using microbial products in commercial aquaculture farms. Indeed, too small a sample size very often leads to difficulties of interpretation, misleading results and inevitably to erroneous conclusions. It is frequent for a farmer to observe large differences in the final survival rate between ponds of the same farm, while no specific treatments were applied. Chim *et al.* (2009) illustrated, for semi-intensive conditions, the within-farm variability among the ponds, and confirmed that the specific characteristics of each pond from the same farm make it difficult to use them as experimental units. These authors proposed the use of floating cages in shrimp culture ponds as an economical, powerful and sensitive experimental tool for shrimp culture studies. Rengpipat *et al.* (2003) and Castex *et al.* (2008) used such experimental designs to assess respectively the effect of *Bacillus* S11 and *Ped. acidilactici* dietary supplementation on *P. monodon* and *L. stylirostris*, respectively. The latter used a sample size of five replicate cages per treatment, repeated in two ponds, and after 3 months there was a significant improvement in survival and FCR, with a statistical power close to 80%.

11.5 CONCLUSION

Probiotics are applied in order to develop and fortify gut microbial communities to promote and sustain the health of the host. The current literature provides a foundation and shows that it is possible to achieve this goal in crustaceans. However, our knowledge of the mechanisms by which probiotics operate in crustaceans requires a great deal of further attention, with a distinct emphasis on the effect of probiotics on the gut microbiota. Moreover, further investigations on the microbial communities in the digestive tract of crustaceans together with a demonstration of their relevance in terms of nutritional contribution and physiological implication (notably immunity) are of utmost importance. The impact of the different rearing techniques of crustaceans on the intestinal microbiota, especially with the development of closed recirculating systems based on microbial floc, must also be investigated. A better understanding of

the principles governing microbial community assembly and maintenance within the intestine of crustaceans is required in order to design the best strategy to manipulate these communities. The development of modern model systems and molecular techniques for the study of host–microbiota interactions will be highly informative in this regard. Such information will be useful in order to optimize practices to manage diseases, but may also reveal new knowledge that could help to address the current challenges in feed formulation with the scarcity of certain raw materials. In addition, even if it is at a very early developmental stage, the use of recombinant probiotics as vehicles for new ‘vaccination’ strategies in crustaceans is also a very interesting approach that will merit further attention.

Probiotics are no longer a ‘concept’ for crustacean aquaculture; the use of probiotics in crustacean farming is in fact becoming an important component of BMPs. Therefore policies and regulations must be designed, and enacted, to regulate these practices and to address safety concerns linked to the use of undocumented autochthonous microorganism strains or artisanal probiotic production.

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12 Can Probiotics Affect Reproductive Processes of Aquatic Animals?

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ABSTRACT

Commercial and ornamental aquaculture production continues to increase but reproductive dysfunctions remain a bottleneck, and a limiting factor, for the production of many fish species. Numerous studies have addressed the efficacy of probiotics in finfish but very few studies have focused on fecundity. However, the results from the limited studies available have demonstrated the positive effect of probiotic administration on freshwater, and marine, teleost reproductive performance. The importance of the results achieved thus far infers a relationship between the gut microbiota and the reproductive processes. This chapter summarizes the present knowledge of this topic in fish.

12.1 INTRODUCTION

The number of aquatic species currently under domestication is rapidly rising, due to the development of both commercial and ornamental aquaculture (Vannucci 2004; Duarte *et al.* 2007). One of the biggest limiting factors in the development of the aquaculture industry is brood-stock management which involves all the appropriate measures to enable a captive group of fish to undergo reproductive maturation, spawning and production of fertilized eggs. Although many cultured fish species achieve successful reproductive performance under cultured conditions there remains a large number of important species which still exhibit reproductive dysfunctions. Among these are freshwater eel (*Anguilla* spp.), Japanese yellowtail and greater amberjack (*Seriola* spp.), some groupers (*Epinephelus* spp.) and bluefin tuna (*Thunnus* spp.) (Ottolenghi *et al.* 2004). These dysfunctions, often due to the combination of captivity-induced stress (Sumpter *et al.* 1994; Pankhurst and Van der Kraak 1997) and the lack of an appropriate natural spawning environment (Zohar 1989a; 1989b; Yaron 1995; Battaglene and Selosse

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1996; Ohta *et al.* 1997), are usually more serious in female broodstocks, and may affect the reproductive system at different levels.

This chapter provides an overview of the fish reproductive system, broodstock reproductive dysfunctions, the existing interplay between reproduction and metabolism, and the effects of probiotics on the reproduction and fecundity of cultured fish.

12.2 THE FISH REPRODUCTIVE SYSTEM

Similar to other vertebrates, the reproductive functions of fish are regulated by the interplay of the three major organs – hypothalamus, pituitary and gonads – which form the so-called hypothalamus–pituitary–gonadal axis (HPG axis) (Peter and Yu 1997; Yu *et al.* 1997). The hypothalamus, integrating internal and external stimuli, releases the gonadotrophin releasing hormone (GnRH) (Zohar *et al.* 2010). Under the influence of the GnRH, the pituitary secretes follicle stimulating hormone (FSH) and luteinizing hormone (LH) which act upon the ovary, regulating oogenesis (Holland *et al.* 2001; Moles *et al.* 2007) (Figure 12.1). During oogenesis, the oocytes grow, differentiate, undergo meiosis and incorporate macromolecules, such as proteins, lipids, sugars, enzymes, vitamins, hormones, growth factors and transcription factors, required to support embryo development (Brooks *et al.* 1997). Oogenesis consists of two distinct stages: (1) the growth stage and (2) the maturation phase. The growth stage, or vitellogenesis, is controlled by the FSH (Figure 12.2). In all oviparous vertebrates, during vitellogenesis, the liver synthesizes and releases vitellogenin (Vtg), a bulky and complex calcium-binding phospho-glycoprotein, under the stimulation of estradiol (E_2) produced by the follicular cells (Wallace and Selman 1990; Carnevali *et al.* 1999; Calp *et al.* 2003; Polzonetti-Magni *et al.* 2004). Once synthesized in the liver, vitellogenin is released into the bloodstream and is sequestered by the growing oocytes via specific receptors (VtgRs)

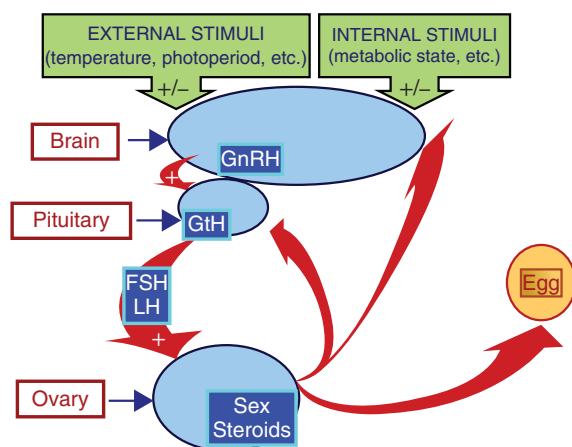


Fig. 12.1 Diagrammatic presentation of the actions of the HPG axis in the control of reproductive function. The hypothalamus, integrating internal and external stimuli, releases the gonadotrophin releasing hormone (GnRH). Under the influence of the GnRH, the pituitary secretes follicle stimulating hormone (FSH) and luteinizing hormone (LH) which act upon the ovary, regulating oogenesis by activating steroidogenesis. For colour detail see Plate 12.

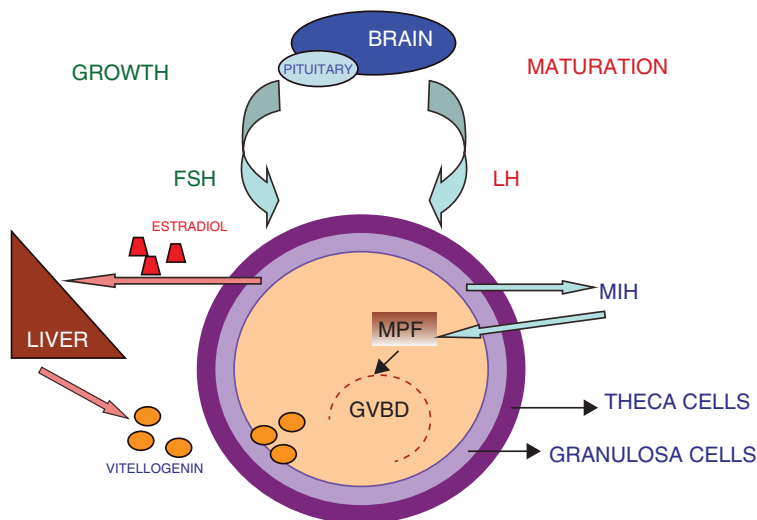


Fig. 12.2 Control of follicle growth and maturation in teleosts. The action of follicle stimulating hormone (FSH) and luteinizing hormone (LH) is depicted in teleost oocytes during growth and maturation phases. A teleost follicle contains an oocyte, one layer of granulosa cells and one layer of theca cells. The basement membrane separating the granulosa and thecal cells and the zona radiata are omitted in the figure. Under the influence of FSH, granulosa cells aromatize testosterone to estradiol, which initiates vitellogenin production in the liver, leading to follicle growth. Under the influence of LH, granulosa cells produce MIH, leading to the oocyte maturation. (Source: The model is based primarily on the work of Clelland and Peng 2009.) For colour detail see Plate 13.

clustered in endocytic clathrin coated pits, giving rise to the formation of Vtg-containing coated vesicles that move into the peripheral oolema (Hiramatsu *et al.* 2006). Vesicles fuse with lysosomes leading to the formation of multivesicular bodies (MVB). The presence of lysosomal enzymes, such as cathepsins, leads to the cleavage of Vtg into the yolk components (lipovitellins, phosvitin, phosvettes and β -components) necessary for embryonic development (Nagahama *et al.* 1995; Okumura *et al.* 2002; Carnevali *et al.* 2006; Le Menn *et al.* 2007).

The maturation phase occurs at the end of vitellogenesis, when the accumulation of mRNAs and the necessary yolk proteins has been completed (Patino *et al.* 2001). Maturation of fish oocytes is a complex phenomenon that is characterized by the resumption of meiosis and is accompanied by several nuclear and cytoplasmic events, including migration of the germinal vesicle towards the micropylar cell followed by germinal vesicle breakdown (GVBD), spindle formation, chromosome condensation and secondary cleavage of yolk proteins to free amino acids (FAAs) which leads to the clearing of the ooplasm as well as to the hydration of the oocyte (Kondo *et al.* 1997; Yamashita 1998; 2000; Le Menn *et al.* 2007; Carnevali *et al.* 2006; 2008; Nagahama and Yamashita 2008). Oocyte maturation in teleosts is stimulated by the release of LH from the pituitary gland (Figure 12.2). In particular, at the ovary level, LH stimulates the production of maturation inducing hormone (MIH) through its receptor (Lhr) (Patino *et al.* 2001). The ability of the follicle cells to produce MIH and the responsiveness of oocytes to MIH represent the acquiring of maturational competence which is a very important stage of oogenesis that signals the transition from the growth phase to the maturation phase. The binding of MIH to its membrane receptors activates the maturation processes (Hanna and Zhu

2009) leading to maturation promoting factor (MPF) formation, by the binding and activation of two subunits, cyclin B and cdc2 (Figure 12.2) (Yoshikuni and Nagahama 1994; Yamashita 1998; 2000; Nagahama and Yamashita 2008).

When maturation is complete the oocyte is expelled from the follicle. The ovulated oocyte (now properly termed egg) becomes fertilizable (Nagahama *et al.* 1994; 1995; Khan and Thomas 1999), traverses the oviducts to the gonopore, and is expelled during the next spawning event (Connaughton and Aida 1999).

12.3 BROODSTOCK REPRODUCTIVE DYSFUNCTIONS

Reproductive problems are usually more serious in female broodstocks and can be classified into three types depending on which phase of the reproductive cycle is affected.

The first and most severe reproductive dysfunction, which is fortunately restricted to a limited number of species, affects the vitellogenic phase. This problem occurs in freshwater eel (*Anguilla* spp.) which fail to undergo vitellogenesis when maintained in captivity. Other fish species with a similar problem include the Mediterranean amberjack (*Seriola dumerili*), a species of great interest for the aquaculture industry (Abellan and Basurco 1999), and the grey mullet (*Mugil cephalus*) (de Monbrison *et al.* 1997).

Another type of reproductive dysfunction affecting cultured females is the lack of final oocyte maturation. At the onset of the spawning season, the post-vitellogenic oocytes fail to undergo maturation and ovulation becoming atretic (Tucker 1994; Berlinsky *et al.* 1997; Larsson *et al.* 1997; Mylonas *et al.* 1997a; 1997b). This is the most common type of reproductive problem encountered in aquaculture and a great deal of research has been focused on this issue (Mylonas *et al.* 2010).

The last type of reproductive dysfunction of female broodstocks is the failure to deliver the ovulated eggs into the water at the end of the reproductive cycle (i.e. failure to spawn). In salmonids, eggs are retained in the abdominal cavity and reabsorbed over the following months (Bromage *et al.* 1992). Females of some marine species, like the striped bass (*Morone* spp.), common dentex (*Dentex dentex*) and white grouper (*Epinephelus aeneus*) may release these eggs at a later time after ovulation (Hassin *et al.* 1997).

For the species presenting these reproductive dysfunctions, the aquaculture industry has dealt with this trouble for many years, nearly exclusively by collecting juveniles or adults directly from the wild, but this conduct is unreliable and unpredictable, and thus inappropriate for industrial scale aquaculture. Additionally, to improve the reliability of spawning, manipulations of various environmental parameters, such as temperature, photoperiod, salinity, tank volume and depth, and substrate vegetation are undertaken (Zohar *et al.* 1989; Munro *et al.* 1990; Yaron 1995). However, in some species such as cyprinids (Kaminski *et al.* 2004), catfish (Wen and Lin 2004) and mullets (Aizen *et al.* 2005), hormonal treatments are used to control reproduction and, over the years, a variety of hormonal approaches have been successfully applied (Mylonas *et al.* 2010).

Finally, more recently an improvement in broodstock nutrition and alimentation has been shown to greatly enhance gamete quality and offspring production, because a large number of dietary nutrients such as proteins, vitamins and fatty acids affect gonadal development and fecundity, particularly in continuous spawners with short vitellogenesis (Izquierdo *et al.* 2001; Watanabe and Vassallo-Agius 2003; Bobe and Labbe 2010).

12.4 REPRODUCTION AND METABOLISM

Broodstock nutrition is without doubt one of the most poorly understood and poorly researched areas of finfish nutrition. To a large extent, this has been due to the necessity of suitable indoor or outdoor culture facilities for maintaining large groups of adult fish and the consequent logistical challenges and higher cost of running and conducting extended broodstock feeding trials. As in other animals, it is also clear that many of the deficiencies and problems encountered during the early rearing phases of newly hatched finfish larvae are directly related to the feeding regime (including the nutrient level and quality) provided to the broodstock. At present, for most cultured fish species, many of the commercially available so-called 'broodstock diets' are just larger sized on-growing diets. In practice, many marine fish hatcheries improve the nutrition of their broodstock by feeding them solely on fresh marine byproducts or in combination with commercial diets.

Despite the lack of specific nutritional understanding in this context, the consequences of nutrition on reproduction are well known and widely reported (Izquierdo *et al.* 2001; Watanabe and Vassallo-Agius 2003; Bobe and Labbe 2010). Nutrition affects all aspects of reproductive events, from pubescence to gametogenesis, in both males and females. The explanation for this close association between nutrition and reproduction is to guarantee that the reproductive event will be intimately aligned with the nutrient supply to assure the survival of new progeny (Scaramuzzi *et al.* 2006). Food restriction itself can seriously affect spawning success. A reduction in feeding rate has been reported to cause an inhibition of gonadal maturation in several fish species, including goldfish (*Carassius auratus*) (Sasayama and Takahashi 1972), European sea bass (*Dicentrarchus labrax*) (Cerdà *et al.* 1994) and male Atlantic salmon (*Salmo salar* L.) (Berglund 1995).

Reduced fecundity, as reported in several marine fish species, could be caused either by the restriction in the availability of biochemical components for egg formation or by the influence of a nutrient imbalance on the brain–pituitary–gonad endocrine system. In fact, in recent years it has been well established that the process of GnRH transcription and secretion is gated by the state of energy reserves in the organism (Hill *et al.* 2008) and is sensitive to different metabolic cues. The neuroendocrine mechanisms responsible for the tight coupling between energy homeostasis and fertility are represented by metabolic hormones and neuropeptides which integrate and interplay at different levels with the HPG axis governing reproduction (Fernandez-Fernandez *et al.* 2006; Castellano *et al.* 2008; Kitahashi *et al.* 2008; Zohar *et al.* 2010). The impact of energy status on the reproductive axis is conveyed through a number of neuropeptide hormones such as KiSS1 and KiSS2, and peripheral metabolic signals, such as leptin, whose nature and mechanisms of action have begun to be deciphered only in recent years in mammals and, to a lesser extent, in fish (Fernandez-Fernandez *et al.* 2006; Castellano *et al.* 2008; Kitahashi *et al.* 2008). In 2003, the understanding of the regulation of reproduction and puberty was revolutionized by the discovery of the KiSS1/GPR54 system (de Roux *et al.* 2003; Seminara *et al.* 2003). It is now confirmed that KiSS1/GPR54 signalling is central to the regulation of GnRH and consequently to LH and FSH secretion, as well as being implicated in a growing list of key biological functions including nutrition, metabolism and response to photoperiodicity (Revel *et al.* 2006; Roa and Tena-Sempere 2007; Carnevali *et al.* 2011). Recently, studies have reported the identification and characterization of a KiSS1 gene from zebrafish *Danio rerio* (van Aerle *et al.* 2008) and medaka *Oryzias latipes* (Kanda *et al.* 2008). Furthermore, recent studies indicate the presence of two genes encoding different

KiSS peptides in zebrafish, medaka and European sea bass (Kitahashi *et al.* 2008; Felip *et al.* 2009), but it seems that the KiSS2 gene was lost in placental mammals. A close association between the GnRH neurons and the kisspeptin fibres present in the median eminence of primates, as well as of fish, has been discovered in the last few years (Parhar *et al.* 2004; Shahab *et al.* 2005).

In addition, the identification of the adipose hormone leptin, which signals the magnitude of energy stores to the hypothalamic centres governing reproduction (Goumenou *et al.* 2003), represented an important step towards understanding the mechanisms controlling this interplay. Leptin, at the central nervous system level, induces reproduction by activating kisspeptin neuropeptide production. Leptin produced by adipose tissue also acts at the pituitary and gonadal levels. In the pituitary, receptors for leptin have been found and a stimulatory action on FSH and LH production has been described (Zieba *et al.* 2005). Concomitantly, leptin may also act at the gonadal level, controlling the gonadal steroidogenesis process (Moschos *et al.* 2002).

12.5 THE EFFECTS OF PROBIOTIC APPLICATIONS ON FISH REPRODUCTION

Despite the exponential growth in the interest in, and use of, probiotics in the fish farming industry, studies concerning their effects on reproduction are scarce. However, a number of recent studies have reported the beneficial effects of a dietary probiotic supplementation on the reproductive performances of freshwater species: guppy (*Poecilia reticulata*, Ghosh *et al.* 2007), Mexican molly (*Poecilia sphenops*, Ghosh *et al.* 2007), green swordtail (*Xiphophorus helleri*, Ghosh *et al.* 2007; Abasali and Mohamad 2011), southern platyfish (*Xiphophorus maculatus*, Ghosh *et al.* 2007; Abasali and Mohamad 2011) and zebrafish (Gioacchini *et al.* 2010a; 2010b; 2010c; 2011; 2012; Giorgini *et al.* 2010). However, such effects have only been investigated in one marine species, the killifish (*Fundulus heteroclitus*, Lombardo *et al.* 2011).

Lipid, protein, fatty acids, vitamins and carotenoids contained in the broodstock diet may influence various reproduction processes such as fecundity, fertilization, hatching and larval development (Izquierdo *et al.* 2001). At the same time, hormones, antibiotics, nutrient mixtures and herbal products usually used as nutrient supplements for broodstock fish might potentially affect microbial abundance and/or activity in the intestinal tract of the breeders (Nayak 2010; Wong and Rawls 2012; Sullam *et al.* 2012; *Chapter 4*). Therefore probiotics could restore and fortify microbial balance and may be used to repair these reproductive deficiencies by increasing the availability of minerals, and vitamins and producing important digestive enzymes or metabolites (Holzapfel *et al.* 1998).

Although the current volume of literature available is limited, the results obtained are so far are encouraging, clearly demonstrating positive effects of probiotic administration on the endocrine and paracrine control of the reproductive processes of female broodstock.

12.5.1 Effect of probiotics on the reproduction of poeciliid livebearing ornamental fish species

Livebearing ornamental fish are amongst the most popular fish with fish hobbyists, because they are brightly coloured, accept a diverse range of food and breed prolifically to produce live free-swimming offspring.

In the first study on this topic, Ghosh and co-workers (2007) demonstrated that probiotic *Bacillus subtilis*, isolated from the intestine of mrigal carp (*Cirrhinus mrigala* Hamilton), improved the reproductive performance of four species of livebearing ornamental fish: guppy, Mexican molly, green swordtail and southern platyfish. The probiont was administered for one year by incorporation in fish feed at four different concentrations (5×10^5 cells g⁻¹, 5×10^6 cells g⁻¹, 5×10^7 cells g⁻¹ and 5×10^8 cells g⁻¹). The results showed that probiotic supplementation significantly improved the gonadosomatic index (GSI: a well-used indicator of ovary development and fecundity) and the fecundity of spawning females in all fish species. In addition, Ghosh and co-workers (2007) observed that both the length and the weight of fry were significantly enhanced by probiotic treatment in all four species. Moreover, the number of dead and deformed fry was reduced in fish fed the probiotic. By analysing the results on the basis of the concentration of probiotics supplemented to the diet, improvements were not dose dependent, with the use of the higher concentration not always leading to significantly improved reproductive performance of the spawners.

In a more recent study, Abasali and Mohamad (2011) evaluated the effects of dietary incorporation of a commercial probiotic mixture, composed of equal portions of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Enterococcus faecium* and *Bifidobacterium thermophilum* (Primalac: Nikandisham Farjad Commerce Corporation, Tehran, Iran), on southern platyfish. After 26 weeks treatment with the probiotic at three different concentrations (0.04%, 0.09% and 0.14% of feed), females showed improved GSI and fecundity. In addition, larvae from treated females showed higher length and elevated survival rate compared to larvae derived from control females. However, weight and deformation rate were not affected by probiotic treatment.

12.5.2 The effect of probiotics on zebrafish reproduction

Due to a number of favourable characteristics, such as its fully sequenced genome, ease of culture, small size and translucent larval stages, the zebrafish is considered an excellent vertebrate model system for studying reproductive genetics and development (Penberthy *et al.* 2002; Keller *et al.* 2006; Shiels *et al.* 2009). Several studies have utilized this model to assess the effect of a commercial *Lactobacillus rhamnosus* probiotic (Synbiotec Srl, Italy) on reproduction. The wide range of results obtained at the molecular, macromolecular and physiological levels provides a strong level of understanding, even if still incomplete, of the possible mechanisms through which probiotics can stimulate reproductive performances.

Gioacchini *et al.* (2010a; 2011) reported that after feeding zebrafish experimental diets containing *Lb. rhamnosus* (at 10^6 CFU g⁻¹) for 10 days, the daily numbers of ovulated eggs rose significantly compared to control levels, starting from the second day of administration. In particular, probiotic treated females reached the maximum daily ovulated egg production levels on day 4 (a twofold increase with respect to control levels), with egg production remaining high during the following days (Gioacchini *et al.* 2010a; 2011). It was also demonstrated that the embryos produced by zebrafish females fed with the *Lb. rhamnosus* IMC 501® showed higher hatching rate and faster embryonic development, characterized by the fact that embryo hatching was brought forward by 4 h compared to the control group (Gioacchini *et al.* 2010a). In probiotic treated females, both real-time PCR and culture based methods revealed alterations in GI tract microbiota, characterized in the gut by high levels of *Lb. rhamnosus*, which was absent in the control females (Gioacchini *et al.* 2011). At the same time, PCR-DGGE analysis revealed that the probiotic was able not only to populate the GI tract but also to modulate the

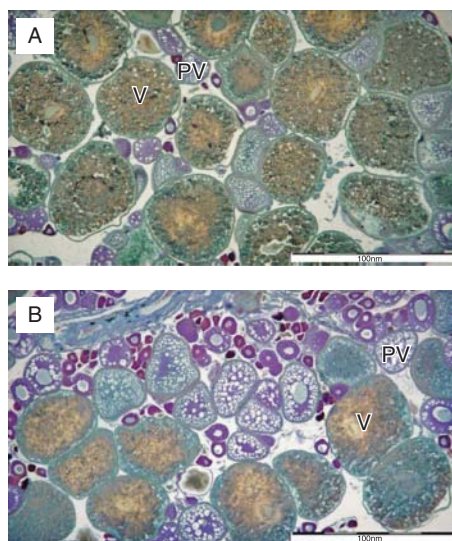


Fig. 12.3 Representative micrographs of ovaries from (A) zebrafish females fed on a *Lb. rhamnosus* supplemented diet and (B) control fed females, revealing an increase in vitellogenic follicles in ovary sections from fish treated with the probiotic. PV = pre-vitellogenic follicles; V = vitellogenic follicles. Scale bars = 100 μ m. [Source: Gioacchini *et al.* 2011.] For colour detail see Plate 14.

microbial communities, causing a clear shift in microbial populations, specifically enhancing the presence of another LAB, *Streptococcus thermophilus* (refer to Figure 8.2 Gioacchini *et al.* 2012).

Regarding the effects of *Lb. rhamnosus* administration on ovary development, the results indicate a positive effect on both growth and maturation phases. The effect of the probiotic on follicle enhancing growth phase was supported by the increase in GSI (Gioacchini *et al.* 2011) along with histological studies evidencing, in ovary sections from fish treated with the probiotic, an increase in vitellogenic follicles (Gioacchini *et al.* 2010a; 2011) (Figure 12.3). Concomitantly, increases were observed in gene expression of cytochrome p 19 (cyp19a), codifying the enzyme responsible for the final conversion of testosterone into E_2 , in the ovary, and of vitellogenin (vtg) and the α isoform of the E_2 receptor (er α) in the liver (Gioacchini *et al.* 2011).

Evidence is also available to demonstrate the stimulating role of *Lb. rhamnosus* on follicle maturation phase. First, *in vitro* maturation assays showed that oocytes isolated from probiotic fed females displayed higher GVBD rates (Gioacchini *et al.* 2010a; 2010b; 2010c). At the molecular level, in the ovary an increase in the transcription of genes codifying for signals which induce the maturation phase (such as lhr, 20 β -hsd, mpr β , cyclin B, activin β A1 and smad2 genes) concomitantly with a reduction in the transcription of genes codifying for local factors which prevent oocyte maturation (tgfb β 1, gdf9 and bmp15) were observed. An SDS–PAGE conducted on zebrafish follicles at different stages of development revealed changes in cytoplasmic proteins induced by probiotic treatment. In particular, major modifications of the electrophoretic pattern were evidenced during maturation, which were also evident

to a lesser extent during vitellogenesis (Giorgini *et al.* 2010). These alterations, due to proteolytic events, controlled by the activity of cathepsins, were in accordance with the significant increase in gene expression and enzymatic activity of cathepsin L (Giorgini *et al.* 2010), which codifies for the enzyme involved in the yolk degradation responsible for FAAs release.

Fourier transform infrared (FT-IR) microspectroscopy has been employed to evaluate macromolecular changes occurring during follicle development (Carnevali *et al.* 2009; Giorgini *et al.* 2010; Gioacchini *et al.* 2012); it is a powerful and well-established technique to study the composition and the macromolecular chemistry of cells and tissues, providing a biochemical fingerprint of the samples under investigation and generating chemical cartograms giving a semi-quantitative evaluation of the distribution of biocomponents (Lasch *et al.* 2002). The identification and correlation of spectral groups (clusters), directly evidenced on the images, can also be achieved by means of multivariate procedures (Tosi *et al.* 2007; Walsh *et al.* 2009). Few reports have utilized spectroscopic studies on fish oocyte

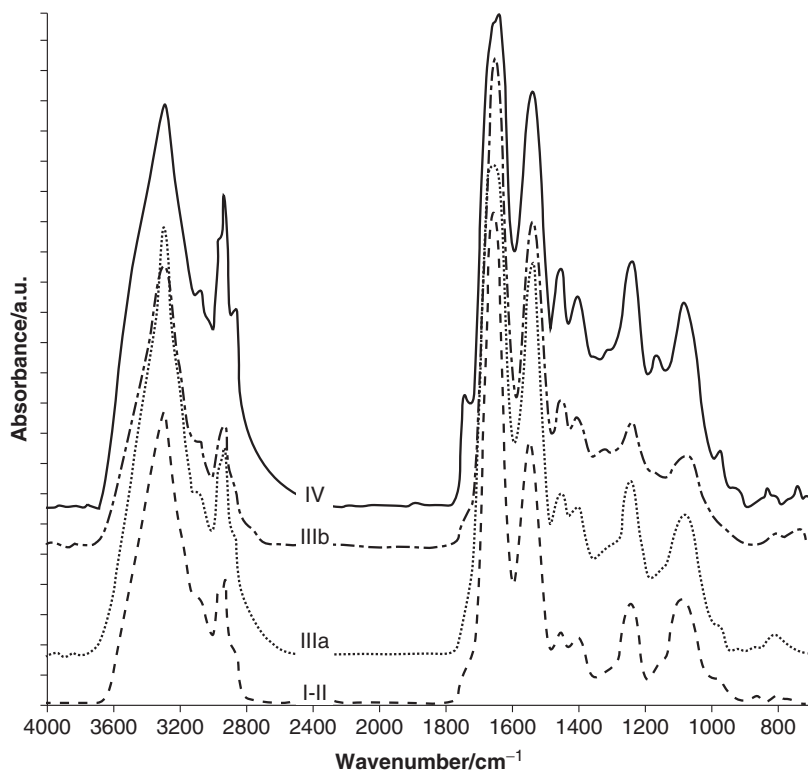


Fig. 12.4 Representative FT-IR spectra in the range 4000–900 cm^{-1} of I–II, IIIa, IIIb and IV class follicles from zebrafish. All spectra were further scaled on amide I band. On going from I–II to IV, some conclusions can be drawn: (i) the vibrational mode at 1737 cm^{-1} relative to phospholipids, found as a shoulder in I–II, becomes evident in III and IV classes; (ii) a small broadening of amide I and II bands indicates changes in protein composition and secondary structure; (iii) an increase in the lipidic chains length is evidenced by the 1452/1392 cm^{-1} intensity band ratio; (iv) the intensity of the bands at 1157 cm^{-1} (relative to carbohydrates) and at 1080 cm^{-1} increases. (Source: Carnevali *et al.* 2009. Reproduced with permission of Elsevier.)

components. Given the unique myriad of biochemical changes associated with oocyte growth, maturation, embryonic development and differentiation, the application of FT-IR can provide a deeper understanding at the single cell level, merging morphological and molecular assays (Singh and Sinclair 2007; Wood *et al.* 2008). Recently, FT-IR analysis of average spectra of zebrafish oocytes at different developmental stages (I-II, IIIa, IIIb and IV) has revealed specific vibrational patterns, correlated with the selective uptake of vitellogenin and with the maturation process (Figure 12.4; Carnevali *et al.* 2009). In particular, ongoing from I-II to IV class oocytes, an increase in the lipidic and glucidic components, an increase in hydration and phosphorylation processes and a modification in protein secondary structures were evidenced by analysing specific vibrational modes (Figure 12.4). In addition, by comparing representative spectra of vitellogenin and lipovitellin with the chemical map acquired on a sample of zebrafish ovary, it was possible to visualize the distribution of these two yolk components (Figure 12.5; Carnevali *et al.* 2009).

This technique was recently utilized with a bidimensional focal plane array (FPA) detector to study the effects of probiotic *Lb. rhamnosus* on zebrafish oogenesis. For all oocyte classes isolated from both probiotic treated and control zebrafish females, total absorbance cartograms and the relative chemical maps were obtained (Figure 12.6; Giorgini *et al.* 2010). The results indicated that the probiotic administration mainly influenced oocytes at the later stages of development, whose representative spectra showed changes in the glucidic and phosphate content as well as changes in protein secondary structures. In addition, earlier initiation of hydration and phosphorylation processes, concomitantly with modifications in protein secondary structures, were registered at the IIIa follicle stage, indicating that *Lb. rhamnosus* acts at this follicle stage affecting the maturation phase (Gioacchini *et al.* 2012).

In a recent study, the effects of *Lb. rhamnosus* on maturational competence acquiring were evidenced, with probiotic administration inducing the responsiveness of incompetent follicles (IIIa) to MIH and their *in vitro* maturation (Gioacchini *et al.* 2012). Very few IIIa follicles isolated from control or probiotic treated females underwent spontaneous maturation after 18 h in the absence of MIH (Table 12.1). However, when exposed to MIH for 18 h a significantly higher GVBD rate was achieved by IIIa follicles isolated from probiotic fed females compared to IIIa follicles isolated from control fed females (Table 12.1). The acquiring of competence of the IIIa follicles was further validated by changes of *lhr*, *mprβ*, *activinβA1*, *tgfb1*, *gdf9* and *oct4* gene expression. From DNA array experiments and from the relative gene ontology (GO) data analysis, numerous prominent and putatively regulated functions and processes were revealed to be regulated by *Lb. rhamnosus* during the follicle development. In class IIIa follicles (maturationally incompetent) the probiotic administration modulated several genes belonging to biological processes (BP) involved in signal transduction and response to chemical stimuli. Interesting also was its effect in regulating transcripts associated with development (regulation of anatomical structure size) and regulation of biological quality. The GO analysis of molecular function (MF) in class IIIa follicles revealed that *Lb. rhamnosus* regulated the expression of two main groups of genes: (1) those involved in the selective interaction of a molecule with one or more specific sites on another molecule, such as heme binding, tetrapyrrole binding, iron ion binding, GTP binding and guanyl ribonucleotide binding; and (2) those involved in oxidoreductase activity and monooxygenase activity. In class IIIb (maturationally competent) follicles both BP and MF analysis highlighted the probiotic regulation of many genes involved in transcription including regulation of transcription DNA-dependent (BP), transcription factor activity (MF), transcription regulator activity (MF) and sequence-specific DNA binding

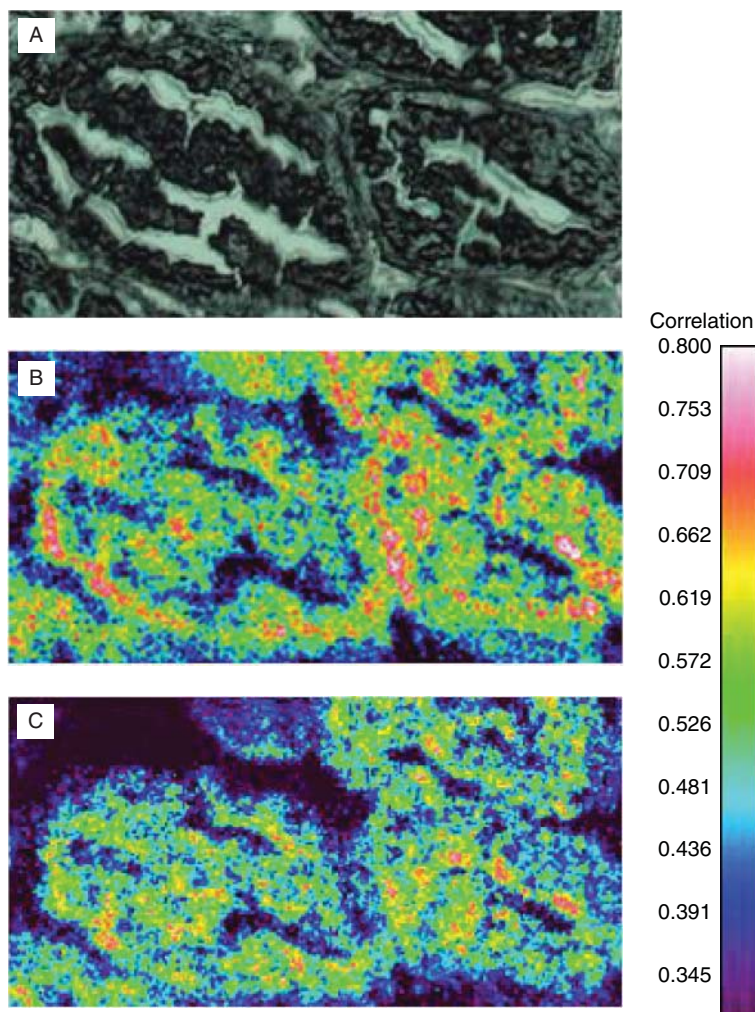


Fig. 12.5 Compare correlation map in the range $1800\text{--}1480\text{ cm}^{-1}$ on an ovary section from zebrafish (A) with (B) VTG and (C) IV representative spectra. The correlation maps obtained suggest that vitellogenin is present mainly in the outer zone of IV class oocytes, with lipovitellin homogeneously distributed in the inner. (Source: Carnevali *et al.* 2009.) For colour detail see Plate 15.

(MF). Moreover, the BP analysis revealed a regulation of transcripts involved in development: developmental process, multicellular organismal development, anatomical structure development and organ morphogenesis.

Finally a very recent paper showed preliminary evidence of the involvement of *Lb. rhamnosus* on autophagy/apoptosis balance modulation during zebrafish follicle development by inhibiting follicular apoptosis and improving follicular survival (Gioacchini *et al.* 2013).

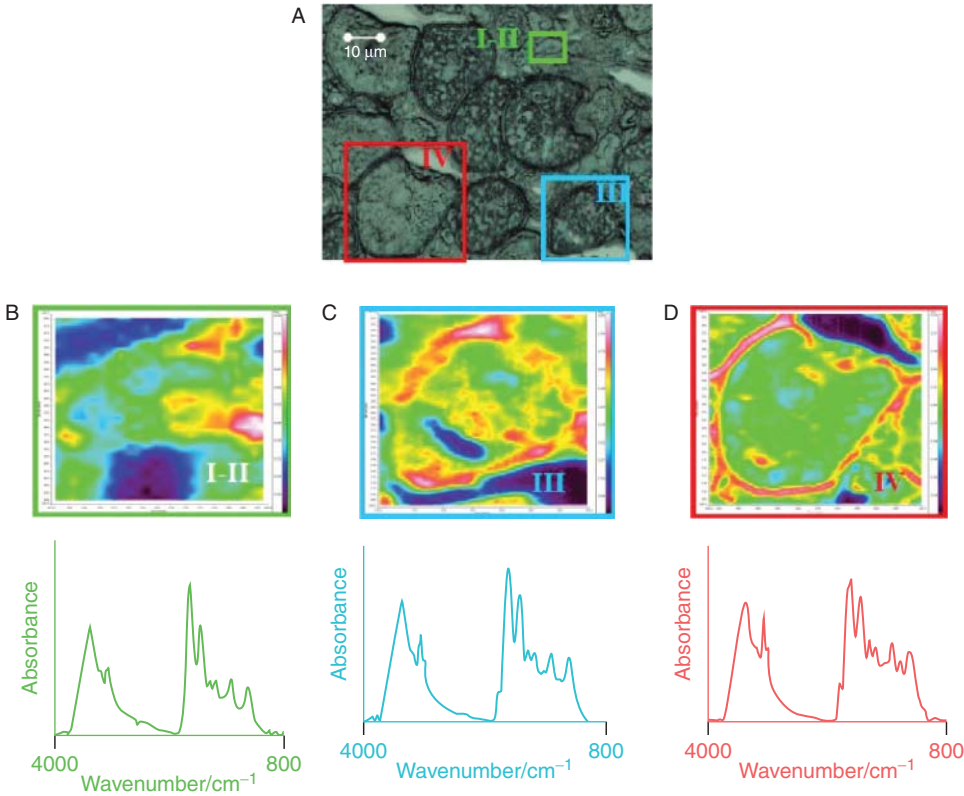


Fig. 12.6 Compare correlation map in the range 1800–1480 cm⁻¹ on an ovary section from zebrafish (A) with representative spectra from (B) class I-II, (C) class III and (D) class IV oocytes. (Source: Carnevali *et al.* 2009.) For colour detail see Plate 16.

Table 12.1 Rate (%) of GVBD of zebrafish IIIa and IIIb follicles isolated from control group ovaries incubated in L15 (CTRL) and L15 + MIH (MIH) (1 µg/ml) and of IIIa and IIIb follicles isolated from probiotic (*Lb. rhamnosus*) group ovaries incubated in L15 (PROBIO) and L15 + MIH (PROBIO+MIH), *n* = 20. Different superscript letters indicate statistically significant differences (*P* < 0.05).

	CTRL	CTRL + MIH	PROBIO	PROBIO + MIH
IIIa	3 ± 1 ^a	20 ± 2 ^b	6 ± 1 ^a	42 ± 3 ^c
IIIb	21 ± 3 ^b	70 ± 4 ^d	25 ± 2 ^b	96 ± 4 ^e

Source: Gioacchini *et al.* 2012.

In fact, by investigating zebrafish preovulatory follicles (stages III and IV) by electron microscopy, an increased number of autophagosomes were observed in follicles from females fed the probiotic. This was confirmed by increased expression of genes involved in the autophagic process (such as *ambra1*, *becn1*, *lc3* and *uvrag*), by increased LC3-II protein levels and by decreased p62 protein levels in preovulatory follicles from females fed the probiotic. Concomitantly a decrease in the apoptotic process in the ovary was indicated from TUNEL assays and confirmed by lower expression of genes involved in apoptosis (i.e. *p53*, *bax*, *apaf* and *cas3*) and higher expression of *igfII* and *igf1r* (Gioacchini *et al.* 2013). During teleost vitellogenesis, apoptosis controls the number of developing oocytes, whereas autophagy provides a source of energy from those oocytes that fail to develop properly (Wood and Van Der Kraak 2003; Santos *et al.* 2008), and therefore the findings following administration of *Lb. rhamnosus* (Gioacchini *et al.* 2013) may infer an elevated efficacy for energy recycling in highly active ovaries to produce an elevated number of mature oocytes and eggs.

12.5.3 Probiotic effects on killifish reproduction

The results obtained by Lombardo and co-workers (2011) represent the only data currently available on the effects of probiotics on the reproduction of marine fish species at this time. These authors administered dietary *Lb. rhamnosus* at 10^6 CFU g⁻¹ to killifish, a euryhaline oviparous teleost that in nature displays semi-lunar spawning cycles synchronized with new and full moon tides (Taylor 1986). Due to its appropriate size and its capacity for successful reproduction under laboratory conditions, this species can be utilized as an excellent fish model for research studying the endocrine control of reproductive cycles in marine teleosts (Shimizu 2003).

After 10 days administration of *Lb. rhamnosus* to killifish breeders, a significantly higher fecundity, in terms of mean numbers of eggs spawned (twofold increase) and a higher GSI (+15%), was observed in females fed the probiotic supplemented diet when compared with females fed the control diet. The hatching rate of killifish occurred at 8 days post fertilization (dpf), which was not affected by *Lb. rhamnosus* administration, but the embryo survival rate at 8 dpf was significantly enhanced by probiotic treatment (Table 12.2). From 10 days post hatching (dph) larvae hatched from the probiotic group showed a significantly higher total length (TL) with respect to the control larvae and the differences remained significant until the end of the experiment (30 dph); indeed, the TL of larvae hatched from the probiotic fed group at 20 dph was the same as the TL of larvae hatched from the control group at 30 dph. Differences in body weight (BW) were significant only at 30 dph. Larval survivorship was not affected by probiotic treatment.

Table 12.2 Reproductive performance of control and probiotic fed groups of *Fundulus heteroclitus*. Mean values per female $\pm$ standard deviation. Different superscript letters indicate statistically significant differences ($P < 0.05$).

	Control	<i>Lb. rhamnosus</i>
Gonadosomatic index (%)	8.0 $\pm$ 1.2 ^a	15.0 $\pm$ 1.5 ^b
Fecundity (number of eggs spawned per day)	107.9 $\pm$ 36.1 ^a	204.3 $\pm$ 49.2 ^b
Embryo survival (at 8 dph)	92.8 $\pm$ 28.0 ^a	181.9 $\pm$ 47.7 ^b
Hatching rate (%)	75.7 $\pm$ 10.8 ^a	79.2 $\pm$ 6.6 ^b

Source: Lombardo *et al.* 2011.

12.6 CONCLUDING REMARKS

It is evident from the available literature that positive effects on fish reproduction can be achieved with dietary probiotic administration, although the mechanisms through which probiotics may enhance reproduction are only partly described. A possible explanation of the effects of *Lb. rhamnosus* on zebrafish reproduction is reported in recent works in which PCR-DGGE analysis revealed that the probiotic was not directly associated with ovaries, excluding its possible locally direct role (Gioacchini *et al.* 2012). In addition, the same authors showed that at both gut and brain levels the probiotic treatment induced a significant increase in leptin gene expression, a key hormone in energy homeostasis and neuroendocrine functions. In the same study, this increase was correlated with a significant rise of KiSS1, KiSS2 and gnrh3 gene expression in the brain. These results could suggest that the probiotic may act indirectly by activating a potent metabolic hormone such as leptin, and that this hormone (alone or with other hormones) may represent the link between metabolic and reproductive systems, through which the probiotic may act.

At present no studies have been conducted on a large scale on marine commercial species affected by reproductive dysfunctions. The results achieved in freshwater species provide a foundation for future studies to progress to experimentation directly conducted in an industrial scale hatchery system with commercially important marine species. Furthermore research should also focus on studying the probiotic effects on male fish reproductive performance and gonadal development.

The significance of the results available today underlines the importance of gut microbes in reproductive processes, supporting the potentiality of feed additives such as probiotics, frequently used in aquaculture practices, as a new technology to improve reproduction.

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13 Issues with Industrial Probiotic Scale-up

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ABSTRACT

Even though more research is necessary to elucidate the exact mechanisms behind the effects of specific probiotics, some promising candidates for applications in aquaculture have been identified and considered for industrial scale-up. In the last two decades aquaculture has embraced the industrial applications of probiotics, and this has truly taken off in some countries in South East Asia, Central and South America and more recently in Europe. However, the technical prerequisites for industrial probiotic scale-up that will be detailed at length in this chapter are overlooked in most cases, resulting in controversies around the actual consistency, efficiency and most importantly the safety of some of the products being used. This therefore highlights a crucial need to review the current probiotic product portfolio, define proper regulations and/or adhere to current regulations, specifically in countries where farmers are currently being presented with a plethora of so-called probiotics or bioremediation products with inconsistent quality and unsubstantiated benefits. The other central question that should be asked is: *how can one go from a promising probiotic candidate identified at laboratory level to a commercial industrial scale application?* This chapter will address these issues and will attempt to present some of the most important steps for optimal industrial scale-up of probiotics for use in aquaculture.

13.1 INTRODUCTION

Probiotics for aquaculture use have been the subject of numerous studies, ranging from the bare fundamentals to more advanced applications. The increasing number of scientific teams working on probiotic applications for aquaculture demonstrates the desire of the scientific community to better understand the gut microbiota of fish and shrimps, the interlink between the microbial make-up and the host, and the best way to steer the fragile balance with the

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aim of improving the general health, well-being and/or performance of aquatic farmed animals. Even though more research is still necessary to better understand the role of the intestinal microbiota in aquatic animal physiology and its underlying mechanisms of actions, some promising candidates for applications in aquaculture have been identified and can be considered for industrial scale-up.

During the last two decades aquaculture has embraced industrial applications, notably of probiotics, and this has truly taken off in some countries in South East Asia as well as in Central and South America. In the European Union (EU), Canada and China, regulatory rules and constraints have greatly impeded the growth of similar uptake as fairly demanding pre-marketing authorization is first required. One could therefore argue that a more thorough knowledge and understanding of the biological and physiological effects of probiotics on aquatic animals could be a prerequisite to enabling the rapid expansion of probiotic applications in aquaculture in these countries. The growing development and application of probiotics in human and terrestrial farmed animal nutrition has helped to elucidate some of the mechanisms of action and thus help demonstrate safety in use of certain strains now widely used in industrial feed applications. These strains are promising candidates for use in aquaculture.

There are several advantages in using probiotics in feed applications:

- First and foremost, at a time of global concern (on human safety grounds) regarding the continued use of antibiotics as growth promoters, probiotics provide ready to use alternatives.
- Second, farmers could become more familiar with the concept of ‘microbial ecosystem management’, which could again lead to a fundamental switch from an anti(biotic) to a pro(biotic) approach generally considered more environmentally friendly and sustainable.
- Third, the use of probiotics would allow farmers to become innovative, accessing newer technologies and/or management tools to tackle certain specific issues for which solutions had not previously been developed and are thus not yet currently available (for example, the use of probiotics to control and guard against deformities in marine fish larvae).

These benefits are nonetheless often overlooked as industrial probiotic scale-up for aquaculture use still poses a number of challenges. These could include the requirement that any probiotics identified for use must be carefully assessed to ensure efficacy as well as optimal protection of the animals, the farmer, the environment and ultimately the consumers. There are also factors such as ensuring maximum and consistent efficiency in use as well as consistency in product quality and composition. These must therefore be carefully considered, and rightly so, by the regulations (pre-marketing authorizations) imposed by certain countries. Unfortunately, regulation and/or better still adhering to regulations has yet to apply fully in the aquaculture sector, where farmers in practice are currently being presented with a plethora of so-called probiotics or bioremediation products with inconsistent quality and unproven beneficial effects. This could therefore call for a crucial need to review the current probiotic product portfolio. The other central question that should be asked is: *how can one go from a promising probiotic candidate identified at laboratory level to a commercial industrial scale application?* We will try to answer some of these questions in this chapter and will certainly attempt to present some of the most important steps for optimal industrial scale-up of probiotics for use in aquaculture.

13.2 SCALING-UP GUIDELINES

In most cases, the putative probiotic candidates reported in scientific publications rarely go on to commercialization and industrial application. This is because isolating and identifying a microorganism/strain as a potential probiotic, following extensive *in vitro* and/or *in vivo* programmes, is only the beginning of the process. The job does not stop there; additional important steps are required for viable industrial scale-up which requires not only substantial financial investment but also contributions from a multidisciplinary team that should include scientists, fermentation engineers and regulatory personnel at the least. Some of these important steps include:

- demonstration of the safety of the potential probiotic
- evaluation of the efficacy at farm level
- optimization of the production at industrial scale, ensuring that it retains the particular properties for the subsequent application
- determination of administration strategies
- pre-market registration.

13.2.1 First step: demonstration of safety

This step is crucial and yet it has been considerably under-evaluated. When developing probiotic strategies for the aquaculture market, one must demonstrate and provide all the scientific information concerning the safety of the strain with regard to the target animal species but also for the environment, the workers handling the product and finally the ultimate consumer. In many countries (USA, EU, Canada, Australia, China etc.), regulations are in place to discourage the use of strains which have not been unequivocally demonstrated as safe. There exist positive lists of microorganisms for which their safety for use in food and feed applications can be robustly assumed. The United States' Food and Drug Administration's (FDA) Generally Recognized as Safe (GRAS) list and the European Food Safety Authority's (EFSA) Qualified Presumption of Safety (QPS) are two examples. For this reason, most of the probiotic strains currently commercially available for feed application use in developed countries are on at least one of these lists. However, when it comes to applications in aquaculture, it would appear that many putative probiotic strains have been isolated from fish and their immediate aquatic environment: this is termed an *autochthonous* approach. These specific microbial strains however may not necessarily share the same historical safety status as their 'traditional' or widely tested counterparts (Adams 1999). With academics and regulators becoming more and more aware of this issue (Wang *et al.* 2008), manufacturers and scientists alike are increasingly looking at considerations of safety in use prior to progressing to investments in product development and commercialization. In fact, many are now prepared to consider working on species isolated from human or agricultural application systems (Gatesoupe 2002a) with possible 'proven records'. Although extensive research has been undertaken on microbial species from the immediate environment of the host, very little attention has been paid to their safety. One of the main reasons for going down the autochthonous strains route relates to the fact that the effectiveness, and the ability to thrive in the host environment, of these species is generally

presumed more efficient (Garriques and Arevalo 1995; Balcázar *et al.* 2007), even though this consideration could be questioned and likely is not always systematically true in the light of some recent findings (Gatesoupe *et al.* 2012).

In 2002, Gatesoupe discussed the advantages of an *allochthonous* approach – in which strains are not isolated from the target species itself or its immediate environment – and concluded that allochthonous probiotics were the best solution in the short term as their use will help to establish the probiotic concept in aquaculture and will generate interest, resources and subsequently a market for the development of next-generation probiotics for aquaculture application. Apart from the fact that some allochthonous species/strains have been shown to demonstrate efficacy in use as probiotics for aquatic organisms, the main reason cited by Gatesoupe (2002a) at the time was linked to the ease of their industrial scale-up. Obviously from a commercial point of view, a compromise should always be found. Probiotics already developed for terrestrial animal species (such as lactic acid bacteria, LAB) are good candidates, as they already offer several advantages (efficiency, adaptable technological properties, optimized industrial productivity, authorization for their use etc.), and have been found, in many cases, to be as efficient for use with aquatic animals as for terrestrial animals (Kozasa 1986; Gatesoupe 2002b; Merrifield *et al.* 2010b; Gatesoupe *et al.* 2012). They should however benefit from comparison with their autochthonous counterparts to maximize potential prior to being validated as potentially useful strains. There are some proven benefits in then using scaled-up allochthonous probiotic strains as positive controls in order to help develop more robustly specific and efficient second-generation probiotics as suggested by Castex (2009) and recently supported by Gatesoupe *et al.* (2012). Interestingly it appears today that the proposition put forward by Gatesoupe (2002a) continues to hold true and should be given serious consideration by entrepreneurs and scientists alike in order to optimize and maximize the development and successful progression to the use of probiotics in aquaculture. At farm level, the empirical use of such microbial strains, isolated from the rearing or source environment and cultivated ‘on site’, appears to be common and is regularly reported to be promising in shrimp hatcheries in China, South East Asia, Madagascar, Mexico and Ecuador, although comprehensive published safety and efficacy data on these are non-existent or very scarce (Garriques and Wyban 1993; Griffith 1995; Garriques and Arevalo 1995; Gomez-Gil *et al.* 2000; Rodriguez *et al.* 2007). For example, the use of probiotics isolated from Ecuadorian shrimp hatcheries and farms has received a lot of interest over recent years and even today seems to be a fairly established means of disease control while sustaining a reasonably high level of production (Cedeño 2007). However, few or no safety data exist as their safety has been ‘assumed’. A survey conducted by the Centro Nacional de Acuicultura e investigaciones Marinas (CENAIM) in the northern part of the Manabi province in Ecuador, which included over 56 farms, showed that 89% were using probiotics with more than 20 products identified, and that in 56.9% of cases the bacterial strains used were of local origin (Cedeño 2007). However the safety of the strains used is not supported by unequivocal and adequate assessment and thus may not provide for sustainable development in the future.

Commercialization of diverse ‘probiotic’ products being sold to aquaculture farmers without associated clear specification regarding the strain (Nimrat and Vuthiphandchai 2011), and for which the safety and the standardization of the production process are also questionable, is a growing problem. It is obvious that, prior to industrial application, more attention should be paid to issues beyond the standard efficacy criteria. First, one must clearly elucidate and identify the strain(s) and then submit them to robust scientific evaluation for their safety in

use prior to their development for wider industrial applications. Specific studies must be conducted and should include assessment of the safety in use in the target animal species, as well as with regards to the safety of the environment and consumers. Having mostly human safety in mind, this initial and essential consideration must demonstrate the absence of toxins and other virulence factors as well as the absence of transferable antibiotic resistance genes. There should also be consideration of the safety of the handler of the product in order that such uses do not elicit allergic or irritative reactions.

Understandably the approach taken in most cases in order to reduce the investment needed to demonstrate the safety of a potential probiotic strain is to develop products based on microbial strains whose safety in use is already well documented and demonstrated (i.e. existing probiotics with a long history of use or as routinely referenced GRAS/QPS species).

13.2.2 Evaluation of the efficacy of the probiotic at the farm level

Only once the proof of safety has been satisfactorily achieved should a probiotic intended for aquaculture use be evaluated at a larger scale, for example on farm. This is particularly important from industrial and commercial perspectives: it enables the validation of technical and other benefits and helps elucidate financial implications with regards to optimization for use at farm level. These trials should also help fine-tune the scaled-up application from a practical point of view (conditions of use, stability under different storage conditions etc.). Field trials must be robust, logical and statistically valid in order prevent inconclusive outcomes and enable better application.

Several studies have been conducted on farms but *en masse* industrial level applications are not frequently reported. It is likely that industrial scale applications are relatively common since entrepreneurs are forced by, for example, regulation and/or their prospective clients to extensively assess their products' efficacy at industrial scale prior to placing them on the market. However, issues such as intellectual property rights limit access to such data by the scientific community. Another limitation to wider access could be the possible impingement from legislative requirements (specifically the EU) demanding that authorizations are first requested prior to trials being undertaken. Commercial companies may therefore not consider applying and thus not carry out the necessary studies.

Finally, with regards to the evaluation of the use of a probiotic in aquaculture, additional criteria need to be recognized. Recent studies in marine fish larvae have demonstrated that the usual efficacy criteria may not always be sufficient to quantify the efficiency of probiotics. It has been suggested that further scrutiny of such effects as the ontogenetic chronology and larval quality properties would also need special attention (Avella *et al.* 2010; Gatesoupe *et al.* 2012; Lamari *et al.* 2012).

13.2.3 Producing quality probiotics is both an art and a science

Once safety and efficacy issues are successfully completed, attention should then be turned to optimization of the industrial production of the probiotic. Progression to field application has been shown to be a real challenge, and among the main issues is the consistency in the quality of the probiotic product. In other words, production of probiotics must be standardized, and more so at industrial scale-up level.

Potential probiotics for aquaculture application produced solely at the small scale laboratory level do not always demonstrate reproducible efficacy at industrial level. For example, LAB produced at laboratory level, then dried and suspended in buffer prior to use, may not necessarily reproduce similar growth and/or viability at industrial level. This basic preparation procedure is far from the required procedure for the industrial scale production. Unfortunately there appear to be very few studies that have paid sufficient attention to the optimization of culture conditions for industrial application (Ochoa-Solano and Olmos-Soto 2006). Probiotics are live (viable) microorganisms and thus differ from chemical substances and must be managed differently. Their efficacy depends not only on the strain of microorganism, but also on its physiological state. The latter is crucial in relation to viability, stability and efficacy/activity at the application stage.

Few studies have compared the effect of a microorganism administered to fish as either viable or killed (non-viable) microorganisms, but it has been demonstrated that viability is an important factor with regards to probiotic efficacy for fish (Brunt and Austin 2005; Panigrahi *et al.* 2005; Taoka *et al.* 2006). Panigrahi *et al.* (2005) concluded that even though the potential benefits of heat-killed cells should not be overlooked, viable forms induced better and possibly reproducible results. Also microorganisms, as is the case with all living organisms, are selective in terms of benefits they could exert and their modes of action; hence not all probiotics are equivalent in the way they act and the ultimate benefit they exert.

A recent study evaluated 12 different commercial probiotics marketed for use in marine shrimp production in Thailand (Nimrat and Vuthiphandchai 2011). Starting from their presentation for sale, only two products provided the necessary information on the label as to the strain names of the live microorganisms or the recommended dose for their use. That notwithstanding, none of the 12 products provided the composition or number of cells of the strains indicated on the label. There were also huge discrepancies with regards to matching the strains in the products to those indicated on the labels (Nimrat and Vuthiphandchai 2011).

The viability of yeast and bacteria depends on the strain's intrinsic and extrinsic properties, although this is often overlooked, and on the quality of the production process, formulation and storage conditions. The production of live bacteria requires expertise and stringent quality controls throughout the process, and only a few companies in the world actually possess the technical knowhow and industrial capability to produce and process the live microorganism in a way that ensures that it is and/or does what it says on the label, and that it is stable with a consistent performance.

If we take the example of bacterial production, several hundred tonnes of products containing hundreds of billions of live bacteria per gram are produced each year. However, production always starts with a few microlitres of the stored cell suspension in a vial at a collection bank. This tiny amount of live bacteria undergoes sequential multiplications in strictly controlled conditions, first in small scale flasks and successively in larger industrial scale fermentors (where the final volume could be as large as 10 cubic metres or more). During this process it is difficult to maintain the strain's integrity without stringent quality control measures at each step. For example, any contaminant or changes in growth conditions could irrevocably affect the bacteria's behaviour and physiology, ranging from changes in the purity of the strain to effects on its activity, viability and efficacy. At each step in the production process, it is imperative that the bacteria's identity, purity and activity are controlled by appropriate testing. These checks ensure that the end-product is safe and efficacious.

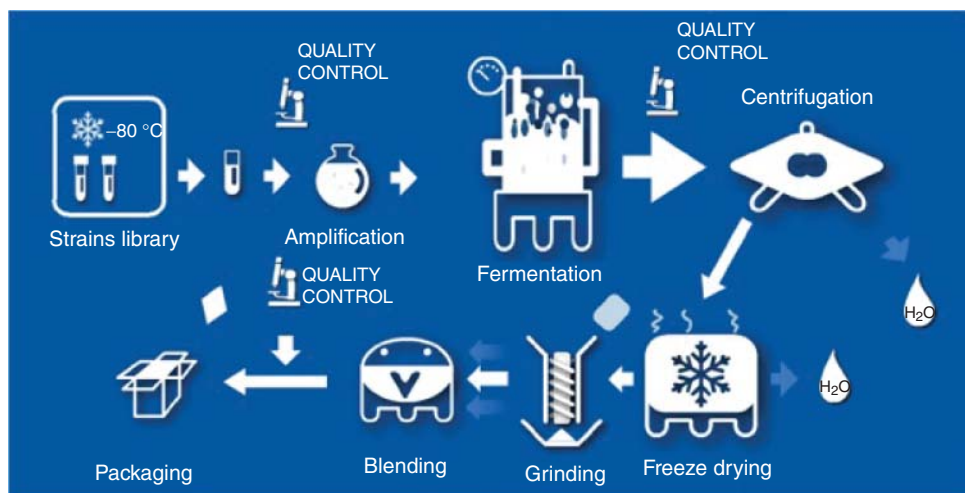


Fig. 13.1 Schematic representation of a probiotic bacteria production process. For colour detail see Plate 17.

One key element to ensure that the strain being grown is strictly identical to the one selected is to ensure consistency with the stock culture. The pure strains, when selected for probiotic use, should be deposited in recognized culture collections which may as part of the contract maintain the pure culture inoculums from which subcultures for industrial scale-up could be produced. It is essential to undertake regular genetic profiling to ensure the purity of the strain, comparing the genetic identity against the deposited strain. As discussed by Wang *et al.* (2008), producers of probiotics for aquaculture use should also consider quick modern molecular techniques to test and ensure the identity of their strains for both quality and safety assurance.

Probiotic production involves unique and complex processes, which require substantial and continuous research and development to fine-tune the fermentation process, the further downstream processing, and the ultimate packaging in terms of conditions and materials. Such parameters should be adapted with regards to the strains concerned. It is also essential that the producer has total control over all of the aspects previously mentioned in order to continue to ensure the safety, composition, purity, stability and quality of the product as well as the traceability and most importantly the efficacy. Some of the pertinent steps that must be considered in producing high quality probiotics, for example bacteria, are illustrated in Figure 13.1.

The process starts with a few microlitres of the probiotic suspension (from cell bank vials) grown in successive scale-ups to eventually larger scale industrial fermentors. This typically starts with the inoculum introduced in a flask containing the appropriate culture medium, under sterile conditions. At their optimal preferred growth conditions, the microorganisms multiply and each batch is successively aseptically transferred to larger containers. The quality and purity of the culture are checked at each stage and the conditions (pH, temperature, pressure etc.) are continuously monitored to ensure optimal growth. Microorganisms have typical growth patterns, but these usually include a lag phase (where the organism is probably adjusting to the growth conditions) then an exponential phase (multiplication) followed by a plateau (the stationary phase) after which there is the death phase. The conditions for optimal

probiotic activity might be related to halting fermentation when the bacteria are at their most active, which does not necessarily equate to maximum yield. Producers who use stationary phase bacteria might maximize production yield but this could be at the expense of quality and viability.

The microorganisms are then usually separated from the culture medium through centrifugation and concentration; around 80–90% of the water is removed, resulting in a concentration of $\times 5$ to $\times 10$. The next step(s) is a further concentration and stabilization process, usually drying. With regards to aquaculture practices, Panigrahi *et al.* (2005) concluded that the freeze drying method merited consideration as an efficient method of probiotic preservation rather than spray drying.

Freeze drying was first used as a method of food preservation some 600 years ago by the Incas. The process was first applied to live bacteria in the 1940s by Institut Rosell in Canada (today part of Lallemand SAS), thus revolutionizing bacterial preservation and industrial applications. Freeze drying involves quickly reaching a very low temperature, with water eliminated by sublimation under low pressure. Given the quick reduction of cell temperature involved, freeze drying is a very delicate process with regards to microorganisms as they are very sensitive to extreme temperatures. The current practice in preserving the viability of bacteria for example is to mix it with a suitable cryo-protectant. As they are live organisms, each strain will behave differently under freeze drying; thus careful consideration should be given to optimizing the cryo-protectant used and the freeze drying parameters (temperature cycle, pressure etc.).

The subsequent treatment of the freeze-dried organism such as grinding, blending and even the final formulation and packing are also crucial steps to monitor. For example the packaging material will affect the subsequent viability and performance of the microorganism; as examples, the live bacteria could be highly sensitive to moisture or oxygen. Packaging materials have varying levels of oxygen and moisture permeability (transmission rates) and this should be considered to meet the needs of the microorganism concerned. There is also a need for stringent control of environmental temperature and humidity during packaging. Storage conditions are also critical with regards to the subsequent probiotic survival, viability and use.

13.3 MODE OF ADMINISTRATION

Another aspect which is vital for consideration in using probiotics in aquaculture is their means of administration (and particularly with regards to the host's digestive tract). Many studies have provided proof of concepts but probiotic administration methods have been shown to vary extensively and are not always practical for fish and shrimp farming. The review undertaken by Merrifield *et al.* (2010a) summarized the most important practical aspects that must be considered and emphasized the need for further research with regards to form of supplementation, vector of administration, dose levels and duration of application; therefore, this chapter will only focus on the vector of administration.

One of the most common routes of administration for probiotics is via incorporation in the feed. For industrial scale application, therefore, the technology associated with incorporation in extruded and pelleted feeds is paramount. In order to exert their effects, probiotics must be viable at their site of action, meaning that the selected microorganism must survive the stressful conditions of feed processing and storage. This is of particular concern in aquaculture since

feed processing conditions are harsher than those for farmed terrestrial animals. One of the main challenges is high temperature, which is seen as a major drawback; this could explain the limited development of in-feed application of probiotics for use in aquaculture and also the disparity in label claims against content as discussed earlier in this chapter.

In order to overcome these issues, novel approaches include for example inducing heat tolerance in the potential microorganism by optimizing the production conditions during the manufacturing process. Altering different parameters such as pH and temperature during the growing phase, application of pre-heat treatment, harvesting at a specific physiological state, growth media characteristics and drying parameters could also assert particular influences that might culminate in the ability of the microorganism to resist heat at a subsequent encounter. In their study of autochthonous and allochthonous probiotics for consideration for use in shrimp feed, Kosin and Rakshit (2010) showed that for several species (including *Lactobacillus plantarum*, *Leuconostoc mesenteroides*, *Bacillus licheniformis* and *Bacillus subtilis*), manipulating the pH of the growth media, the physiological growth phase and the application of heat or osmotic pre-treatment positively induced heat tolerance in these bacteria, when applied on shrimp feed at higher temperatures. For instance, the authors reported that an *Lb. plantarum* strain showed 48% survival after exposure to 60 °C, after storage for 4 weeks at 4–5 °C, when the bacteria were prior heat-treated (50 °C) and exposed to 0.3 M NaCl.

Lallemand SAS (France) and BioMar AS have successfully developed an industrial method for the inclusion of Bactocell® (*Pediococcus acidilactici* CNCM 18/5MA) in commercial fish feeds such as EFICO Vital® and INICIO Plus® (Castex and Aarestrup 2011). This breakthrough followed some in-depth research and development centred on optimizing the production parameters of the probiotic and of the commercial feed. The resulting conditions were optimal for the probiotic's incorporation in extruded feeds with a recovery rate of 96.2% and good stability during storage.

It is important to highlight that some bacteria naturally exhibit a better resistance to heat, specifically spore forming bacteria. Spores are produced in nature by some microorganisms as a means to survive extreme environmental conditions (i.e. UV radiation, extreme heat, chemicals such as solvents, hydrogen peroxide, lysozyme etc.) and to enable longer term survival in conditions that could otherwise kill the vegetative cell (Nicholson *et al.* 2000). Spores can germinate under favourable conditions (appropriate nutrients and water) (Moir 2006) and thus could be ideal candidates for probiotic use in aquaculture. Therefore spore forming bacteria, particularly *Bacillus* species, are often interesting potential probiotics for feed applications: they can be heat stable, conferring fairly good viability after pelleting, and have high resistance to gastric conditions (Casula and Cutting 2002; Hong *et al.* 2005; Cutting 2011). However their survivability during the higher temperature pelletization or extrusion encountered in shrimp feeds is questionable. Suggestions to include spore forming bacteria directly in aquaculture feeds before pelletization and/or extrusion may not be ideal. Temperatures could be as high as 85 to 90 °C during aquafeed pelletization and could even exceed 100 °C in the case of extrusion. Spore forming bacteria will not resist such harsh conditions. Kosin and Rakshit (2010) evaluated two heat tolerant *Bacillus* sp. (*B. licheniformis* and *B. subtilis*) for their viability after spraying onto shrimp feed pellets subjected to 80 °C and 70 °C, respectively. They reported a survival rate (CFU) for *B. licheniformis* and *B. subtilis* of 40% and 30%, respectively – less than that obtained with the heat adapted *Lactobacillus*. In conclusion therefore, where feed application of probiotics in aquaculture is concerned, attention must be paid to the heat tolerance and stability of each potential probiotic as well as their

optimal feed incorporation conditions. European Commission guidelines for the approval of feed additives require applicants to provide evidence of the optimal conditions for the incorporation of the probiotic microorganism in the feed in addition to a demonstration of its stability over at least a 3 month period.

Protecting viable probiotic cells, that is providing a physical barrier against adverse environmental conditions, is receiving considerable attention. One could consider, for example, micro-encapsulation of microbial cells as detailed by Kailasapathy (2002), and patented micro-encapsulated bacteria or yeasts are currently commercially available (Ziggers 2005; US patent 7157258 B2). Recent improvements with the technology in commercially available live yeast applications have led to reports where encapsulated products have been shown to resist temperatures as high as 80–90 °C (Ziggers 2005). Despite this, applications to shrimp feeds or extruded fish feeds are still at an early stage, necessitating approaches where feed manufacturers are looking internally for options involving optimization of their processes and/or the equipment used.

Post-pelleting applications, for example by spraying, are generally alternatives that have shown some promise in terms of homogeneity, stability and conformity at both laboratory and industrial levels (Ziggers 2010; Lallemand unpublished results). Such applications with salmon feeds have shown promising results in terms of probiotic survival, including exposure to harsher pressure, temperature and flow rate, for several months at both laboratory and industrial levels (Ziggers 2005). Significant financial investments are required in order to implement post-pelleting applications; however, given the increased usage of feed additives (not only biotics) in aquafeeds, it appears that the use of drum or vacuum coaters, as currently used in salmonid and/or marine fish feeds in Europe, is here to stay. Nevertheless, for the future of probiotic development, alliance and transversal experience gained from biological technology and industrial processing applications should inform innovative and easy to use solutions for the feed industry.

Administration of probiotics to fish and shrimp via rearing water is also a common practice which has received much attention. From a technological point of view, probiotics developed for such applications must be formulated with certain specific features: (1) good stability in water, (2) float control in the tanks/ponds, and (3) high concentration favouring a low daily dosage to avoid possible contamination by the carrier (when used in excess, certain carriers in probiotic formulations can promote undesirable growth of non-beneficial microorganisms in the tanks/ponds). This entails specifically formulated products. In practice, sophisticated applications via water have been mainly considered for fish and shrimp hatcheries (larval tanks and live prey cultures) for obvious practical reasons, while ‘bio-remediation’ strategies are mainly implicated in live microorganisms for shrimp and freshwater fish ponds.

There are limited data comparing the effectiveness of water based versus feed based administration of probiotics to fish and shrimp (Merrifield *et al.* 2010a). Water application is not a practical way to supply probiotics in open systems such as flow-through and sea/lake cages, except if used for bath treatments, which themselves have clear limitations. The development of recirculating aquaculture systems might of course offer new possibilities; however there could be regulatory constraints and/or welfare issues that might pose enormous challenges. As an example, one of the critical factors with water application is the low level of control over the quantity of probiotics actually ingested by the animal, which may lead to concern about precision with regards to the minimal/maximal dose of use.

13.4 PROBIOTIC REGISTRATION

Probiotics can be used as feed additives/ingredients and/or as drugs, with both requiring pre-marketing authorization prior to their use. With regards to feed for livestock, probiotics are usually required to be marketed and used only after they have undergone pre-marketing assessment based on their quality, composition, safety and efficacy. The same applies to drugs, with of course extensive requirements for additional clinical trials to support efficacy.

In some countries, however, the efficacy of probiotics is not always of such concern as it is widely believed that the market will dictate. This appears to be the position in India and to some extent the US (allowing GRAS microorganisms to be used as direct fed microbials without necessarily any other pre-marketing authorization). However, with regards to most other countries including the EU, Canada, Brazil and Chile, improvement or optimization of certain performance parameters (e.g. feed efficiency, feed digestibility, daily growth rate etc.) encountered in normal animal husbandry production (which may or may not improve welfare) are usually acceptable claims. Health claims including survivability clearly linked to disease conditions are not acceptable for feed additives and must be considered as 'drug-like' and/or 'medicinal' claims. This is not to be confused with using probiotics to optimize normal husbandry conditions because of the quite frequent references to use as 'replacements of antibiotics'.

Probiotics are usually administered to optimize normal animal husbandry, especially prior to attaining undesirable situations that irreversibly degenerate into diseases. Drugs are used for preventive or therapeutic purposes and their registration, in comparison with registration as feed additives, is usually much more prohibitive in costs; thus there are few or no reported probiotic registrations for typical medicinal use purposes.

Finally, one of the main stumbling blocks from an industrial point of view is that potential probiotic species/strains must satisfy stringent regulations. Safety and efficacy considerations are increasingly taken into account when considering the development of probiotics. As an example, the EU regulates the authorization, marketing and use of probiotics as feed additives under the Regulation of the European Parliament and of the Council (Regulation EC no. 1831, the Feed Additive Regulation). In accordance with this Regulation, the Commission, having first consulted the European Food Safety Authority (EFSA), has established rules (Commission Regulation EC no. 429/2008) concerning the preparation and the presentation of applications. The registration of a probiotic requires the preparation of a dossier with data and studies demonstrating the efficacy and safety of the product for the animals, the product handler, the consumer and the environment. These safety concerns are of the utmost importance in aquaculture since, as mentioned by Wang *et al.* (2008), new species and specific strains identified as potential probiotics for aquatic species do not share the historical safety of traditional or widely tested strains such as LAB (Adams 1999).

A long term research and development project, initiated from 2001 through the OFIMER program with BioMar AS and several research institutions, has resulted in the first EU approval of the use of probiotics for salmonids in August 2009, quickly followed by an authorization for use in shrimps. The goal of seeking and obtaining this authorization allowed BioMar to develop an innovative dietary probiotic concept, resulting in the introduction of the first approved industrial trout and salmon feed containing probiotics in 2010 (Castex and Aarestrup 2011). In 2012, the same probiotic product formulated with the strain *P. acidilactici* MA18/5M received another positive scientific opinion from the EFSA Panel on Additives and Products or

Substances used in Animal Feed (FEEDAP) for the extension of use as a feed additive in all fish species (EFSA 2012). This positive opinion results from the examination of a technical dossier containing a documentation of the mode of action of the probiotic strain in the fish gastrointestinal tract, together with zootechnical data showing the efficacy of the probiotic, notably to reduce deformities and improve mineralization in marine fish. Fish deformities represent a major issue for fish hatcheries (especially marine fish hatcheries) and very few solutions are available to the farmers. This achievement is an important milestone for the development of the probiotic concept for aquaculture in Europe.

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14 Prebiotics in Finfish: An Update

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ABSTRACT

Worldwide interest in prebiotics has increased in endothermic animals as well as in fish. Various sectors of the aquaculture industry would benefit if cultured organisms were conferred with improved growth performance, feed efficiency, disease resistance, modulation of the gut microbiota and enhanced immune responses. According to recent reviews, the potential benefits to health and performance in various endothermic animals are documented in numerous studies while the use of prebiotics in aquaculture has been less investigated. However, the studies carried out on prebiotics in fish and shellfish have investigated the following parameters: effect on growth performance, feed conversion, gut microbiota, gut morphology, cell damage in the gut, resistance against pathogenic bacteria and innate immune parameters. This review discusses the results from studies published post 2009–2010. Readers with an interest in probiotic studies published prior to 2009–2010 are referred to review papers previously published. If the use of prebiotics leads to health responses becoming more clearly manifested in fish and shellfish, then prebiotics might have the potential to increase the efficiency and sustainability of aquaculture production. However, gaps of knowledge exist and in order to fully elucidate the beneficial effects of adding prebiotics to fish diets the topic merits further investigations.

14.1 INTRODUCTION

To the authors' knowledge the first study on prebiotics in aquaculture was reported by Hanley *et al.* (1995). Since then numerous papers have been published and the most common prebiotics used in fish are inulin, fructooligosaccharides (FOS), short-chain fructooligosaccharides (scFOS), oligofructose, mannanoligosaccharides (MOS), *trans*-galactooligosaccharides (TOS),

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galactooligosaccharides (GOS), xylooligosaccharides (XOS), arabinoxylooligosaccharides (AXOS), isomaltoligosaccharides (IMO) and various commercial products containing multiple prebiotic combinations. Although the above mentioned plant-derived additives and fibres are not naturally present in fish diets, especially not carnivorous fish, the potential of prebiotics may have interesting applications in aquaculture in order to improve growth performance, survival, feed conversion, digestibility, gastrointestinal (GI) enzyme activities, immune functions and the presence of beneficial gut bacteria as well as the suppression of potentially pathogenic bacteria. In addition, several papers have investigated the effect of prebiotics on GI morphology (Pryor *et al.* 2003; Genç *et al.* 2006; 2007; Yilmaz *et al.* 2007; Barbu *et al.* 2008; Dimitroglou *et al.* 2009; 2010a; 2010b; Ringø *et al.* 2010; Sweetman *et al.* 2010; Dimitroglou *et al.* 2011a; 2011b; 2011c).

The numbers of scientific publications dealing with prebiotics in finfish has increased since the reviews of Burr *et al.* (2005), Gatlin III *et al.* (2006), Denev *et al.* (2009), Yousefian and Amiri (2009), Ganguly and Mukhopadhyay (2010), Merrifield *et al.* (2010), Ringø *et al.* (2010), Sweetman *et al.* (2010) and Dimitroglou *et al.* (2011a) were published. Readers with a special interest in prebiotic studies published prior to 2010 are referred to these reviews and the recent review of Torrecillas *et al.* (2014). The aim of the present review is to summarize the effects of prebiotics recently published, and data from previous investigations not cited in the aforementioned reviews, on the following important fish: salmonids (Salmonidae), cyprinids (Cyprinidae), sturgeons (Acipenseridae), catfishes (Siluriformes), European sea bass (*Dicentrarchus labrax*), gilthead sea bream (*Sparus aurata*), white sea bream (*Diplodus sargus*), hybrid striped bass (*Morone chrysops* × *Morone saxatilis*), Nile tilapia (*Oreochromis niloticus*), red drum (*Sciaenops ocellatus*), Senegalese sole (*Solea senegalensis*), turbot (*Scophthalmus maximus* L., *Psetta maxima* L.), sorubims (*Pseudoplatystoma* sp.) and Poeciliidae. Readers with a special interest in prebiotics in crustaceans are referred to Chapter 15.

The results cited in the present review include work on prebiotics in finfish published in peer-reviewed scientific journals, peer-reviewed open access scientific journals, and books, as well as minimally circulated investigations available as short communications, and abstracts presented in books from international conferences. The latter is done in order to indicate that there are numerous interesting investigations ongoing albeit not yet fully published in scientific journals. The information presented is organized into fish families according to the current recognized taxonomical structure (FishBase 2013).

14.2 SALMONIDAE

Salmonids such as Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss* W.) are amongst the most well documented fish species in respect to prebiotic applications. Indeed, MOS, GOS, FOS, inulin, and commercial products containing multiple prebiotic combinations have been investigated in studies on Atlantic salmon, brook trout (*Salvelinus fontinalis*), rainbow trout and Arctic charr (*Salvelinus alpinus*) (Table 14.1). Readers with an interest in prebiotic applications in salmonids are referred to the review of Merrifield *et al.* (2010) and Ringø *et al.* (2010). Beyond these reviews, some further information is also available.

Table 14.1 Prebiotic application in Salmonidae.

Species	Prebiotic	Dose and length of administration	Results	References
Atlantic salmon	MOS	0 and 4 g kg ⁻¹ – 14 weeks	↑ proximate carcass analysis, liver histology, intestinal histology and sea lice attachment → growth parameters, nutrient digestibility and flesh pigmentation	Dimitroglou <i>et al.</i> (2011b)
	MOS	10 g kg ⁻¹ – 4 months	↓ oxygen consumption ↓ protein and ↑ energy concentration in the whole body	Grisdale-Helland <i>et al.</i> (2008)
	GOS	10 g kg ⁻¹ – 4 months	Nitrogenous and energy losses in the non-faecal nitrogen excretion	Grisdale-Helland <i>et al.</i> (2008)
	FOS	10 g kg ⁻¹ – 4 months	→ feed intake, growth or digestibility	Grisdale-Helland <i>et al.</i> (2008)
	Inulin	75 g kg ⁻¹ – 3 weeks	→ intestinal cell damage ↑ intestinal growth and relative mass of the GI tract → hydrolytic and absorptive capacity → TVC	Refslie <i>et al.</i> (2006)
	Inulin	75 g kg ⁻¹ – 3 weeks	Microbiota – control: ↓ <i>Marinilactibacillus psychrotolerans</i> , <i>Carnobacterium maltaromaticum</i> , <i>Enterococcus faecalis</i> Microbiota – inulin: ↓ <i>Pseudalteromonas</i> , <i>Micrococcus</i> → intestinal cell damage ↑ SGR, survival and lymphocytes ↓ neutrophils, basophils, FCR ↑ growth gain, serum lysozyme and complement activities ↓ FCR ↑ intestinal histology and changes in gut microbiota	Bakke-McKellep <i>et al.</i> (2007)
Brook trout	MOS	2 g kg ⁻¹ – 35 weeks		Sara <i>et al.</i> (2010)
Rainbow trout	MOS	2 g kg ⁻¹ – 90 days		Staykov <i>et al.</i> (2005)
	MOS	2 g kg ⁻¹ – 111 and 58 days		Dimitroglou <i>et al.</i> (2009)
	MOS	2 g kg ⁻¹ – 90 days	↑ growth and survival ↑ antibody titre and lysozyme activity in one trial → bactericidal activity	Staykov <i>et al.</i> (2007)

MOS	1.5, 3 or 4.5 g kg ⁻¹ – 90 days	1.5 g kg ⁻¹ ↑ growth rate 1.5 g and 3 g kg ⁻¹ ↑ intestinal villi → FC, HSI, intestinal morphology ↑ survival of fish after experimental challenge with IHNV → weight gain, FCR, carcass composition or immune gene expression and non-specific immune response ↑ SGR, CF and PER → FCR Carcass composition (protein and lipid), gut microbiota (TVC LAB levels) were affected when feeding 15, 20, 25 and 30 g kg ⁻¹ ↑ lysozyme, IgM, leukocyte levels and resistance against <i>Streptococcus</i> sp. challenge ↑ SGR when the fish were fed the basal diet (not supplemented with inulin) → on FCR, PER, CF and SR ↑ BW → on body chemical composition (moisture, CP, TL and ash) ↓ FCR Affect some gut microbiota → SGR, FCR and survival ↓ serum creatinine, Na ⁺ and ALP Intestinal cell damage ↓ TVC Microbiota – control: <i>Pseudomonas</i> , <i>Psychrobacter glacincola</i> , <i>Carnobacterium divergens</i> , <i>Micrococcus</i> , <i>Staphylococcus</i> , <i>Streptococcus</i> Microbiota – inulin: <i>Bacillus</i> , <i>Carnobacterium maltaromaticum</i> , <i>Staphylococcus</i> , <i>Streptococcus</i> . Different colonization pattern on enterocytes surface	Yilmaz et al. (2007)
GroBiotic®-A	20 g kg ⁻¹ – 9 weeks		Sealy et al. (2007)
GroBiotic®-A	5, 10, 15, 20, 25 and 30 g kg ⁻¹ – 12 weeks		Azari et al. (2011a; 2011b)
Inulin	5 and 20 g kg ⁻¹ – 8 weeks		Sheikholesami et al. (2007)
Inulin	10, 20 and 30 g kg ⁻¹ – 8 weeks		Akrami et al. (2009a)
Inulin and FOS	5 and 10 g kg ⁻¹ – 7 weeks		Ortiz et al. (2012)
FOS	1 g kg ⁻¹ – 105 days		Rehulka et al. (2011)
Arctic charr	Inulin Inulin	150 g kg ⁻¹ – 4 weeks 150 g kg ⁻¹ – 4 weeks	Olsen et al. (2001) Ringø et al. (2006)

Symbols represent an increase (↑), no effect (→) or decrease (↓) in the parameter of the prebiotic relative to the control.

14.2.1 Atlantic salmon

In a recent study, Dimitroglou *et al.* (2011b) evaluated the effect of dietary MOS (4 g kg^{-1}) on sea cage reared Atlantic salmon smolts ($\sim 47 \text{ g}$) fed diets containing high levels of soy protein concentrate, extracted soy protein and sunflower meal in a 14 week experiment. Growth performance parameters were not affected by MOS supplementation; however, body protein composition was significantly increased. Additionally, histological analyses revealed that glycogen deposition in liver tissue increased in the MOS fed fish and the absorptive surface area of the anterior intestine was increased. Scanning electron microscopy revealed that the microvilli density was also significantly higher in the anterior intestine in the MOS fed fish when compared to the control fed fish. Similar results were observed in the posterior intestinal region using transmission electron microscopy, where microvilli length increased from $1.10 \pm 0.18 \text{ }\mu\text{m}$ in the control group to $1.41 \pm 0.19 \text{ }\mu\text{m}$ in the MOS fed fish. Furthermore, the total counts of sea lice (*Lepeophtheirus salmonis*) and salmon lice (*Caligus elongates*) attached to fish, and total numbers of fish infected by lice, were significantly lower in the MOS fed fish. At present it is not fully understood how dietary MOS supplementation can affect lice infection, but it is hypothesized that it may relate to MOS induced mucus secretion on the fish epidermis where a higher volume of mucus or altered mucus quality may prevent or retard lice attachment. In any case, further research is warranted.

14.2.2 Brook trout (*Salvelinus fontinalis*)

To the authors' knowledge only one prebiotic study has been published on brook trout (Sara *et al.* 2010). Although no indication of statistical significance was indicated, from this 35 week study it was reported that dietary inclusion of MOS (2 g kg^{-1}) improved specific growth rate (SGR), feed conversion ratio (FCR) and survival, reduced the percentage of neutrophils and basophils, and increased the percentage of lymphocytes.

14.2.3 Rainbow trout

Staykov *et al.* (2005) reported higher growth gain and improved FCR of rainbow trout ($\sim 30 \text{ g}$) reared in net cages and raceways with the inclusion of dietary MOS (2 g kg^{-1}). In addition, the authors demonstrated increased serum lysozyme and complement activities of fish fed MOS in net cages and raceways.

In a later study on rainbow trout (initial weight $\sim 14 \text{ g}$), Sealy *et al.* (2007) concluded that inclusion of GroBiotic®-A (a mixture of partially autolysed brewer's yeast, dairy ingredient components and dried fermentation products) at 20 g kg^{-1} had no negative impacts on weight gain, FCR, carcass composition, immune gene expression or non-specific immune responses. However, dietary GroBiotic®-A improved survival of fish after experimental challenge with infectious haematopoietic necrosis virus. In contrast to this study, two studies on rainbow trout ($\sim 11 \text{ g}$) by Azari *et al.* (2011a; 2011b) reported that the inclusion of GroBiotic®-A significantly improved SGR, condition factor (CF) and protein efficiency ratio (PER). Furthermore, the carcass protein level was significantly higher and the lipid content was significantly lower in fish fed high levels (15, 20, 25 and 30 g kg^{-1}) of GroBiotic®-A. At the end of the 12 week feeding trial the allochthonous gut microbiota was investigated. The aerobic bacterial levels (g^{-1}) were unaffected but a significant increase in the anaerobic and lactic acid bacteria (LAB) levels were observed. Unfortunately, no further identification was carried out.

The immunomodulatory effects of dietary inulin on rainbow trout were investigated by Sheikholeslami *et al.* (2007). Fish (34 ± 4 g) were fed inulin at two inclusion levels (5 and 20 g kg^{-1}) for 8 weeks. At the end of the trial, immune parameters including serum lysozyme activity, IgM, peripheral leukocyte levels and resistance against *Streptococcus* sp. were measured. Their results confirmed significant increases of lysozyme activity IgM as well as elevated leukocyte levels and resistance against *Streptococcus* sp. in the prebiotic groups. In a later study, Akrami *et al.* (2009a) reported the lowest SGR of juvenile rainbow trout after 8 weeks when the fish were fed inulin supplemented diets (at 10, 20 and 30 g kg^{-1}) compared to those on the basal diet (not supplemented with inulin). Furthermore, the authors reported no significant difference in FCR, feed efficiency (FE), PER, CF and survival.

In a recent study, Rehulka *et al.* (2011) reported results from a 105 day experiment, where the effect of dietary scFOS on rainbow trout (initial weight 240 ± 34.9 g) growth and plasma biochemical parameters was determined. Inclusion of scFOS at 1 g kg^{-1} did not significantly affect SGR, FCR and survival. The results of the biochemical parameters indicated significant differences in creatinine, Na^+ and alkaline phosphatase (ALP, involved in intestinal mucosal defence). Recently, Ortiz *et al.* (2012) evaluated the effects of inulin and FOS on growth performance, body chemical composition and some intestinal bacteria in rainbow trout reared in raceways for 7 weeks. The prebiotic supplementation significantly improved body weight compared with the control while FCR decreased with prebiotics inclusion. However, fillet crude protein content decreased in the prebiotic fed fish ($P = 0.009$). The effect of prebiotics on some culturable gut bacteria isolated from the intestinal contents was also investigated. Neither inulin nor FOS supplementation had any significant effect on *Aeromonas*, *Pseudomonas* and Gram-positive bacterial levels. In contrast *Vibrio* spp. were not detected when trout were fed inulin. As no effort was made to identify the gut bacteria to species level, we recommend that this is included in further studies.

14.3 GADOIDS

To our knowledge the only study evaluating the effect of prebiotics (MOS) on Atlantic cod (*Gadus morhua* L.) was carried out by Lokesh *et al.* (2012). In this study, the expression of inflammatory (IL1 β , IL8 and IFN γ) and anti-inflammatory (IL10) cytokine genes in anterior intestine, posterior intestine and rectum were investigated using qPCR both prior, and post, challenge with *Vibrio* (*Listonella*) *anguillarum*. Compared to the control fed fish, MOS fed fish (1 g kg^{-1} of diet) displayed significantly higher levels of IFN γ mRNA in the anterior intestine prior to challenge and significantly higher IL1 β mRNA levels in the posterior intestine post challenge. Furthermore relative to the pre-challenge levels, MOS fed fish displayed significantly higher expression of IL1 β in the posterior intestine and rectum and IL8 in the rectum, post challenge. This was not the case for the control fed fish. These results indicate that MOS may influence the localized intestinal immune readiness of cod, and the authors concluded that further studies are needed to clarify the mechanisms linked to these pathways.

14.4 ACIPENSERIDAE

With the decline in wild sturgeon populations there has been great interest in farming various sturgeon species for restocking, caviar and meat. Total sturgeon aquaculture production has

more than doubled in the past 5 years, exceeding 50,000 tonnes in 2011 (FAO FIGIS 2013). Several studies have assessed the impact of dietary prebiotics (MOS, AXOS and inulin) on the gut microbial community abundance and/or composition of sturgeons (Pryor *et al.* 2003; Mahious *et al.* 2006a; Rurangwa *et al.* 2008; Delaedt *et al.* 2008); these findings are summarized in Table 14.2 and discussed in the review of Ringø *et al.* (2010). More recently the effect of prebiotics on beluga (*Huso huso*) and Siberian sturgeon (*Acipenser baerii*) growth performance, immune response and GI microbiota has been evaluated in a number of studies (Pryor *et al.* 2003; Mahious *et al.* 2006a; Delaedt *et al.* 2008; Rurangwa *et al.* 2008; Akrami *et al.* 2009b; Mohajer Esterabadi *et al.* 2010; Hoseinifar *et al.* 2011a; 2011b; 2011c; Ta'ati *et al.* 2011a; 2011b; Geraylou *et al.* 2012; Razeghi Mansour *et al.* 2012).

14.4.1 Beluga

Akrami *et al.* (2009b) investigated the effects of inulin supplementation (10, 20 and 30 g kg⁻¹) on beluga juveniles (16.14 ± 0.38 g). Compared to the control fed fish, dietary inulin did not affect growth performance or most of the haematological parameters investigated. Intestinal bacterial analyses of the whole intestines (with contents) were carried out at 0, 4 and 8 weeks of feeding. No significant difference in the level of CFU g⁻¹ was present when using tryptic soy agar medium but significantly lower levels were observed in the intestine of fish fed 3 g inulin kg⁻¹ on nutrient agar plates at week 4. However, this effect was not observed after 8 weeks of feeding. After 4 weeks of feeding, LAB levels were significantly lower in the control group and in the group fed 30 g inulin kg⁻¹ compared to fish fed 20 g inulin kg⁻¹. In contrast, LAB levels after 8 weeks feeding were significantly higher in fish fed 10 g inulin kg⁻¹ compared to control fish and fish fed 30 g inulin kg⁻¹. The authors concluded that inulin is not an appropriate dietary supplement for beluga. However, oligofructose, which is obtained by partial enzymatic hydrolysis of inulin, showed different results on beluga juveniles (Hoseinifar *et al.* 2011a; 2011b). In two 8 week studies with beluga juveniles (18.77 ± 0.76 g), Hoseinifar *et al.* (2011a; 2011b) studied the effect of dietary oligofructose (10, 20 and 30 g kg⁻¹) on growth performance, haematological and serum biochemical parameters and intestinal microbiota. Compared to the control, no significant effects were observed on growth performance. LAB levels in the gut were significantly higher in fish fed 20 g kg⁻¹ and the elevated LAB level was able to persist for at least 1 week after reverting the prebiotic group back to a control diet (Hoseinifar *et al.* 2011b). Haematological assessments revealed no significant effects on blood cell counts, mean corpuscular volume, mean cellular haemoglobin, mean cell haemoglobin concentration or serum lactate dehydrogenase, ALP, alanine aminotransferase and aspartate aminotransferase activities. However 20 g dietary oligofructose kg⁻¹ significantly increased haemoglobin concentration, leukocyte levels and the proportion of lymphocytes and decreased serum cholesterol level (Hoseinifar *et al.* 2011a). The difference between the results of the studies of Hoseinifar *et al.* (2011a; 2011b) and Akrami *et al.* (2009b) can possibly be attributed to the prebiotic types and their degree of polymerization which directly affect fermentation by the intestinal microbiota. However, as this has not been elucidated the topic merits further investigations.

Hoseinifar *et al.* (2011c) evaluated the effects of dietary inactive yeast (*Saccharomyces cerevisiae* var. *ellipsoideus*) product (10 and 20 g kg⁻¹) on intestinal microbiota, haematology, serum biochemical indices and growth performance of beluga juveniles (11.44 ± 0.56 g). After 6 weeks of feeding, beluga fed 20 g *S. cerevisiae* var. *ellipsoideus* kg⁻¹ showed significantly improved final weight, weight gain, SGR and FCR compared to the control treatment.

Table 14.2 Prebiotics in Acipenseridae.

Species	Prebiotic	Dose and length of administration	Results	References
Beluga	Inulin	10, 20 and 30 g kg ⁻¹ – 8 weeks	→ survival, body composition, haematological and serum biochemical parameters and gut microbiota (TVC, LAB levels) ↓ growth performance with increasing prebiotic levels	Akrami <i>et al.</i> (2009b)
	Oligofructose	10, 20 and 30 g kg ⁻¹ – 8 weeks	↑ leukocyte count, percentage lymphocytes and levels of gut LAB when fed 10 and 20 g inulin kg ⁻¹ → growth performance, body composition, gut microbiota (TVC), serum biochemical parameters, enzymes and haematological parameters ↓ growth performance when fed 30 g kg ⁻¹	Hoseinifar <i>et al.</i> (2011a; 2011b)
	Inactive yeast (<i>Saccharomyces cerevisiae</i> var. <i>ellipsoideus</i>) Immunoster and ImmunoWall	10 and 20 g kg ⁻¹ – 8 weeks 10 and 30 g kg ⁻¹ – 8 weeks	↑ growth performance and intestinal LAB levels → survival, haematological and serum biochemical parameters and autochthonous gut microbiota (TVC) ↑ growth performance and haematological parameters	Hoseinifar <i>et al.</i> (2011c) Ta'ati <i>et al.</i> (2011a; 2011b)
	MOS	2 and 4 g kg ⁻¹ – 46 days	→ carcass protein content, innate immune parameters (lysozyme activity and IgM) ↑ carcass lipid levels	Razeghi Mansour <i>et al.</i> (2012)
	Immunogen	5, 10, 20 and 40 g kg ⁻¹ – 8 weeks	→ growth performance, survival rate, haematological parameters and intestinal LAB levels ↑ growth performance and CF (5 and 10 g kg ⁻¹ – inclusion level) → survival rate	Mohajer Estarabadi <i>et al.</i> (2010)

(continued overleaf)

Table 14.2 (continued)

Species	Prebiotic	Dose and length of administration	Results	References
Siberian sturgeon	AXOS	0.2 g AXOS-3:0.25 kg ⁻¹ and 2 g AXOS-32:0.30 kg ⁻¹	↑ ACH50, PO and SCFAs (AXOS-32:0.30) → weight gain, SGR, FCR, lysozyme and SO anions Inclusion of AXOS modulate the allochthonous hindgut microbiota → growth → acetate, propionate and total SCFA production → butyrate production Microbial community in the hindgut was affected → total SCFA and lactate ↓ butyrate	Geraylou <i>et al.</i> (2012)
	AXOS	10 and 20 g kg ⁻¹ – 10 weeks		Rurangwa <i>et al.</i> (2008)
	AXOS Inulin	10 g kg ⁻¹ – 18 weeks 20 g kg ⁻¹ – 1 month		Delaedt <i>et al.</i> (2008) Mahious <i>et al.</i> (2006a)
Gulf sturgeon (<i>Acipenser oxyrinchus desotoi</i>)	MOS	3 g kg ⁻¹ – 5 weeks	↑ gas production → growth performance, FCR, and gross gastrointestinal morphology	Pryor <i>et al.</i> (2003)

Symbols represent an increase (↑), no effect (→) or decrease (↓) in the parameter of the prebiotic relative to the control.

However the survival rate, haematological and serum biochemical parameters were not significantly affected by dietary yeast. Although no substantial effects were observed on the total autochthonous intestinal heterotrophic bacterial levels, the autochthonous LAB levels were significantly elevated in beluga fed 20 g yeast kg⁻¹.

Feeding 10 and 30 g kg⁻¹ of Immunoster™, a commercial prebiotic containing MOS and β -glucans derived from the cell wall of a single source of brewer's yeast, or ImmunoWall, a prebiotic mixture of polysaccharides derived from the cell wall of *S. cerevisiae*, to beluga juveniles (ca. 95 g) in two 8 week trials, Ta'ati *et al.* (2011a; 2011b) showed that these prebiotic products significantly increased growth performance. Although some haematological parameters were affected (e.g. MCV, MCH, lymphocytes and eosinophils), no significant effects were observed in the immune parameters investigated (IgM and serum lysozyme) (Ta'ati *et al.* 2011a).

Razaghi Mansour *et al.* (2012) reported results from a study evaluating dietary inclusion of MOS at 2 and 4 g kg⁻¹ on growth performance, haematological parameters and intestinal LAB levels of beluga juveniles (46.89 $\pm$ 2.57 g). The results of this study showed that MOS did not influence growth performance, haematological parameters or LAB levels in the gut.

Mohajer Esterabadi *et al.* (2010) tested four inclusion levels (5, 10, 20 and 40 g kg⁻¹) of Immunogen®, a commercial prebiotic containing 30% glucans and 18% MOS, on growth performance on beluga in an 8 week experiment. The authors reported significant improvement in SGR, length and CF in fish fed diets containing 0.5 and 1% Immunogen compared to the control fish.

14.4.2 Siberian sturgeon

Recently, Geraylou *et al.* (2012) tested AXOS (AXOS-3-0.25, low degree of polymerization; and AXOS-32-0.30, high degree of polymerization) on juvenile Siberian sturgeon growth performance, immune responses and allochthonous hindgut microbiota. Both supplementations enhanced the phagocytic activity of macrophages, while the alternative haemolytic complement activity and total serum peroxidase content improved only in fish fed AXOS-32-0.30. Neither AXOS preparation affected growth performance, serum lysozyme activity and superoxide dismutase. Inclusion of AXOS however modulated the allochthonous hindgut microbiota: for example, *Clostridium colicanis*, *Clostridium baratii*, *Lactobacillus aviaries*, *Lactococcus raffinolactis*, *Lactococcus lactis* and *Eubacterium budayi* were uniquely present in fish fed AXOS-32-0.30. The production of short chain fatty acids, the end products of microbial fermentation, were significantly higher in fish fed AXOS-32-0.30 than in AXOS-3-0.25 and the control group. Based on their results the authors concluded that AXOS improved sturgeon health through prebiotic actions, but the induced effect is dependent on the degree of polymerization.

14.5 CYPRINIDAE

Carp represent the largest group of cultured fish globally. In 2011, total carp production exceeded 23 million tonnes with production predominantly in China, India and to a lesser degree Eastern Europe. As such, prebiotic investigations have been undertaken with common carp (*Cyprinus carpio*), koi carp (*Cyprinus carpio koi*), crucian carp (*Carassius auratus gibelio*), gibel carp (*Carassius auratus*) and Indian carp (*Catla catla*) as well as a number of other cyprinids: Caspian roach (*Rutilus rutilus*), kutum (*Rutilus frisii kutum*), goldfish

(*Carassius auratus auratus*), golden shiner (*Notemigonus crysoleucas*), fathead minnow (*Pimephales promelas*) and blunt snout bream (*Megalobrama amblycephala*) (Ringø *et al.* 2010; Table 14.3).

14.5.1 Common carp

Staykov *et al.* (2005) reported higher weight gain when common carp (initial ~140 g) were fed dietary MOS (2 g kg⁻¹) for 60 days. In addition the authors reported increased serum lysozyme and complement activities of fish fed prebiotic with highest activities after 30 days of feeding. Ebrahimi *et al.* (2011) investigated the effects of Immunogen (inclusion levels: 0.5, 1, 1.5 and 2.5 g kg⁻¹) on common carp fingerling (11.12 ± 0.55 g) growth performance, carcass composition and resistance against *Aeromonas hydrophila*. Although weight gain was not affected, improved FCR and PER were observed in the 1.5 g Immunogen kg⁻¹ treatment. Inclusion of Immunogen at 2.5 g kg⁻¹ significantly increased carcass protein content, haematological parameters and plasma total protein concentration. At the end of the experiments, intestinal samples (entire intestine including contents) were tested for total culturable bacterial counts. The bacterial levels were significantly higher in fish fed the control diet compared to the prebiotic treatments, and the lowest level was recorded when fish were fed 2.5 g Immunogen kg⁻¹. In the challenge test dietary Immunogen significantly elevated resistance of carp fingerling against *A. hydrophila*. Mazurkiewicz *et al.* (2008) investigated the effect of Fermacto, a commercial feed supplement based upon primary fermentation (*Aspergillus*), in common carp fry, and observed that inclusion of 3 g Fermacto kg⁻¹ had a positive effect on SGR, FCR and PER, while lower inclusion levels of 1 and 2 g kg⁻¹ did not have a similar effect. In a study with juvenile carp, the effects of MOS (1, 2 and 3 g kg⁻¹) were investigated on growth, survival, body composition and some haematological parameters (Akrami *et al.* 2012a). Inclusion of 1 g MOS kg⁻¹ significantly enhanced haematocrit and lymphocyte levels, while total leukocyte levels, erythrocyte levels, haemoglobin concentration and eosinophil levels were not significantly affected. Furthermore, growth performance and carcass composition were improved, but were not significantly different compared to the other treatment groups.

14.5.2 Crucian carp

Little information is available with regards to prebiotics in crucian carp and to the authors' knowledge only three studies have been carried out (Xu *et al.* 2009; Zhou *et al.* 2009a; Liu *et al.* 2013). Xu *et al.* (2009) investigated the effects of XOS on growth performance and digestive enzymes activity of allogynogenetic crucian carp (*Carassius auratus gibelio*) (16.88–17.56 g). Three levels of XOS were included in the diets; 50 mg kg⁻¹, 100 mg kg⁻¹ and 200 mg kg⁻¹, respectively. The results revealed that dietary XOS inclusions significantly increased growth performance as well as digestive enzyme (protease and amylase) activities. However survival rate was not affected. In the study of Zhou and co-authors, fish (initial weight ~40 g) were fed MOS (1 and 2 g kg⁻¹) in combination with Chinese traditional medicines. Consequently growth performance and serum lysozyme and superoxide dismutase activities were increased by dietary supplementation. Furthermore, inclusion of 2 g oligosaccharides kg⁻¹ improved resistance of crucian carp against *A. hydrophila*. In a more recent study, Liu *et al.* (2013) evaluated the effect of MOS on physiological responses, HSP70 gene expression and resistance to *A. hydrophila* infection in allogynogenetic crucian carp. Supplementation of MOS (240 and 480 mg kg⁻¹) significantly increased serum ALP and serum globulin concentration prior to

Table 14.3 Prebiotics applications in Cyprinidae.

Species	Prebiotic	Dose and length of administration	Results	References
Caspian roach (<i>Rutilus gibelio</i>)	FOS	10, 20 and 30 g kg ⁻¹ – 8 weeks	↑ growth performance, digestive enzymes activity (amylase, lipase, protease), innate immune parameters (lysozyme, Ig, ACH50) and resistance against salinity stress test → carcass composition → growth performance, FCR, survival and salinity stress test	Soleimani <i>et al.</i> (2012)
Kutum (<i>Rutilus frisii kutum</i>)	MOS	1.5, 3 and 4.5 g kg ⁻¹ – 8 weeks		Akrami <i>et al.</i> (2010)
Common carp (<i>Cyprinus carpio</i>)	GraBiotic®A	10, 20 and 30 g kg ⁻¹ – 15 days	↑ total protein, albumin, creatinine, C3, C4 and IgM → growth performance and survival	Yousefian <i>et al.</i> (2012)
	MOS	2 g kg ⁻¹ – 60 days	↑ growth gain, serum lysozyme and complement activities	Staykov <i>et al.</i> (2005)
	MOS	1, 2 and 3 g kg ⁻¹ – 45 days	↑ of haematocrit and lymphocyte – 1 g MOS kg ⁻¹ → growth performance and carcass composition	Akrami <i>et al.</i> (2012a)
Koi carp (<i>Cyprinus carpio koi</i>)	Immunogen	0.5, 1, 1.5 and 2.5 g kg ⁻¹ – 8 weeks	↑ FE, PER, carcass protein levels, haematological, serum total protein and resistance against <i>A. hydrophila</i> → weight gain	Ebrahimi <i>et al.</i> (2011)
	Fermacto	1, 2 and 3 g kg ⁻¹ – 50 days	↓ gut microbiota (TVC) ↑ SGR and PER ↓ FCR	Mazurkiewicz <i>et al.</i> (2008)
	COS	2 g kg ⁻¹ – 8 weeks	↑ SGR, total leukocyte counts, respiratory burst, phagocytic capacity, lysozyme activity, SOD and resistance against <i>A. veronii</i> ↓ FCR	Lin <i>et al.</i> (2012)
	Dairy-yeast prebiotic	30 g kg ⁻¹ – 10 weeks	↑ weight gain and FE in the presence of phytoplankton and zooplankton → proximal or distal gut microbiota and disease resistance against <i>A. hydrophila</i> → microbiota (digesta) ↓ ADC of protein, lipid and carbohydrate, lowest values were in fish fed MOS	Savolainen and Gatlin III (2009) Raggi and Gatlin III (2012)

(continued overleaf)

Table 14.3 (continued)

Species	Prebiotic	Dose and length of administration	Results	References
Crucian carp (<i>Carassius auratus gibelio</i>)	XOS	50, 100 and 150 mg kg ⁻¹ – 45 days	↑ growth performance and digestive enzymes activities → survival rate	Xu <i>et al.</i> (2009)
	MOS	60, 120, 240 and 480 mg kg ⁻¹ – 10 weeks	↑ serum ALP, serum globulin concentration (240 and 480 mg MOS kg ⁻¹ ; prior to infection) ↑ serum T3, T4, globulin, ALP, SOD, HSP70 gene expres- sion and disease resistance against <i>A. hydrophila</i> (240 and 480 mg MOS kg ⁻¹) ↓ serum globulin concentration 2 days after infection (240 and 480 mg MOS kg ⁻¹)	Liu <i>et al.</i> (2013)
Gibel carp (<i>Carassius auratus</i>)	MOS and Chinese medicines	1 and 2 g kg ⁻¹ – 56 days	↑ growth performance, serum lysozyme and SOD activi- ties. Inclusion of 2 g kg ⁻¹ MOS improve resistance against <i>A. hydrophila</i>	Zhou <i>et al.</i> (2009a)
	XOS	0.15, 2.1 and 3.2 g kg ⁻¹ – 45 days	↑ growth → survival ↑ enzymatic activity	Xu <i>et al.</i> (2009)
	Saccharoculture	2 and 3 g kg ⁻¹ – 56 days	↑ SGR ↓ FCR Modulate the autochthonous gut microbiota → non-specific immunity factors	He <i>et al.</i> (2011)
	MOS	1.5, 3 and 4.5 g kg ⁻¹ – 8 weeks	↑ immune responses (lg, lysozyme activity and ACH50) 4.5 g MOS kg ⁻¹ → growth performance	Akrami <i>et al.</i> (2012b)
Indian carp (<i>Labeo rohita</i>)	MOS	1, 2 and 4 g kg ⁻¹ – 60 days + 7 days (challenge)	↑ growth, blood and serum parameters and resistance against <i>A. hydrophila</i> (1 g MOS kg ⁻¹)	Andrews <i>et al.</i> (2009)
Fathead minnow (<i>Pimephales promelas</i>)	GroBiotic®A	20 g kg ⁻¹ – 8 weeks	↑ FC and <i>Acinetobacter</i> sp. ↓ whole-body Na indepen- dently of water hardness	Lochmann <i>et al.</i> (2011)

Golden shiner (<i>Notemigonus crysoleucas</i>)	Dairy-yeast product	20 g kg ⁻¹ – indoor (14 weeks); outdoor (10 weeks)	Indoor trial: ↑ growth ↓ FCR → whole body composition Outdoor trial: No clear effect ↑ resistance against <i>F. columnare</i> was noticed in stressed fish → individual weight gain, FC, SR, haemoglobin, lysozyme and alternative complement activity → survival ↑ resistance against <i>F. columnare</i> ↑ resistance against <i>F. columnare</i>	Lochmann <i>et al.</i> (2010a)
	Dairy-yeast product	20 g kg ⁻¹ – 7 weeks + 2 weeks (challenge)		Lochmann <i>et al.</i> (2010b)
	GroBiotic®-A	20 g kg ⁻¹ – 10 weeks and thereafter exposed to <i>F. columnare</i>		Sink and Lochmann (2008)
	GroBiotic®-A	20 g kg ⁻¹ – 16 weeks and thereafter exposed to <i>F. columnare</i>		Sink <i>et al.</i> (2007)
Grass carp (<i>Ctenopharyngodon idellus</i>)	Inulin	IP 10 mg kg ⁻¹ body weight – 2 weeks	→ susceptibility against <i>A. hydrophila</i> and <i>E. tarda</i>	Wang and Wang (1997)
Blunt snout bream (<i>Megalobrama amblycephala</i>)	FOS	0.5, 1, 2, 4 and 8 g kg ⁻¹ – 8 weeks	↑ SGR and survival [4 and 8 g MOS kg ⁻¹], crude lipid (8 g MOS kg ⁻¹), activities of amylase [4 and 8 g MOS kg ⁻¹] and protease (1–8 g MOS kg ⁻¹), microvilli length in mid intestine (all MOS levels) ↓ FCR, moisture [4 g MOS kg ⁻¹], lipase activity	Wu <i>et al.</i> (2013)

Symbols represent an increase (↑), no effect (→) or decrease (↓) in the parameter of the prebiotic relative to the control.

infection. Furthermore, survival was significantly improved when the fish were fed doses of 240 and 480 mg MOS kg⁻¹ compared to the control group.

14.5.3 Gibel carp

Gibel carp is an important fish species in China, due to its rapid growth and excellent taste (Xue and Cui 2001). Recently, He *et al.* (2011) reported that Saccharoculture, a commercial fermented yeast product with a high MOS content, improved SGR, FCR and the autochthonous gut microbiota of gibel carp. The non-specific immune parameters assessed (serum lysozyme activity and complement activity (C3 and C4)) were not significantly affected, however. On the contrary, the application of a more refined MOS product has been reported to improve gibel carp immune responses (Ig levels, lysozyme and ACH50 activities) when fed at 4.5 g MOS kg⁻¹ (Akrami *et al.* 2012b). However, no significant differences were observed in growth or feeding parameters.

14.5.4 Indian carp

The effects of MOS on the Indian carp growth performance, haematological parameters and resistance to *A. hydrophila* were investigated by Andrews *et al.* (2009). SGR was significantly improved and the highest leukocyte and erythrocyte levels, haemoglobin and respiratory burst activity, and serum protein parameters (total protein, globulin, albumin and albumin/globulin ratio) were observed in the MOS fed fish. Furthermore, inclusion of 1 g MOS kg⁻¹ improved resistance to *A. hydrophila*. However, the positive effects present at the lower MOS inclusion level (1 g kg⁻¹) were not observed at the higher inclusion level (4 g kg⁻¹).

14.5.5 Koi carp

Chitosan oligosaccharides (COS) are made by chemical and enzymatic hydrolysis of chitosan. Recently, Lin *et al.* (2012) conducted an 8 week feeding study with koi fed COS to evaluate the effect on the growth, immunity and disease resistance against *Aeromonas veronii*. Inclusion of 2 g COS kg⁻¹ significantly improved SGR, total leukocyte counts, respiratory burst, lysozyme and superoxide dismutase activities, and increased protection against infection in the challenge experiment. Based on their results, the authors suggested that information on the cellular and molecular mechanisms merits further evaluation.

14.5.6 Goldfish

Goldfish, one of the earliest domesticated fish species, is one of the most popular ornamental species globally. Savolainen and Gatlin III (2009) evaluated supplementation of GroBiotic®-A (20 g kg⁻¹) by dry weight in the diet of juvenile goldfish in the presence or absence of phytoplankton and zooplankton. The prebiotic tended to improve weight gain and feed efficiency in the presence of phytoplankton and zooplankton. However, prebiotic supplementation did not modulate the proximal or distal allochthonous gut microbiota and supplementation did not improve disease resistance of goldfish intraperitoneally (IP) challenged with *A. hydrophila*. In a more recent study, Raggi and Gatlin III (2012) evaluated the effect of GroBiotic®-A, MOS, GOS and FOS on apparent digestibility coefficients (ADC) of protein, lipid, organic matter and carbohydrate and the faecal microbiota of goldfish after 8 weeks of feeding. Supplementation of the prebiotics decreased ADC values, and the general trend was that MOS inclusion had

the lowest ADC values. The authors stated that the bacterial communities of fish fed the various prebiotics were not significantly modulated. This lack of intestinal modulation is contrary to the effects of MOS reported in other fish species, which might be suggestive of a species-dependent effect or could be due to the different rearing conditions or possibly the use of Cr_2O_3 as internal indicator; previous studies have demonstrated that Cr_2O_3 affects the intestinal bacterial community of Arctic charr (Ringø 1993). Further studies should assess the influence of dietary markers on the gut microbiota of fish with particular reference to studies using feed additives such as prebiotics.

14.5.7 Golden shiner

The golden shiner, a native North American species which is widely pond cultured in the USA, has been used in a number of prebiotic studies (Table 14.3). Previously it has been reported that dietary GroBiotic®-A can improve golden shiner resistance to *Flavobacterium columnare* (Sink *et al.* 2007; Sink and Lochmann 2008). More recently the effects of GroBiotic®-A on golden shiner growth performance, immune status and disease resistance have been investigated (Lochmann *et al.* 2010a; 2010b).

The aim of the study of Lochmann *et al.* (2010a) was to determine the effects of a dairy-yeast prebiotic (20 g kg^{-1}) on growth, survival and non-specific immune response of golden shiners held in indoor tanks and outdoor ponds. Growth and FCR were marginally improved by prebiotic application when the fish were reared indoors, while growth and FCR were not affected in the outdoor trial. The authors suggested that these differences were reflective of the influence of natural food. In the indoor experiment, whole body composition and alternative complement activity were not affected by prebiotic supplementation. In another study using golden shiners reared in ponds, Lochmann *et al.* (2010b) evaluated the effect of GroBiotic®-A (20 g kg^{-1}) on growth, feed conversion (FC), survival, haemoglobin, non-specific immune response and resistance against *F. columnare*. Individual weight gain, FC, survival, haemoglobin, serum lysozyme and alternative complement activities were not affected by dietary prebiotic. However, improved resistance against *F. columnare* was observed in confinement stressed fish. The latter result is in accordance with previous studies showing improved survival against *F. columnare* when the fish were fed GroBiotic®-A (Sink *et al.* 2007; Sink and Lochmann 2008).

14.5.8 Other Cyprinidae

In Caspian roach fry ($0.67 \pm 0.03 \text{ g}$), growth performance, innate immune parameters (lysozyme, Ig, ACH50), digestive enzyme activities (amylase, lipase and protease) and resistance to salinity stress were significantly improved with increasing levels of dietary FOS ($10, 20$ and 30 g kg^{-1}) (Soleimani *et al.* 2012).

To our knowledge only two studies using prebiotics in kutum have been carried out (Akrami *et al.* 2010; Yousefian *et al.* 2012). In the study of Akrami *et al.* (2010), $1.5, 3$ and $4.5 \text{ g MOS kg}^{-1}$ were fed to kutum ($\sim 742 \text{ g}$) but no significant differences in growth performance (WG, SGR, FCR) or salinity stress resistance were reported. On the other hand, the authors stated that fish fed $1.5 \text{ g MOS kg}^{-1}$ had significant higher survival and intestinal LAB counts. In their study with kutum fed GroBiotic®-A ($1, 2$ and 3 g kg^{-1}), Yousefian *et al.* (2012) reported that the clearest effect was observed by feeding the fish ($\sim 0.8 \text{ g}$) the highest

GroBiotic®-A level, as significant effects were noticed on total protein, albumin, creatinine, C3, C4 and IgM. On the other hand, prebiotic supplementation (all inclusion levels) had no effect on growth performance and survival.

Recently, Wu *et al.* (2013) investigated the effect of FOS (0.5, 1, 2, 4 and 8 g kg⁻¹) on growth performance, body composition, intestinal enzyme activities and histology of blunt snout bream (*Megalobrama amblycephala*; also known as Wuchang bream) fingerlings. SGR and survival were significantly improved by FOS at 4 and 8 g kg⁻¹. In addition, FOS at 8 g kg⁻¹ significantly increased the body crude lipid level and decreased moisture content. Of the intestinal enzyme activities, amylase and protease activity was significantly higher in fish fed 8 g kg⁻¹ FOS. Microvilli length in mid intestine was significantly higher when fish were fed FOS.

Lochmann *et al.* (2011) investigated the effect of GroBiotic®-A (20 g kg⁻¹) on growth performance, mineral composition and gut microbiota of fathead minnow in recirculating systems with low (35 mg CaCO₃ l⁻¹) or moderate (141 mg CaCO₃ l⁻¹) water hardness. Dietary prebiotic application enhanced feed conversion which was attributed to potentially enhanced nutrient absorption. GroBiotic®-A decreased whole-body Na independently of water hardness. The entire intestinal tract with content was used for bacteriological evaluations. Six hundred and twenty-six isolates were identified, but only the most predominant genera were presented; *Acinetobacter* was more abundant in fish fed with inclusion of prebiotic in the diet.

14.6 SILURIFORMES

In contrast to the well documented effects of probiotics in catfish there is a paucity of information regarding prebiotic applications (refer to Table 14.4).

Peterson *et al.* (2010) tested MOS (2 g kg⁻¹) in two basal dietary formulations – one which was not extruded and did not float (MOS-sink) and one extruded floating diet (MOS-float) (Brian Peterson, personal communication 2013). Both diet types were also produced without MOS inclusion. Channel catfish (*Ictalurus punctatus*) growth, FCR, survival and disease resistance against *Edwardsiella ictaluri* were investigated. The results showed that survival after *Ed. ictaluri* challenge was improved by dietary MOS-sink while no effect was noticed in the MOS-float study. Growth and FCR were not improved by MOS inclusion. These results are in agreement with the study of Welker *et al.* (2007), who also reported no differences in growth performance in MOS fed channel catfish.

Silver catfish, also as known South American catfish (*Rhamdia quelen*), is a commercially important species in Argentina and Brazil, and is mainly reared in intensive or semi-intensive production systems. Recent investigations have sought to investigate the gut microbiota (Pedrotti *et al.* 2013) and to test the efficacy of prebiotics applications (Hernández *et al.* 2012) in this species. Hernández *et al.* (2012) investigated the effect of MOS (2 g kg⁻¹) on silver catfish (0.11 ± 0.015 g) growth performance and survival, and reported no effect on growth performance but a significant improvement of survival.

Readers with a specific interest in the gut microbiota of channel catfish and the initial studies of prebiotics in catfish species are referred to the reviews of Ringø *et al.* (2010) and Burr *et al.* (2012).

Table 14.4 Prebiotics applications in catfishes.

Species	Prebiotic	Dose and length of administration	Results	References
Channel catfish	MOS-sink and MOS-float MOS	2 g kg ⁻¹ – 6 weeks + 3 weeks (challenge) 2 g kg ⁻¹ – 4 weeks	↑ resistance against <i>E. ictaluri</i> – by MOS-sink → weight gain, SGR and FCR → growth performance, haematology, immune function, resistance against <i>E. ictaluri</i> ↓ plasma cortisol → survival against <i>E. ictaluri</i> → SGR	Peterson <i>et al.</i> (2010) Welker <i>et al.</i> (2007)
African catfish (<i>Clarias gariepinus</i>)	MOS AXOS	2 g kg ⁻¹ – 60 days 10 and 20 g kg ⁻¹ – 10 weeks	↑ survival → growth ↑ acetate, propionate and total SCFA production → butyrate production	Hernández <i>et al.</i> (2012) Rurangwa <i>et al.</i> (2008)

Symbols represent an increase (↑), no effect (→) or decrease (↓) in the parameter of the prebiotic relative to the control.

14.7 MORONIDAE

14.7.1 European sea bass

The European sea bass is the second most important fish species in the Mediterranean region with an estimated production volume of over 70,000 tonnes in 2011 (FAO FIGIS 2013). To our knowledge, MOS is the only prebiotic which has been tested on the European sea bass at present (Table 14.5). The effect of two levels of MOS supplementation (2 and 4 g kg⁻¹) was evaluated on European sea bass juveniles (35 g) in a 9 week study which showed that MOS did not affect body proximate composition, fatty acid composition, growth parameters (except SGR which was increased), blood serum alternative complement activity, lysozyme activity or intestinal histology (Torrecillas *et al.* 2007). However, differences were reported in liver lipid vacuolization and head kidney (HK) macrophage phagocytic activity. Furthermore, MOS supplementation was able to improve disease resistance against enteric *Vibrio alginolyticus* infection when fish were challenged by either cohabitation with infected fish or direct bacterial inoculation of the gut. In the cohabitation trials, the presence of *V. alginolyticus* in sea bass HK was 33% for the control group but only 8% and 0% for fish fed 2 and 4 g MOS kg⁻¹, respectively. In the trials with direct gut inoculation with *V. alginolyticus* the number of infected fish in the control group was double the level of the MOS groups within 48 h. Furthermore, the number of the infected fish in the MOS groups remained the same at 24 and 48 h post infection. A later trial, using multiple sub-experiments with European sea bass fed diets with 2, 4 and 6 g MOS kg⁻¹, revealed that MOS supplementation improved FCR, reduced hepatic lipogenic enzymes activity, lowered hepatic lipid vacuolization and improved hepatocyte morphology (Torrecillas *et al.* 2011a). Body proximate analysis revealed significant reduction in crude lipids as well as moisture content. HK leukocyte phagocytic activity was also increased in fish fed 4 and 6 g MOS kg⁻¹ after 30, 45 and 60 days of feeding. In addition, intestinal morphological analyses showed a dose-dependent enhancement in the number of the acid mucin producing goblet cells, which may have been related to the improved bacterial resistance observed in the previous study (Torrecillas *et al.* 2007) and in later studies whereby a significant reduction of intestinal *V. anguillarum* translocation, after *in vivo* and *ex vivo* exposures, was observed in European sea bass (Torrecillas *et al.* 2011b; 2012; 2013). In addition Torrecillas *et al.* (2011b) observed improved intestinal morphology in the anterior intestine, posterior intestine and rectum (fold height and surface area) as well as elevated acid mucin producing goblet cells, higher infiltration of mixed leukocyte populations (mainly eosinophilic granulocytes), and increased intestinal mucus lysozyme activity in MOS fed fish. Torrecillas *et al.* (2012) reported that European sea bass fed 4 g MOS kg⁻¹ displayed improved disease resistance against *Vibrio anguillarum* and improved innate immune response and decreased stress (confinement) induced changes on the posterior gut microbiota. In addition improved growth, SGR and FCR were observed in MOS fed fish. More recently, Torrecillas *et al.* (2013) investigated the intestinal epithelial barrier health status of European sea bass fed 4 g MOS kg⁻¹ in an 8 week study. Significant higher weight gain, total length, CF and SGR were reported with MOS feeding. Furthermore, in the posterior intestine, prostaglandins production, cholesterol/sterols, polar lipid fraction (due to phosphatidylethanolamine and phosphatidylcholine increase), goblet cell levels and eosinophilic granulocyte levels increased with MOS feeding. However, lipid class analysis of the posterior intestine revealed a reduction in the total neutral lipid fraction, particularly due to a reduction in triacylglycerol content.

Table 14.5 Prebiotic applications in Moronidae.

Species	Prebiotic	Dose and length of administration	Results	References
European sea bass	MOS	20 and 40 g kg ⁻¹ – 9 weeks	↑ SGR, disease resistance against enteric <i>V. alginolyticus</i> infection and HK macrophage phagocytic activity → body proximate composition, fatty acid composition, growth parameters, blood serum alternative complement activity, lysozyme activity and intestinal histology ↓ liver lipid vacuolization	Torrecillas <i>et al.</i> (2007)
	MOS	4 g kg ⁻¹ – 4 and 8 weeks, 14 months	↑ growth parameters, carcass proximate analysis, liver enzyme activity, histology, intestinal histology and immune parameters → digestibility and sensory analysis	Torrecillas <i>et al.</i> (2011a)
	MOS	4 g kg ⁻¹ – 8 weeks	↑ intestinal morphology, acid mucins production goblet cells, eosinophilic granulocytes densities, intestinal mucus lysozyme activity ↓ intestinal bacteria translocation <i>in vivo</i> and <i>ex vivo</i> exposure	Torrecillas <i>et al.</i> (2011b)
	MOS	4 g kg ⁻¹ – 8 weeks	↑ disease resistance against <i>V. anguillarum</i> and innate immune response ↓ effects of stress on gut microbiota diversity	Torrecillas <i>et al.</i> (2012)
	MOS	4 g kg ⁻¹ – 8 weeks	↑ SGR, CF, cholesterol/sterols and polar lipid fraction, prostaglandins production, goblet cells, eosinophilic granulocytes in posterior intestine	Torrecillas <i>et al.</i> (2013)
Hybrid striped bass (<i>Morone chrysops</i> × <i>Morone saxatilis</i>)	GroBiotic®-A, MOS, GOS, inulin	<i>in vitro</i> : 5 and 10 g kg ⁻¹ – 48 h <i>in vivo</i> : 10 g kg ⁻¹ – 8 weeks	↓ total neutral lipids and triacylglycerol in posterior intestine ↑ butyrate, intestinal microbiota and protein body composition → weight gain, FCR, PER, propionate, acetate, total VFA or body composition (ash, moisture and lipids)	Burr <i>et al.</i> (2010)
	GroBiotic®-A	10 and 20 g kg ⁻¹ – 2 weeks (trial 1) and 4 weeks (trial 2)	↑ HK macrophage extracellular superoxide anion production and resistance against <i>S. iniae</i>	Li and Gatlin III (2004)
	GroBiotic®-A	20 g kg ⁻¹ – 16 weeks Week 16–21 <i>in situ</i> infection of <i>M. marinum</i>	↑ resistance against <i>M. marinum</i>	Li and Gatlin III (2005)

Symbols represent an increase (↑), no effect (→) or decrease (↓) in the parameter of the prebiotic relative to the control.

These studies indicate that dietary supplementation of MOS can influence several aspects: intestinal lipid, intestinal morphology, localized immunological responses and potentially intestinal microbiota, which provide protection against enteric bacterial pathogens in fish.

14.7.2 Hybrid striped bass (*Morone chrysops* × *Morone saxatilis*)

Hybrid striped bass is an important game fish and food fish in the United States and commercial production has steadily increased over the past 25 years to 4000–6000 tonnes per annum during the past decade (FAO FIGIS 2013). Several studies have investigated the application of prebiotics in hybrid striped bass (Table 14.5). It has been reported that dietary GroBiotic®-A can improve hybrid striped bass HK macrophage extracellular superoxide anion production and resistance to *Streptococcus iniae* and *Mycobacterium marinum* (Li and Gatlin III 2004; 2005). More recently *in vitro* and *in vivo* experiments have been conducted by Burr *et al.* (2010) in order to investigate the effect of four prebiotics (GroBiotic®-A, MOS, GOS and inulin) on hybrid striped bass. In the *in vitro* experiment, the intestines of three fish fed the commercial diet (without prebiotic supplementation) were aseptically removed inside an anaerobic chamber. The intestinal contents were diluted and incubated with the experimental feeds at 25 °C. The experimental diets were based on the commercial diet with an inclusion of 5 g kg⁻¹ and 10 g kg⁻¹ of each of the prebiotics. After 24 and 48 h of incubation at 25 °C, supernatants were removed for volatile fatty acid (VFA) analysis and DNA was extracted for PCR-DGGE analysis. The results showed that propionate production was low compared to acetate and butyrate for all samples. Additionally, there were no significant differences in propionate, acetate and total VFA among the samples after 48 h incubation. Compared to the control, butyrate production significantly increased after 48 h but only for samples containing 5 g kg⁻¹ GroBiotic®-A, 10 g kg⁻¹ MOS, and 5 g kg⁻¹ GOS. Microbial cluster analysis showed that community composition in all samples had high similarity coefficients (>80%) at 24 and 48 h and the dominant bands were identified as *Clostridium perfringens* and Fusobacteria. In the *in vivo* experiment, fish were fed experimental diets supplemented with 10 g kg⁻¹ of each prebiotic for 8 weeks. Weight gain, FCR and PER were unaffected by prebiotic supplementation. Similarly, body proximate analysis revealed that ash, moisture and lipid levels remained unaffected by prebiotic supplementation; however, body protein content was significantly higher in the GroBiotic®-A fed fish compared to the control fed fish. PCR-DGGE analysis revealed that microbial patterns of fish fed inulin, MOS and GroBiotic®-A had very high similarity indices. Similarity coefficients of fish fed GOS were less than 80% compared with the other prebiotic treatments, and the microbial community of the fish fed the control diet was unique compared to fish fed prebiotic supplemented diets. The main genera identified were: *Clostridia*, *Bacillus*, *Enterococcus*, *Lysinibacillus* and *Staphylococcus*. This study showed that prebiotics did not affect the growth performance of the cultivated fish but the prebiotics modulated the intestinal microbiota to some extent.

14.8 SPARIDAE

14.8.1 Gilthead sea bream

The gilthead sea bream is probably the most important fish species in the Mediterranean region with an estimated 97,000 tonnes produced in 2011 (FAO FIGIS 2013). Despite this, very few

prebiotics have been tested in sea bream (Cerezuela *et al.* 2008; Dimitroglou *et al.* 2010a; 2011a; Gültepe *et al.* 2011; Tapia-Paniagua *et al.* 2011; Cerezuela *et al.* 2012; 2013a; 2013b) (Table 14.6). Cerezuela *et al.* (2008) fed gilthead sea bream (~ 175 g) two levels of inulin inclusion, 5 and 10 g kg⁻¹, for both 1 and 2 weeks. The results showed a significant inhibition of leukocyte phagocytosis and respiratory burst activities after 1 week but levels returned to control levels at week 2. An *in vitro* study, where HK leukocytes were incubated with inulin, showed no significant effects on leukocyte peroxidase, phagocytic, respiratory burst or natural cytotoxic activities. Based on these findings, the authors suggested that gilthead sea bream leukocytes do not have inulin receptors and inulin supplementation seems to be inappropriate in gilthead sea bream rearing.

On the other hand, the use of MOS supplementation (2 and 4 g kg⁻¹) in gilthead sea bream (~ 105 g) showed alterations of circulating leukocyte proportions as well as increased total leukocyte levels within 2 weeks of feeding (Dimitroglou *et al.* unpublished data). Serum lysozyme and alternative complement activity remained unaffected however. Microbial analysis revealed that MOS supplementation reduced the aerobic culturable intestinal microbial load without altering the relative abundance of the identified bacterial species. A longer nutrition study, supplementing MOS (2 and 4 g kg⁻¹) into diets using either fishmeal (FM) as the sole protein source, or partial replacement of FM with soybean meal (SBM), showed that body proximate composition, growth parameters and feed conversion were unaffected by MOS (Dimitroglou *et al.* 2010a). The hepatosomatic index (HSI) was significantly reduced by the addition of MOS in diets where FM was the protein source. Histological evaluation of liver showed no significant changes in glycogen deposition among experimental groups but the posterior intestinal mucosal fold (villi) absorptive surface was significantly improved by MOS in the fish fed the FM based diets. Electron microscopy revealed that dietary MOS had a pronounced effect at the ultrastructural level, as microvilli density was increased with MOS supplementation in both FM based and SBM based diets. Microbial analysis using PCR-DGGE demonstrated that the effect of dietary MOS on the allochthonous microbial populations was more distinctive in FM based diets, characterized by increased species diversity, richness and reduced similarity, compared to the SBM fed fish, where species diversity and richness remained unaffected and similarity between treatments was higher. It was suggested that the presence of SBM oligosaccharides could have influenced the impact of MOS on the intestinal microbial community.

In a 12 week feeding trial, Gültepe *et al.* (2011) investigated the effects of two levels (2 and 4 g kg⁻¹) of dietary MOS on the growth performance and digestive capacity of gilthead sea bream (initial weight ~ 170 g). The results showed that MOS significantly increased growth performance, feed utilization and ADCs of protein and carbohydrate. However, no significant effect was observed on lipid digestibility. Later, Gültepe *et al.* (2012) evaluated MOS (2 and 4 g kg⁻¹) supplementation on haematological parameters and histological evaluation of the liver of gilthead sea bream. Administration of MOS did not affect the haematological parameters investigated (RBC, WBC, Thr, Ht, Hb, MCH, MCHC and MCV) and did not influence liver histology. All samples displayed normal characteristics: homogeneous-sized hepatocytes with slightly vacuolated cytoplasm and large, spherical, centrally located nuclei.

In a study with gilthead sea bream, Cerezuela *et al.* (2012) revealed that inclusion of 10 g inulin kg⁻¹ stimulated serum complement activity and phagocytic ability. However, these effects were not observed at higher inclusion levels. Furthermore, inclusion of 10 g inulin kg⁻¹ had no effect on the expression of immune-related genes in the head kidney (HK). A challenge

Table 14.6 Prebiotic applications in Sparidae.

Species	Prebiotic	Dose and length of administration	Results	References
Gilthead sea bream	Inulin	5 and 10 mg kg ⁻¹ – up to 2 weeks	→ HK leukocyte peroxidase, phagocytic activity, respiratory burst and natural cytotoxic activities ↓ phagocytosis and respiratory burst of leukocytes ↑ serum complement activity, phagocytic ability → phagocytic capacity, expression of immune-related genes in HK → resistance against <i>P. damselae</i> subsp. <i>piscicida</i> (10 g inulin kg ⁻¹)	Cerezuela <i>et al.</i> (2008)
	Inulin	10, 15 and 30 g kg ⁻¹ – 4 weeks	→ villi height, intestine diameter, IEL ↓ goblet cells, microvilli height Modulate the gut microbiota	Cerezuela <i>et al.</i> (2012)
	Inulin	10 g kg ⁻¹ – 4 weeks	↑ gene expression of IL-8, β-actin, occludin and transferrin Expressions of the other genes were not significantly affected	Cerezuela <i>et al.</i> (2013a)
	MOS	2 and 4 g kg ⁻¹ – 9 weeks	↑ growth parameters, intestinal histology and intestinal microbiota → liver and proximate carcass content	Cerezuela <i>et al.</i> (2013b)
	MOS	2 and 4 g kg ⁻¹ – 2 weeks	↑ increased total leukocyte number/alterations in circulating proportions → blood serum lysozyme, alternative complement haemolytic activity, relative abundance of the identified bacterial species ↓ aerobic culturable intestinal microbial load	Dimitroglou <i>et al.</i> (2010a)
	MOS	2 and 4 g kg ⁻¹ – 12 weeks	↑ growth parameters, feed utilization and apparent protein and carbohydrate digestibility → carcass protein levels	Dimitroglou <i>et al.</i> (2011a)
	MOS	2 and 4 g kg ⁻¹ – 12 weeks	No effect on hematological parameters and liver histology	Gülepe <i>et al.</i> (2011)
White sea bream	MOS incorporated in <i>Artemia</i>	4 g kg ⁻¹ – 12 weeks 20 g kg ⁻¹ – 20 days	↑ intestinal histology, salinity challenge tolerance → larval growth performance, mortality	Gülepe <i>et al.</i> (2012)
Sharpsnout sea bream	MOS and FOS	8 g kg ⁻¹ – 114 days	→ growth performance, ADC of dry matter, protein, lipid and energy	Dimitroglou <i>et al.</i> (2010b)
	MOS and inulin	8 g kg ⁻¹ – 150 days	↑ moisture → growth performance, crude protein and ash ↓ crude lipid Some fatty acid (16:0, 18:1 n-9 and 20:5 n-3) of total lipid were significantly affected	Piccolo <i>et al.</i> (2011) Piccolo <i>et al.</i> (2012)

Symbols represent an increase (↑), no effect (→) or decrease (↓) in the parameter of the prebiotic relative to the control.

study with *Photobacterium damsela* subsp. *piscicida* (IP) displayed no significant effect of inulin (10 g kg^{-1}) compared with the control group (non-supplemented diet). In a recent study, Cerezuela *et al.* (2013a) investigated the effect of inulin (10 g kg^{-1}) on intestinal morphology and allochthonous intestinal microbiota. Intestinal morphology evaluations by light and transmission electron microscopy revealed signs of damage in the anterior intestine (disruption and damage to the intercellular space and microvilli and enterocyte vacuolization); these results are in accordance with those reported in Arctic charr fed inulin (Olsen *et al.* 2001) but contrary to those reported for Atlantic salmon (Bakke-McKellep *et al.* 2007). Dietary administration of inulin significantly increased villi height, intestinal diameter and the number of intraepithelial leukocytes, but reduced the number of goblet cells and microvilli height. The increased villi height is in accordance with that reported in gilthead sea bream fed MOS (Dimitroglou *et al.* 2010a). DGGE analyses revealed that the intestinal microbiota of the fish fed inulin contained significantly fewer (12 ± 0.43) operational taxonomic units (OTUs) than detected in control fed fish (17.33 ± 0.94).

Cerezuela *et al.* (2013b) investigated the effect of inulin administration on intestinal gene expression in gilthead sea bream, and revealed that IL8 (proinflammatory cytokine), β -actin, occludin and transferrin expression were significantly increased with inulin supplementation. The authors suggested that the increase in IL8 is related to modulation in the intestinal microbiota; however, as no information exists on this topic in fish, further investigations are needed.

In a study with sharpsnout (*Diplodus puntazzo* Cetti, 1777; $\sim 100 \text{ g}$), Piccolo *et al.* (2011; 2012) assessed the effect of MOS, FOS and inulin on growth, ADC, somatic parameters, body composition and fillet fatty acid composition. Supplementation of MOS and FOS (8 g kg^{-1} of both prebiotics) did not affect growth performance (Piccolo *et al.* 2011) and similar results were reported when MOS and inulin were used (Piccolo *et al.* 2012). ADC of dry matter, protein, lipid and energy was not influenced by MOS and FOS supplementation (Piccolo *et al.* 2011). Somatic parameters – viscerosomatic index (VSI) and mesenteric fat index (MFI) – decreased but not significantly (Piccolo *et al.* 2012). Crude lipid decreased significantly while crude protein and ash were not affected. Some fatty acids (16:0, 18:1 n-9 and 20:5 n-3) were significantly affected by MOS and inulin; otherwise no clear effect on fatty acid composition was apparent.

14.8.2 White sea bream

The white sea bream is considered one of the ‘new species’ in the Mediterranean aquaculture industry. In the study of Dimitroglou *et al.* (2010b), white sea bream larvae were fed 20 g kg^{-1} MOS supplementation (incorporated in *Artemia*) from 24 to 43 days post hatching (dph). The results indicated that larval growth performance and survivability were not affected by the MOS supplementation at the early life stage. However, light microscopy and TEM evaluations revealed that MOS supplementation significantly improved the intestinal morphology by increasing villi surface area and microvilli length by 12% and 26% respectively, compared to the control. Salinity challenge experiments showed that MOS significantly increased larval stamina and survival (higher LT_{50}) at both 0 mg l^{-1} and 60 mg l^{-1} salinity by 13% and 23%, respectively. The reason for this observation was not fully elucidated but the authors suggested a possible explanation may be related to improved gut morphology. In marine teleosts the GI tract plays an important role in osmoregulation, thus the better stamina of MOS fed larvae in the salinity challenge may be related to improving intestinal morphology. This improved

osmoregulation capability is very useful in the context of intensive hatchery production where larval quality is a key factor for successful production.

14.9 CICHLIDAE

The family Cichlidae is one of the largest vertebrate families, containing a number of important ornamental species, game species and food fish. After carps, tilapias account for the second largest production volume of food fish with total global production approaching 4 million tonnes in 2011 (FAO FIGIS 2013). The Nile tilapia (*Oreochromis niloticus*), Nile-blue hybrid (*Oreochromis niloticus* × *Oreochromis aureus*) and blue tilapia (*Oreochromis aureus*) are the most popular food fish species and as such some information is available on prebiotic applications in these species (Table 14.7). Benefits have been reported with the use of MOS (Sado *et al.* 2008; Samrongpan *et al.* 2008; Shelby *et al.* 2009), inulin (Ibrahim *et al.* 2010) and GroBiotic®-A (Zheng *et al.* 2011) in Nile tilapia, and inulin (Wang and Wang 1997) and FOS (He *et al.* 2003), scFOS (Hui-Yuan *et al.* 2007; Zhou *et al.* 2009b) and MOS (He *et al.* 2003; Genç *et al.* 2007) in hybrid tilapia.

In addition, Ibrahim *et al.* (2010) reported that dietary supplementation of inulin (5 g kg⁻¹) increased SGR, survival and nitroblue tetrazolium, and improved resistance of Nile tilapia against *A. hydrophila*. However, haematocrit and lysozyme activity was not affected. Shelby *et al.* (2009) measured the effect of 2 g MOS kg⁻¹ on serum components, including protein and Ig concentrations as well as lysozyme and complement activities, in two studies of juvenile Nile tilapia (12.1 and 14.9 g) fed, but no significant effects were observed. Furthermore, no protective effect was observed when the fish were IP injected with *St. iniae*. Zheng *et al.* (2011) evaluated the effect of GroBiotic®-A (4, 8 and 12 g kg⁻¹) on growth, muscle composition, immune response and resistance towards *A. hydrophila* in an 8 week feeding trial of Nile tilapia (~18 g). Inclusion of GroBiotic®-A significantly increased weight gain and reduced FCR but had no effect on VSI or muscle moisture, lipid, protein content or catalase, and lysozyme and superoxide dismutase. However, neutrophil oxidative production (NBT test) and survival after *A. hydrophila* challenge were increased with prebiotic supplementation. Readers with an interest in prebiotic applications for tilapia are referred to the review of Ringø *et al.* (2010).

14.10 SCIAENIDAE

A number of studies have assessed the efficacy of using prebiotics to modulate the gut microbiota of red drum, an important fish species, and the subsequent effects on growth performance, immunity and disease resistance (Table 14.8).

Burr *et al.* (2008a) examined the effect of four prebiotics (GroBiotic®-A, MOS, GOS and inulin) on red drum (~500 g) digestibility of SBM based diets. Six experimental diets were formulated and administered for 3 weeks: control diet without SBM (diet 1); basal diet where 35% of FM was replaced by SBM (diet 2); basal diet + 10 g kg⁻¹ GroBiotic®-A supplementation (diet 3); basal diet + 10 g kg⁻¹ MOS supplementation (diet 4); basal diet + 10 g kg⁻¹ GOS supplementation (diet 5); and basal diet + 10 g kg⁻¹ inulin supplementation (diet 6). The results showed that in SBM based diets GroBiotic®-A, MOS and GOS supplementation increased ADCs of protein and energy on a par with those of the FM based diet (diet 1). Additionally,

Table 14.7 Prebiotic applications in tilapia.

Species	Prebiotic	Dose and length of administration	Results	References
Nile tilapia (<i>Oreochromis niloticus</i>)	MOS	2 g kg ⁻¹ – 4 and 2 weeks + 11 days (challenge)	→ serum protein and IgM, lysozyme and complement activities and resistance against <i>St. initae</i>	Shelby <i>et al.</i> (2009)
	MOS	2, 4, 6, 8 and 10 g kg ⁻¹ – 45 days	→ haematological parameters	Sado <i>et al.</i> (2008)
	MOS	2, 4, and 6 g kg ⁻¹ – 3 weeks	↓ daily feed consumption with increasing level ↑ weight, length and average daily growth of fish fed 4 and 6 g	Samrongpan <i>et al.</i> (2008)
	Inulin	5 g kg ⁻¹ – 2 months	↑ survival against <i>Streptococcus agalactiae</i> ↑ SGR, survival, growth, nitroblue tetrazolium, and resistance against <i>A. hydrophila</i>	Ibrahim <i>et al.</i> (2010)
Blue tilapia (<i>Oreochromis aureus</i>)	GroBiotic®-A	4, 8 and 12 g kg ⁻¹ – 8 weeks	→ haematocrit and lysozyme activity ↑ weight gain, neutrophil oxidative production, and resistance towards <i>A. hydrophila</i>	Zheng <i>et al.</i> (2011)
	Inulin	IP 10 mg kg ⁻¹ body weight – 2 weeks	→ moisture, lipid, protein in muscle, catalase, lysozyme and SOD ↓ FCR → susceptibility against <i>A. hydrophila</i> and <i>Ed. tarda</i>	Wang and Wang (1997)
Hybrid tilapia (<i>Oreochromis niloticus</i> × <i>Oreochromis aureus</i>)	FOS	2 and 6 g kg ⁻¹ – 58 days	→ growth rate ↑ survival	He <i>et al.</i> (2003)
	scFOS	0.8 and 1.2 g kg ⁻¹ – 8 weeks	↑ non-specific immunity ↑ growth rate, feed intake, <i>Vibrio parahaemolyticus</i> , <i>A. hydrophila</i> , <i>Lactobacillus</i> spp., <i>Streptococcus faecalis</i> ↓ FCR	Hui-Yuan <i>et al.</i> (2007)
	scFOS	1 g kg ⁻¹ – 56 days	→ survival and condition factor	
	MOS	2 and 6 g kg ⁻¹ – 58 days	↑ uncultured bacterium clones and <i>Thiothrix eikelboomii</i> → growth rate ↑ survival	Zhou <i>et al.</i> (2009b) He <i>et al.</i> (2003)
	MOS	1.5, 3 and 4.5 g kg ⁻¹ – 80 days	↑ non-specific immunity → growth parameters and body indices Dry matter and protein contents of fillets increased with increasing rates of MOS	Genc <i>et al.</i> (2007)

Symbols represent an increase (↑), no effect (→) or decrease (↓) in the parameter of the prebiotic relative to the control.

Table 14.8 Prebiotic applications in red drum.

Prebiotic	Dose and length of administration	Results	References
GOS, MOS, inulin, GroBiotic®-A	10 g kg ⁻¹ – 3 weeks	↑ apparent nutrient digestibilities	Burr <i>et al.</i> (2008a)
Brewer's yeast, FOS, GroBiotic®-A	20 g kg ⁻¹ – 48 h (<i>in vitro</i>)	↑ acetate, butyrate, total VFA and intestinal microbiota → propionate	Burr <i>et al.</i> (2008b)
GroBiotic®-A, inulin	10 g kg ⁻¹ – 8 weeks and 10 g kg ⁻¹ – 6 weeks	↑ moisture body composition → weight gain, FCR, PER, intestinal microbiota or body composition (ash and lipids) ↓ protein body composition	Burr <i>et al.</i> (2009)
FOS, MOS, TOS, GroBiotic®-A	10 g kg ⁻¹ – 4 and 6 weeks	↑ FE, serum lysozyme, superoxide anion production and survival after <i>Amyloodinium ocellatum</i> challenge	Buentello <i>et al.</i> (2010)
FOS, GOS, MOS, Previda™	10 g kg ⁻¹ – 8 weeks	↑ weight gain and immunological responses by Previda™ ↑ microvilli heights by prebiotics → hepatosomatic index, muscle ratio, CF and morphological changes of the gut (fold – and enterocyte height) ↓ lower survival by MOS feeding	Zhou <i>et al.</i> (2010)

Symbols represent an increase (↑), no effect (→) or decrease (↓) in the parameter of the prebiotic relative to the control.

ADCs of organic matter and total carbohydrates were significantly higher for GroBiotic®-A, MOS and GOS supplemented diets compared with the basal diet (diet 2). The inulin supplemented diet did not show any improvement on ADCs of protein, energy, organic matter and total carbohydrates compared with the basal diet (diet 2). Fish fed all four prebiotics had significantly lower ADC of lipid compared to fish fed diet 1 or diet 2. The findings of this study suggest that prebiotics can improve ADCs in diets with high levels of SBM inclusion.

Burr *et al.* (2008b) evaluated the effects of brewer's yeast, FOS, and GroBiotic®-A on the intestinal microbial community of red drum. Intestinal contents were aseptically removed from three fish fed the same commercial diet inside an anaerobic chamber. Samples were diluted and incubated *in vitro* in one of four liquid media: control diet (commercial diet); control diet + 20 g kg⁻¹ GroBiotic®-A; control diet + 20 g kg⁻¹ brewer's yeast; and control diet + 20 g kg⁻¹ FOS. After 24 and 48 h of incubation at 25 °C, supernatants were removed for volatile fatty acid (VFA) analysis and PCR-DGGE analysis. The intestinal content incubated with GroBiotic®-A had significantly higher acetate, butyrate and total VFA concentrations after 24 h of incubation compared to the other treatments. Propionate production remained unaffected in all culture media. DGGE fingerprint analysis demonstrated that GroBiotic®-A and brewer's yeast were able to modulate the microbial community. These findings combined with the findings from Burr *et al.* (2008a) suggest that prebiotics, especially GroBiotic®-A, play an important role in red drum nutrition.

In a later study, Burr *et al.* (2009) evaluated the effect of 10 g kg⁻¹ GroBiotic®-A and 10 g kg⁻¹ inulin supplementation in feeds containing either FM as protein source or FM with 35.5% SBM included. In the first sub-trial, prebiotics were tested using FM based diets in an 8

week trial in a closed circulation system. In the second sub-trial, prebiotics were tested using diets with FM and SBM inclusion in 6 week trials conducted in either closed (the same circulation system as sub-trial 1) or open (with independent biofilters for each tank) circulation systems. In sub-trial 2, the authors wanted to examine whether the circulation system interacted with the effect from the prebiotics supplementation. The results from the first sub-trial showed that growth parameters such as weight gain, FCR and PER remained unaffected for both prebiotics. Whole body proximate composition showed that ash and lipid contents were similar for all diets. Protein content was reduced for both prebiotics compared with the basal diet. Inulin supplementation increased the moisture content compared to the basal diet. The results from the second sub-trial revealed that weight gain, FCR, PER and whole body proximate composition remained unaffected by the prebiotic supplementations. Similarly, the two circulation systems did not affect any of these parameters. Microbial fingerprint was also examined in sub-trial 2. Intestinal samples from fish and samples from systems biofilters were analysed using PCR-DGGE. There were no differences in the intestinal microbiota between the feeding regimes.

Buentello *et al.* (2010) evaluated the effect of supplementation of FOS, MOS, TOS and GroBiotic®-A to SBM based diets on weight gain, feed efficiency and non-specific immunity of red drum after challenge with *Amyloodinium ocellatum*, more commonly known as marine velvet, which is one of the most frequently encountered pathogens affecting tropical marine ornamental fishes. Prebiotics supplementation significantly improved feed efficiency, serum lysozyme, intracellular superoxide anion production and survival after challenge.

Zhou *et al.* (2010) evaluated four different prebiotics; FOS, GOS, MOS and Previda™. Each prebiotic was supplemented to a basal diet at a level of 10 g kg⁻¹. Fish fed Previda™ had significantly higher weight gain compared to fish fed the basal diet or MOS supplemented diet. Fish fed MOS displayed lower survival rates than the fish fed the other four diets. HSI, muscle ratio, CF and morphological changes of the gut (fold and enterocyte height) were not affected by prebiotics. Microvilli heights in the pyloric caeca, proximal intestine and mid intestine were significantly increased by all prebiotics, but the microvilli heights in the distal intestine were not affected.

14.11 OTHER FISH SPECIES

Beyond the aforementioned fish species, the effects of prebiotics on various other species have been reported (Table 14.9). For example, raftilin, raftilose, lactosucrose, MOS and chitosan oligosaccharides (COS) have been investigated in flatfish (Mahious *et al.* 2006b; Dimitroglou *et al.* 2011c; Cui *et al.* 2012). In an 8 week feeding trial, Cui *et al.* (2012) evaluated the effect of feeding turbot (~12 g) several graded inclusion levels (0.075, 0.15, 0.3, 0.6 and 1.2 g kg⁻¹) of COS on growth, innate immune responses and resistance against IP injection with *Edwardsiella tarda*. The results showed that an inclusion level of 0.3 g COS kg⁻¹ improved SGR, FCR, phagocytic index, serum superoxide dismutase activity, and survival against *Ed. tarda* challenge. However, no effect was observed in malondialdehyde and metallothionein. In a Senegalese sole study, vaccinated and unvaccinated fish (~45 g) were fed 4 g MOS kg⁻¹ after a natural outbreak of pasteurellosis, caused by *Photobacterium damsela* subsp. *piscicida* (Dimitroglou *et al.* 2011c). After 10 weeks the mortality was significantly lower in MOS fed fish and histological examination of the anterior and the posterior intestine using light microscopy

Table 14.9 Prebiotic applications in other fish species.

Species	Prebiotic	Dose and length of administration	Results	References
Platy	Immunogen	5, 10 and 15 g kg ⁻¹ – 26 weeks	↑ GSI, relative fecundity, fry production per female and fry survival when fish were fed 15 g Immunogen kg ⁻¹	Abasali and Mohamad (2011a)
Swordtail	Immunogen	5, 10 and 15 g kg ⁻¹ – 26 weeks	↑ growth when the fish were fed 10 and 15 g Immunogen kg ⁻¹ and GSI in fish fed 10 g Immunogen	Abasali and Mohamad (2011b)
Hybrid sorubims	Inulin	5 g kg ⁻¹ – 15 days	↑ total immunoglobulin and gut LAB → total gut bacteria, <i>Vibrio</i> and immunological parameters	Mouriño <i>et al.</i> (2012)
Turbot	COS	0.75, 0.15, 0.3, 0.6 and 1.2 g kg ⁻¹ – 8 weeks	↓ gut <i>Pseudomonas</i> ↑ 0.3 g kg ⁻¹ – SGR, PI, SOD ↑ survival after <i>Ed. tarda</i> challenge → MDA, MT ↓ FCR (0.3 g kg ⁻¹)	Cui <i>et al.</i> (2012)
Sole	FOS and inulin	20 g kg ⁻¹ – 1 month	↑ growth rate Effects on gut microbiota (<i>Bacillus</i> and <i>Vibrio</i>)	Mahious <i>et al.</i> (2006b)
	MOS	4 g kg ⁻¹ – 10 weeks	↑ survival against <i>Photobacterium damsela</i> subsp. <i>piscicida</i> and improved intestinal morphology	Dimitroglou <i>et al.</i> (2011c)
Cobia	MOS	2 g kg ⁻¹ – 13 dph	↑ larval survival ↑ microvilli alignment ↓ supranuclear vacuoles	Salze <i>et al.</i> (2008)

Symbols represent an increase (↑), no effect (→) or decrease (↓) in the parameter of the prebiotic relative to the control.

revealed that MOS supplementation increased villi folding compared to the control fish. Scanning electron microscopy revealed that MOS promoted microvilli structures of better quality and with greater density in the anterior part of the intestine. These results were in agreement with the previous MOS applications in fish and strengthen the suggestion that MOS produce a positive effect in the overall intestinal morphology, function and efficiency.

In Poeciliidae, Abasali and Mohamad (2011a; 2011b) carried out studies to evaluate if dietary Immunogen (5, 10 and 15 g kg⁻¹) affected platy (*Xiphophorus maculatus*) and swordtail (*Xiphophorus helleri*) reproductivity. Gonadosomatic index (GSI), relative fecundity, fry production per female and fry survival were significantly higher when platy were fed 15 g Immunogen kg⁻¹. Swordtail showed significantly higher growth when fed 10 and 15 g Immunogen kg⁻¹, while the highest GSI was observed in fish fed 10 g kg⁻¹ Immunogen. The authors suggested that their findings were attributed to balanced production of essential nutrients. However, no convincing experiments have been conducted so far to validate this hypothesis and, as the authors suggested, further studies are needed to clarify the mechanisms involved.

Recently, Mouriño *et al.* (2012) evaluated the effect of dietary inulin (5 g kg⁻¹) on the autochthonous gut microbiota, haematological and immunological parameters of hybrid sorubims (*Pseudoplatystoma corruscans* and *Pseudoplatystoma fasciatum*). Inclusion of inulin did not affect total gut bacterial levels or *Vibrio* levels; however, *Pseudomonas* levels were decreased and LAB levels increased. None of the haematological parameters investigated were affected by dietary inulin and, of the immunological parameters evaluated, only total immunoglobulin was affected, which was increased from 1.80 ± 0.58 mg ml⁻¹ in the control group compared to 4.17 ± 0.84 mg ml⁻¹ in the inulin group.

14.12 SYNBIOTICS

Synbiotics are nutritional supplements combining a mixture of probiotics and prebiotics in a form of synergism, which beneficially affect the host by improving the survival and implementation of live microbial dietary supplements in the GI tract of the host (Andersson *et al.* 2001). Since the first study on synbiotics in fish published in 2009 (Rodriguez-Estrada *et al.* 2009) there has been a growing interest in the use of synbiotics in aquaculture and recently Cerezuela *et al.* (2011) published a review on this topic. Since this review was published several further synbiotic studies have emerged (Table 14.10). These studies have assessed growth performance, feed utilization, digestive enzyme activities, body composition, immunological responses, haematological/serum biochemical parameters, disease resistance, survival rate and gut microbiota of synbiotic fed finfish, shellfish and echinoderms. Readers with a special interest in prebiotics and synbiotics in crustaceans are referred to *Chapter 15*. To avoid duplication, the studies reviewed comprehensively by Cerezuela *et al.* (2011) are not discussed in this section and readers with a special interest are referred to the original review.

Firouzbaksh *et al.* (2012) evaluated the effect of Biomin IMBO (*Enterococcus faecium* and FOS; 0.5, 1, 1.5 g kg⁻¹) on rainbow trout SGR, FCR, feed conversion efficiency (FCE), survival and disease resistance towards *Saprolegnia parasitica*. All inclusion levels significantly improved SGR, FCE, survival and resistance against *S. parasitica* while FCR and CF were reduced. Similar improvements in growth, and in some cases survival, have been observed with the application of commercial synbiotic Biomin IMBO to kutum (Haghighi *et al.* 2010), angelfish (*Pterophyllum scalare*) and zebrafish (*Danio rerio*) (Nekoubin *et al.* 2012b).

Table 14.10 Synbiotic applications in fish.

Fish species	Probiotic	Prebiotic	Results	References
Rainbow trout	<i>E. faecalis</i>	MOS, PHB	↑ body weight, SGR, haematocrit, phagocytic index and mucus production ↓ mortality, FCR	Rodriguez-Estrada <i>et al.</i> (2009) ^a
	<i>E. faecium</i>	FOS	↑ body weight, weight gain, SGR, FCE, SR, crude protein and serum protein	Mehrabi <i>et al.</i> (2012) ^a
	<i>E. faecium</i>	FOS	↑ SGR, FCE and disease resistance against <i>Saprolegnia parasitica</i>	Firouzbakhsh <i>et al.</i> (2012)
Atlantic salmon	<i>Ped. acidilactici</i>	scFOS	↓ FCR and CF ↑ intestinal immune response, serum lysozyme activity and intestinal morphology → growth, SGR and FCR	Abid <i>et al.</i> (2013)
Cobia	<i>B. subtilis</i>	Chitosan	↑ SGR, lysozyme, ACP, phagocytosis and respiratory burst ↓ mortality	Geng <i>et al.</i> (2011) ^a
Gilthead sea bream	<i>D. hansenii</i>	Inulin	↑ intestinal microbiota, peroxidase activity and gene expression → immune parameters	Tapia-Paniagua <i>et al.</i> (2011)
	<i>B. subtilis</i>	Inulin	↑ some immune related gene response ↓ disease resistance against <i>P. damsela</i> Modulate gut microbiota	Cerezuela <i>et al.</i> (2012)
	<i>B. subtilis</i>	Inulin	Effect on gut histology	Cerezuela <i>et al.</i> (2013a)
	<i>B. subtilis</i>	Inulin	↑ gene expression of β-actin and occludin Expression of the other genes were up-regulated but not significantly affected	Cerezuela <i>et al.</i> (2013b)
Japanese flounder (<i>Paralichthys olivaceus</i>)	<i>B. clausii</i>	MOS, FOS	↑ body weight, weight gain, crude protein, lipid, lysozyme, protease and amylase activities ↓ FCR, TG and LDL-C	Ye <i>et al.</i> (2011) ^a
Yellow croaker (<i>Larimichthys crocea</i>)	<i>B. subtilis</i>	FOS	↑ SGR, FER, lysozyme and SOD ↓ mortality	Ai <i>et al.</i> (2011) ^a
Kutum	<i>E. faecium</i>	FOS	↑ body weight, SGR, FCR, PER	Haghighi <i>et al.</i> (2010)
Angelfish	<i>E. faecium</i>	FOS	↑ SGR and FCE → hatching rate	Nekoubin <i>et al.</i> (2012a)

Zebratfish	<i>E. faecium</i>	FOS	↑ SGR and FCE ↓ FCR and CF	Nekoubin <i>et al.</i> (2012b)
Koi carp	<i>B. coagulans</i>	COS	↑ SGR, total leukocyte counts, respiratory burst, phagocytic capacity, lysozyme activity, SOD and resistance against <i>A. veronii</i>	Lin <i>et al.</i> (2012)
Hybrid sorubims	<i>W. cibaria</i>	Inulin	↓ FCR ↑ level of midgut LAB, erythrocytes, total IgM by synbiotic treatment → blood glucose, serum protein or lysozyme levels by synbiotic treatment ↓ <i>Pseudomonas</i> spp. and <i>Vibrio</i> spp. and circulating neutrophils in the synbiotic treatment	Mouriño <i>et al.</i> (2012)

^aStudies discussed in the review of Cerezuela *et al.* (2011).

Bacterial abbreviations: *B.* = *Bacillus*; *E.* = *Enterococcus*; *Ped.* = *Pediacoccus*; *W.* = *Weissella*.

Symbols represent an increase (↑), no effect (→) or decrease (↓) in the parameter of the synbiotic relative to the control.

In a study by Tapia-Paniagua *et al.* (2011) the authors evaluated modulation of the intestinal allochthonous microbiota of gilthead sea bream (~80 g) by administration of *Debaryomyces hansenii* in combination with inulin. Experimental fish were fed either a commercial diet (control diet) or adiet supplemented with *D. hansenii* strain L2 (10^6 CFU g⁻¹) plus 3% inulin (experimental diet II) for 4 weeks. After 2 and 4 weeks of feeding, samples of the whole intestine were aseptically removed for allochthonous microbiota analysis using PCR-DGGE and sequence analysis. Additionally, the expression of 12 selected genes related to the immune response (IgM, MHCII α , MHCII β , C3, IL-1 β , TLR9A, TNF α , CSF-1R, NCCRP-1, Hep, TCR β and CD8) from the skin, intestine, liver and HK tissue were analysed by real-time PCR. Samples of blood and HK were obtained for the determination of humoral and cellular immune parameters. The results showed that fish fed the experimental diet had lower intestinal microbial species richness and greater similarity indices compared with fish fed the control diet for 4 weeks. It is of interest to note that *Pseudomonas* spp. dominated the intestinal microbiota in both experimental groups. Peroxidase activity was the only haematological parameter that was significantly increased in fish fed the synbiotic diet. RT-PCR revealed that a number of the selected immune-related genes were generally up-regulated in the skin and intestine after 2 weeks of feeding the synbiotic diet. The maximum transcript levels were reported in the intestine for the major histocompatibility complex (MHC) genes MHC I and MHC II, which were significantly up-regulated. After 4 weeks of feeding, relatively lower gene transcript levels were recorded in the skin and intestine, but higher levels of complement 3 (C3), the pro-inflammatory cytokine TNF α and colony stimulating factor 1 receptor (CSF-1R), a receptor for a cytokine which controls macrophage production, differentiation and function, were observed in the intestine. In addition, at week 4 a greater effect was observed in the HK than at week 2. This was especially prominent in the up-regulation of C3, the pro-inflammatory cytokine IL-1 β , CSF-1R and non-specific cytotoxic cell receptor protein 1 (NCCRP-1, a surface protein which functions in target cell recognition and cytotoxicity). Similar localized intestinal effects of synbiotics have been observed in Atlantic salmon fed *Pediococcus acidilactici* (10^6 CFU g⁻¹) and scFOS (7 g kg⁻¹) for 63 days (Abid *et al.* 2013). The synbiotic application modulated the allochthonous gut microbiota, including the presence of *Ped. acidilactici*, and subsequently up-regulated the gene expression of pro-inflammatory cytokines (IL1 β , TNF α and IL8) and antiviral proteins (toll-like receptor 3 (TLR3) and Myxovirus resistance protein (MX-1)). Both of these studies indicate elevated immune responsiveness and the potential for improved disease resistance. Indeed, Lin *et al.* (2012) observed elevated peripheral total leukocyte counts, respiratory burst, lysozyme and superoxide activities, which afforded increased protection against *A. veronii* infection in koi fed a synbiotic application of *Bacillus coagulans* and COS. In addition, the inclusion of the synbiotic significantly improved SGR and FCR.

In three recent studies with gilthead sea bream, Cerezuela and colleagues evaluated the effect of *Bacillus subtilis* and inulin on immune-related gene expression and disease resistance against *P. damsela* subsp. *piscicida* (Cerezuela *et al.* 2012), gut microbiota and gut histology (Cerezuela *et al.* 2013a), and expression of different genes in the anterior intestine (Cerezuela *et al.* 2013b). Synbiotic administration significantly increased complement activity following 4 weeks of feeding, but not after 2 weeks of feeding. Respiratory burst activity was not affected after 2 and 4 weeks feeding. Serum IgM level was significantly higher after 2 weeks of feeding but not after 4 weeks. The expression of immune-related genes in the HK of fish fed synbiotic for 2 weeks displayed no significant effect, although the expressions of

all the genes were up-regulated compared with the control group. Surprisingly, the challenge experiment with *P. damsela* subsp. *piscicida* (IP) revealed that cumulative mortality of fish fed synbiotic was significant higher than that in the control group. The study of Cerezuela *et al.* (2013a), investigating the effect on intestinal morphology and allochthonous intestinal microbiota, revealed signs of damage in the anterior intestine similar to that reported in fish fed inulin (Olsen *et al.* 2001). Synbiotic administration significantly increased villi height and intestinal diameter, but reduced the number of goblet cells and microvilli height, compared to control fed fish. Intestinal microbiota evaluation by DGGE revealed that number of OTUs in fish fed synbiotic was significantly lower (6.0 ± 0.0) than the number (17.3 ± 0.9) detected in control fed fish. Cerezuela *et al.* (2013b) investigated the effect of synbiotic administration on intestinal gene expression in gilthead sea bream, and revealed that only β -actin and occludin were significantly affected by synbiotic supplementation, although the expressions of nearly all the genes were up-regulated compared with the control group. The conclusions of these studies are that the synbiotic application of *B. subtilis* and inulin increases some immune parameters, had a negative effect on gut morphology and gut microbiota, had less effect on intestinal gene expression in anterior intestine and had a negative effect on disease resistance towards *P. damsela* subsp. *piscicida*. Further investigations are warranted to ascertain if benefits can be achieved with optimized inclusion levels.

14.13 CONCLUDING REMARKS AND FURTHER PERSPECTIVES

Prebiotic administration in aquafeeds and their positive effects are becoming increasingly documented (Burr *et al.* 2005; Gatlin III *et al.* 2006; Denev *et al.* 2009; Yousefian and Amiri 2009; Ganguly *et al.* 2010; Merrifield *et al.* 2010; Ringø *et al.* 2010; Sweetman *et al.* 2010; Dimitroglou *et al.* 2011a; Gatlin III and Peredo 2012). However, the influence on the intestinal microbial communities (abundance, diversity and richness) and the ability of the intestinal microbiota to ferment selected prebiotics is in its infancy. The fermentability of a prebiotic is a key factor which influences its effects on fish growth performance, intestinal physiology and health, which is at least partially related to its degree of polymerization (DP). Thus, fermentation of prebiotics with various DPs should be investigated in *in vitro* and *ex vivo* studies in future. These types of studies will help to elucidate suitable prebiotics, and optimum levels of inclusion, for each different species dependent on our increasing knowledge of beneficial endogenous intestinal microbial communities. Promoting beneficial intestinal bacteria species with the use of prebiotics is a promising method for improving the overall condition of all animals. Altering the intestinal microbiota in favour of the beneficial bacteria may increase the production of beneficial metabolic products and at the same time may reduce the production of toxins. Hence, as has been reported in numerous studies, improved intestinal morphology is promoted which can lead to elevated nutrient absorption and increased growth performance.

Even though several comprehensive reviews have been published (most recently, Torrecillas *et al.* 2014), currently there are gaps in existing knowledge on prebiotic applications to fish and shellfish including various aspects of digestion, absorption, digestive enzymes, carcass composition, metabolism, and influences on physiological responses, especially expression of immunological genes, localized and systemic immunoglobulin production, gut morphology, gut microbiota and disease resistance in challenge studies. Additional information is

also needed in regard to age- and size-related responses, appropriate doses and timing of administration. Thus further research in these areas should be pursued.

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15 Prebiotic Applications in Shellfish

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ABSTRACT

The current research demonstrates great potential for the dietary application of prebiotics in the culture of larval, post larval and juvenile stages of shrimps and prawns (Penaeidae), freshwater crayfish (Astacidae and Parastacidae) and lobsters (Nephropidae and Palinuridae). Specific prebiotics used in such applications include mannanoligosaccharides (MOS), fructooligosaccharides (FOS), isomaltooligosaccharides (IMO), xylooligosaccharides (XOS) and inulin. Prebiotic applications have been demonstrated to increase growth and survival during culture, improve feed conversion, enhance development, elevate survival and improve immune status and/or stress tolerance. These benefits are mediated by modulated gastrointestinal (GI) microbiology and enhanced physical GI structures, which may account for improved feed conversion and consequently growth of the host species. This review thus demonstrates that by making positive changes in GI structure and bacterial community composition combined with influencing immune status, culture success can be enhanced through dietary prebiotic supplementation. However, it is clear that prebiotic efficiency is dependent on a number of independent and interacting factors specific to the culture conditions and host species; further research is required to elucidate these factors in order to improve prebiotic applications.

15.1 INTRODUCTION

Growing consumer awareness of the issues of sustainability has resulted in diverse efforts to improve the productivity of crustacean aquaculture. In 2008 a total of 142.3 million tonnes of seafood entered the market, of which 52.5 million tonnes originated from aquaculture (FAO 2010). Despite the fact that crustacean aquaculture accounted for only 5 million tonnes of this, crustacea have the second highest market value of all cultured seafood (FAO 2010). Therefore improving shellfish culture is paramount to increasing aquacultural outputs, thus keeping up

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with consumer demand and sustaining natural stocks. However increasing production creates complications as intensification can decrease water quality, increase stress and increase bacterial loading which ultimately reduces culture success and can lead to epidemic losses in stock (Robohm *et al.* 2005). Aquatic borne diseases are a major problem facing the development of commercial aquaculture and have resulted in significant economic losses and many industrial failures (Balcázar *et al.* 2006); indeed, disease problems caused Thai shrimp production to drop by 40% between 1994 and 1997 (Moriarty 1999). Alongside the quest to control bacterial epidemics by means other than chemotherapeutics, recent years have seen the search for alternative strategies to help prevent infectious aquatic diseases in crustacean species. The use of probiotics – live dietary supplements which can positively affect host health through manipulation of gut microbiota (Fuller 1989) – has been explored (*Chapters 7 and 11*). However, due to the limitations of probiotics, such as the viability of live bacteria in feed products and the viability and colonization of bacteria within the GI tract, the prebiotic concept has been developed (Roberfroid 2007). Prebiotics have shown potential for increasing growth and survival of shellfish by improving feed conversion, immune status and/or stress tolerance. Furthermore prebiotics have been shown to modulate the GI microbiology and enhance GI morphology, which may enable improved feed conversion and consequently growth. This chapter provides an overview of the types of prebiotics used in shellfish culture to date and their effects on organism development and physiology. Most research on shellfish has been conducted on penaeids due to the size and value of the penaeid culture industry, constituting more than half of all crustacean culture (FAO 2010).

15.2 USE OF PREBIOTICS IN SHELLFISH AQUACULTURE

Compared to the diverse range of prebiotics used in finfish aquaculture (*Chapter 14*), there are relatively few prebiotics documented in shellfish aquaculture. The prebiotics used in shellfish culture to date include oligosaccharides such as mannanoligosaccharide (MOS), fructooligosaccharide (scFOS and FOS), oligofructose, isomaltooligosaccharide (IMO) and xylooligosaccharide (XOS) and the polysaccharide inulin (Ringø *et al.* 2010; *Chapter 14*) (Table 15.1). Studies have applied these prebiotics via incorporation into dry feeds (e.g. Genc *et al.* 2007; Sang *et al.* 2009; Hai and Fotedar 2009) and live feed such as *Artemia* and rotifers (e.g. Daniels *et al.* 2010; Hoseinifar *et al.* 2010).

15.2.1 Mannanoligosaccharides

MOS is the most well documented prebiotic in shellfish culture and has shown potential in a range of shellfish species including shrimp, freshwater crayfish and lobsters (Genc *et al.* 2007; Hai and Fotedar 2009; Sang *et al.* 2009; 2011a; 2011b; 2014; Daniels *et al.* 2010; 2013; Sang and Fotedar 2010; Mazlum *et al.* 2011; Zhang *et al.* 2012). While relatively few studies have been conducted, these studies have demonstrated improved growth and/or survival with the dietary addition of MOS at different stages of development. In the freshwater crayfish (*Cherax destructor*), MOS was supplemented to juveniles via a formulated feed and administration increased specific growth rate (SGR) and average weight gain (Sang *et al.* 2011b). Similar results have been observed in post larval tiger shrimp (*Penaeus semisulcatus*) (Genc *et al.* 2007), juvenile black tiger prawn (*Penaeus monodon*) (Sang *et al.* 2014) and juvenile

Table 15.1 Prebiotic applications in shellfish species.

Crustacean species	Develop-mental stage	Prebiotic	Dose	Route of adminis-tration	Trial duration	Result	Reference
Shrimp							
Black tiger prawn <i>Penaeus monodon</i>	Juvenile (0.4 ± 0.06 g)	MOS	1, 2, 4 and 8 g kg ⁻¹	Dietary	63 days	↑ final weight, ↑ SGR, ↑ wet tail muscle index, ↓ dry tail muscle index, ↑ tail muscle protein, ↑ total haemocyte count, ↑ epithelial layer and ↑ epithelial cell density	Sang <i>et al.</i> (2014)
Pacific white shrimp <i>Litopenaeus vannamei</i>	Juvenile (2.52 ± 0.01 g)	MOS	1, 2, 4, 6 and 8 g kg ⁻¹	Dietary	56 days	↑ final weight (1–8 g kg ⁻¹), ↓ FCR (1–8 g kg ⁻¹), ↑ weight gain (2–8 g kg ⁻¹), ↑ SGR (2–8 g kg ⁻¹), ↑ microvilli length (2–8 g kg ⁻¹), ↑ survival (NH ₃ stress) (2–8 g kg ⁻¹)	Zhang <i>et al.</i> (2012)
Shrimp <i>Penaeus japonicus</i>	Juvenile (5.20 ± 0.15 g)	IMO + <i>Bacillus</i> spp.	2 g kg ⁻¹ in combination with different <i>Bacillus</i> spp. (<i>B. licheniformis</i> , <i>B. subtilis</i>)	Dietary	42 days	↑ total bacterial counts, ↓ <i>Vibrio</i> counts, ↑ phenoloxidase activity, ↑ lysozyme, ↑ nitric oxide synthase, ↑ superoxide dismutase activity, ↑ resistance against <i>V. alginolyticus</i>	Zhang <i>et al.</i> (2011)
Indian white shrimp <i>Fenneropenaeus indicus</i>	Mysis stage 1–3 and post larval stage 1–8	Inulin	60 mg l ⁻¹ at mysis stages and 50 mg l ⁻¹ at post larval stages	Enriched live feed – <i>Artemia nauplii</i>	14 and 22 days	→ mysis and post larvae growth performance, ↑ survival in post larvae	Hoseinifar <i>et al.</i> (2010)
Pacific white shrimp <i>Litopenaeus vannamei</i>	Fingerlings (0.0528 g)	XOS	0.1, 0.3 and 0.5 g kg ⁻¹	Dietary	56 days	→ final weight, → weight gain ratio, → SGR, ↓ FCR, ↑ feed efficiency (0.3 g kg ⁻¹), ↑ peroxidase, ↑ superoxide dismutase, ↑ lysozyme and ↑ phenoloxidase activity, ↑ serum albumin levels, ↑ body protein (0.1 g kg ⁻¹)	Wang <i>et al.</i> (2010)
Western king prawn <i>Penaeus latisulcatus</i>	Juveniles (4.63 ± 0.39 g)	MOS	5 g kg ⁻¹	Dietary	84 days	↑ SGR, ↓ FCR, → survival, ↓ % hyaline cells, ↑ % granular and semi granular cells, ↓ haemolymph clotting time	Hai and Fotedar (2009)
Pacific white shrimp <i>Litopenaeus vannamei</i>	Average weight = 1.75 g	IMO + <i>Bacillus</i> OJ	2 g kg ⁻¹ IMO + 10 ⁶ , 10 ⁸ CFU g ⁻¹	Dietary	28 days	↑ immune parameters (10 ⁸ + 2 g kg ⁻¹), ↑ immune parameters (2 g kg ⁻¹ IMO), ↑ protection from the white spot syndrome virus (WSSV) (10 ⁸ + 2 g kg ⁻¹)	Li J. <i>et al.</i> (2009)

(continued overleaf)

Table 15.1 (continued)

Crustacean species	Developmental stage	Prebiotic	Dose	Route of administration	Trial duration	Result	Reference
Pacific white shrimp <i>Litopenaeus vannamei</i>	Post larvae (1.68 ± 0.18 g)	Grobiotic-A® and dried <i>Saccharomyces cerevisiae</i>	20 and 50 g kg ⁻¹ Grobiotic-A® + 20 and 50 g kg ⁻¹ <i>Saccharomyces cerevisiae</i>	Dietary	28 days	→ weight gain, → feed efficiency, → bacterial clearance from haemolymph, → THC → haemolymph protein, → phenoloxidase activity, → haemocyte respiratory burst, ↑ survival (20 g kg ⁻¹ Grobiotic-A®, salinity stress)	Li P. <i>et al.</i> (2009)
Pacific white shrimp <i>Litopenaeus vannamei</i>	Average weight 7 g	Inactive yeast cell wall	1 and 2 g kg ⁻¹	Dietary	28 days	→ FCR, → SGR, → final weight, → feed intake, → protein efficiency ratio, → survival, ↑ THC (2 g kg ⁻¹), ↑ GHC (1 and 2 g kg ⁻¹)	Chotikachinda <i>et al.</i> (2008)
Tiger shrimp <i>Penaeus semisulcatus</i>	Post larvae (0.34 ± 0.01 g)	MOS	1.5, 3.0 and 4.5 g kg ⁻¹	Dietary	48 days	→ FCR, → SGR, → hepatopancreas tissue, in 3 g kg ⁻¹ treatment, ↑ final weight and ↑ survival, ↓ whole body protein (4.5 g kg ⁻¹)	Genc <i>et al.</i> (2007)
Pacific white shrimp <i>Litopenaeus vannamei</i>	adult (75.4 ± 0.8 g)	scFOS	0.25, 0.5, 0.75, 1, 2, 4 and 8 g kg ⁻¹	Dietary	42 days	→ weight gain, feed conversion and survival, scFOS affected gut microbiota, → haemocyte phenoloxidase, ↑ THC (1 and 8 g kg ⁻¹) and ↑ haemocyte respiratory burst (1 and 8 g kg ⁻¹)	Li <i>et al.</i> (2007)
Pacific white shrimp <i>Litopenaeus vannamei</i>	Juvenile (0.17 g)	scFOS	0, 0.4, 0.8, 1.2 and 1.6 g kg ⁻¹	Dietary	56 days	↑ growth rate, feed intake, feed conversion, scFOS affected gut microbiota	Zhou <i>et al.</i> (2007)
Freshwater crayfish							
<i>Asiatasus leptodactylus</i>	Juvenile (1.25 ± 0.43 g)	MOS	1.5, 3.0, and 4.5 g kg ⁻¹	Dietary	60 days	→ final weight and final length, → FCR, ↑ SGR, → survival, → body composition	Mazlum <i>et al.</i> (2011)
<i>Cherax tenuimanus</i>	4.44 ± 0.20 g	MOS	0.5, 1, 2, 4 and 8 g kg ⁻¹	Dietary	112 days	↑ survival (2 and 4 g kg ⁻¹), ↑ biomass increment (2 and 4 g kg ⁻¹), ↑ wet tail muscle index (0.5–8 g kg ⁻¹), ↑ dry tail muscle index (0.5–8 g kg ⁻¹), ↑ osmoregulatory capacity (1, 2 and 4 g kg ⁻¹), ↓ bacteraemia (0.5–8 g kg ⁻¹), ↑ THC (0.5–4 g kg ⁻¹) ↓ haemolymph clotting time (2 and 4 g kg ⁻¹) and ↑ % granular cells (1–4 g kg ⁻¹)	Sang <i>et al.</i> (2011a)

<i>Cherax destructor</i>	Adult (35.14 ± 0.48 g)	MOS	4 g kg ⁻¹	Dietary	56 days	→ survival, ↑ weight, ↑ SGR, ↑ weight gain, ↑ THC, ↑ granular cells and semi-granular cells, ↑ protease and amylase activities Sang <i>et al.</i> (2011b)
<i>Cherax tenuimanus</i>	Juvenile (4.44 ± 0.20 g)	MOS	2 and 4 g kg ⁻¹	Dietary	30, 42 and 112 days	↑ survival, → THC (<i>Vibrio</i> , transportation), ↑ THC (NH ₃ stress), → <i>Vibrio</i> spp. in haemolymph, ↓ haemolymph clotting time, ↓ neutral red retention time Sang <i>et al.</i> (2009)
Lobster						
European lobster <i>Homarus gammarus</i> L.	Larval stage I–III (12.8–14.6 mg)	MOS and <i>Bacillus</i> spp.	12 mg l ⁻¹	Enriched live feed – <i>Artemia</i> nauplii	12 days	↑ weight, ↑ carapace length, ↑ salinity stress tolerance, ↑ microbial species richness, ↑ diversity, ↓ GI <i>Vibrio</i> levels Daniels <i>et al.</i> (2013)
European lobster <i>Homarus gammarus</i> L.	Larval stage I–IV (average 11.05–12.00 mg)	MOS and <i>Bacillus</i> spp.	12 mg l ⁻¹	Enriched live feed – <i>Artemia</i> nauplii	30 days	↑ growth performance, ↑ SGR, ↓ FCR, ↑ survival, ↑ gut absorptive surface area, ↑ microvilli length and density, affect gut microbiota Daniels <i>et al.</i> (2010)
Tropical spiny lobster <i>Panulirus ornatus</i> , Fabricius 1798	Juvenile (1.28 ± 0.01 g)	MOS	4 g kg ⁻¹	Dietary	56 days	↑ SGR, ↑ weight gain, ↑ survival, ↑ wet tail muscle index, wet hepatosomatic index and dry tail muscle index, ↑ total aerobic and <i>Vibrio</i> spp. in GIT, ↑ GIT absorptive surface area, ↑ THC and granular cell proportion, ↓ bacteraemia, ↑ resistance against bacterial infection Sang and Fotedar, (2010)
European lobster <i>Homarus gammarus</i> L.	Larval stage I–IV, juvenile IV–VIII	MOS	0.072, 0.72 and 7.2 g l ⁻¹	Enriched live feed – <i>Artemia</i> nauplii		↑ survival (0.072 and 0.72 g l ⁻¹), ↓ survival (7.2 g l ⁻¹), ↑ growth (0.72 g l ⁻¹), ↓ bacterial counts in <i>Artemia</i> culture Daniels <i>et al.</i> (2006)

Symbols represent an increase (↑), decrease (↓) or no effect (→) of prebiotic administered.

FOS = fructooligosaccharide; scFOS = short chain fructooligosaccharide; IMO = isomaltooligosaccharide; MOS = mannanoligosaccharide; XOS = xylanoligosaccharide.

western king prawn (*Penaeus latisulcatus*) (Hai and Fotedar 2009) fed MOS through formulated feeds. MOS provided to larval European lobster (*Homarus gammarus*), via *Artemia*, improved growth and survival in a dose-dependent manner. Inclusion of MOS at levels of 0.072 and 0.72 g l⁻¹ of *Artemia* culture water increased survival and growth compared to the control larvae, though higher concentrations resulted in decreased survival and growth (Daniels *et al.* 2006). Later studies on larval European lobster also showed significant improvements in SGR, feed conversion and survival with MOS supplementation at 12 mg l⁻¹ of *Artemia* culture water (Daniels *et al.* 2010).

MOS is a glucomannan-protein complex derived from mannans and presents mannose-binding sites (Newman 1994; Miguel *et al.* 2002; Fritts and Waldroup 2003). Attachment and colonization of bacteria on the epithelial cells of the intestine are mediated by specific lectins on the bacterial cell surface (Newman 1994). A number of important intestinal Gram-negative pathogens often present mannan-specific fimbriae that will bind to the mannan receptors on MOS which is therefore hypothesized to reduce pathogen adhesion to intestinal epithelial cells (Spring *et al.* 2000; Ferket *et al.* 2002; Pryor *et al.* 2003). As well as preventing pathogen attachment, MOS has also been shown to actively displace pathogens from epithelial cells on the mucosal surface (Newman 1994). The dietary application of MOS is well documented to provide beneficial changes in GI microbial populations in finfish (Dimitroglou *et al.* 2011; Chapter 14) though little work is documented on GI microbial changes in shellfish. A recent study on the dietary supplementation of MOS in European lobster showed stabilized bacterial communities and a reduction in total heterotrophic bacterial counts (Daniels *et al.* 2010). The results of this study also demonstrated that MOS improved GI structure by enhancing GI absorptive surface area and increasing the uniformity, length and density of microvilli (Figure 15.1). This effect on GI morphology and modulation of the bacterial community may improve digestive function and nutrient absorption, thus leading to improved feed conversion and growth as observed elsewhere (Genc *et al.* 2007; Hai and Fotedar 2009). Furthermore, Sang *et al.* (2011b) observed enhanced amylase and protease activities in the mid gut and hepatopancreas of MOS fed freshwater crayfish which may also contribute towards improved nutritional utilization and growth.

Enhanced innate immunity has been achieved by dietary application of MOS in shellfish species; for example, enhanced immune response was observed in Pacific white shrimp (*Litopenaeus vannamei*) with the administration of dietary MOS at 2 and 4 g kg⁻¹ (Fisher *et al.* 2001). Chotikachinda *et al.* (2008) also reported enhanced immune status, as demonstrated by increased total haemocyte counts (THC) and granular haemocyte counts in the blood of *L. vannamei* with the dietary inclusion of inactive yeast cell wall (a source of MOS and β -glucans) at 1 and 2 g kg⁻¹. A more recent study by Sang *et al.* (2009) on freshwater crayfish (*Cherax tenuimanus*) also reported enhanced immunity, as measured by THC, differential haemocyte counts (DHC), haemolymph clotting time and reduced haemolymph *Vibrio* load after *Vibrio mimicus* challenge, with the dietary addition of MOS at 2 g kg⁻¹ and 4 g kg⁻¹ of the diet. This study also showed improved immune capability measured by THC and haemolymph clotting time in the presence of environmental stressors (such as exposure to NH₃ and air) and bacterial infections. Similar enhancements in immunological parameters (specifically THC) have also been observed in juvenile *P. monodon* fed MOS supplemented diets (Sang *et al.* 2014).

These studies demonstrate the potential for the applications of MOS to improve crustacean immune status, gut microbiota and gut morphology, increasing nutrient uptake and feed conversion, improving growth and enhancing survival.

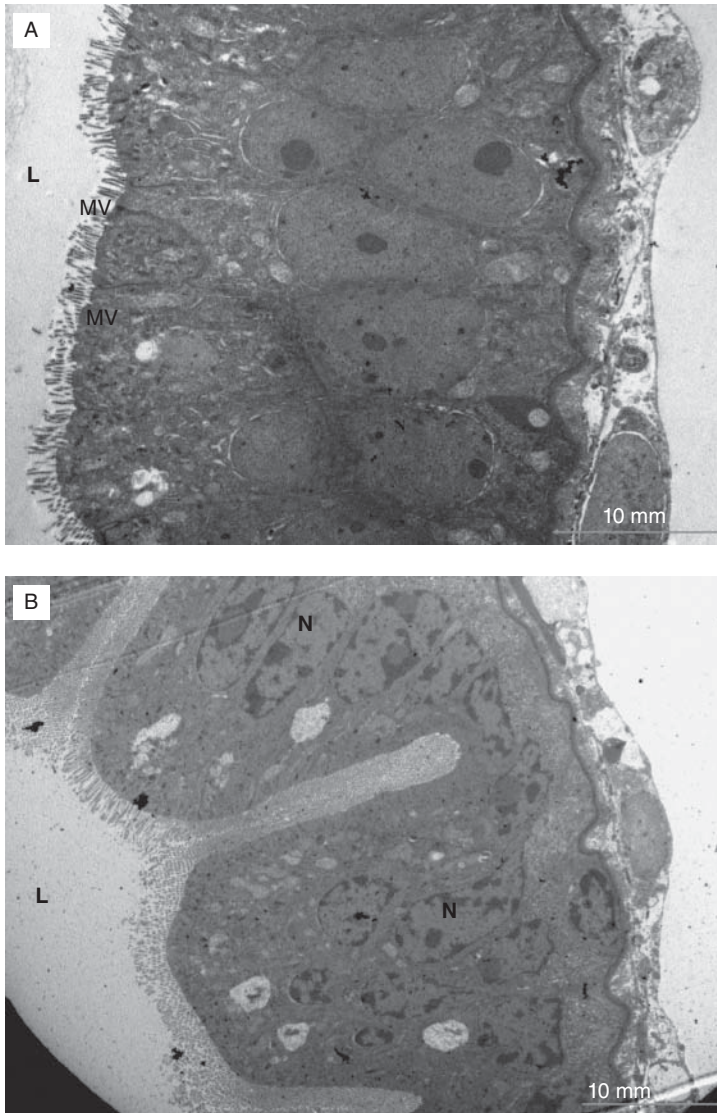


Fig. 15.1 Transmission electron micrographs of stage II larval lobsters showing the variation in the degree of mucosal folding present at the apical brush border and the microvilli structure of (A) control fed and (B) MOS fed individuals. Key: L, lumen; MV, microvilli; N, nuclei. Some stain precipitation is visible on micrographs. Scale bar = 10 μ m. (Source: Daniels *et al.* 2010.)

15.2.2 Fructooligosaccharides

Fructooligosaccharides (FOS) and oligofructose are naturally present in a variety of foods such as banana, barley, garlic, honey, onion, wheat, rye, triticale and Jerusalem artichokes. Commercial scale FOS mixtures are produced from inulin degradation or transfructosylation processes (Van Loo *et al.* 1995). FOS have been commonly studied in the shrimp industry, though relative to finfish culture (Ringø *et al.* 2010; Dimitroglou *et al.* 2011; *Chapter 14*) their application is still comparatively scarce in shellfish. In shellfish, improvements in growth rates (SGR), feed intake and feed conversion have been documented with the dietary application of a commercial short chain fructooligosaccharide supplement (scFOS; Profeed®) at 0.4–1.6 g kg⁻¹ in Pacific white shrimp (Zhou *et al.* 2007). However, a study by Li *et al.* (2007) observed no significant enhancements in weight gain or feed efficiency with the dietary supplementation of a different scFOS (Fortifeed®), at concentrations of 0.25 to 8 g kg⁻¹, in the same species. Of the shellfish studies presently available (Li *et al.* 2007; Zhou *et al.* 2007), neither have documented the significant increases in survival often reported in finfish fed FOS (Ringø *et al.* 2010; *Chapter 14*). However, enhanced immunity has been documented in Pacific white shrimp with the dietary supplementation of scFOS whereby elevated levels of both THCs and haemocyte respiratory burst activity were observed when fed at 1 and 8 g kg⁻¹ feed (Li *et al.* 2007). Changes in immunity were concomitant with changes in GI microbiota, causing distinct shifts in the intestinal tract microbial populations from individuals fed basal diets without scFOS supplementation. However, the changes in bacterial populations seen in *L. vannamei* were potentially negative at low level scFOS supplementation (0.4 and 0.8 g kg⁻¹), with increases in the presumptive levels of *Vibrio parahaemolyticus* in the GI tract; this was not observed in *L. vannamei* fed the highest scFOS concentrations (Zhou *et al.* 2007).

15.2.3 Isomaltooligosaccharides

Isomaltooligosaccharides (IMO) are glucosyl saccharides with α -(1,6)-glucosidic linkages. They occur naturally in several fermented foods and are commercially produced from starch by enzymatic processes (Kuriki *et al.* 1993). There are limited studies available concerning IMO applications in shellfish. To the authors' knowledge information is restricted to two shrimp studies (Li J. *et al.* 2009; Zhang *et al.* 2011). Li J. *et al.* (2009) reported that dietary IMO (2 g kg⁻¹) did not affect Pacific white shrimp immune response, disease resistance or intestinal microbiota. Survival of shrimp was not affected in either study (Li J. *et al.* 2009; Zhang *et al.* 2011). However, synergistic interactions were observed when IMO was used in combination with *Bacillus* (as a synbiotic) promoting enhanced immune response and disease resistance as indicated by phenoloxidase activity, haemocyte phagocytic activity, respiratory burst activity and phosphatase activity. Similar synbiotic effects, including increased immune status, improved survival after *Vibrio alginolyticus* challenge, and modulated GI microbiota (i.e. reduced *Vibrio* levels) were observed with dietary supplementation of IMO (2 g kg⁻¹) in combination with *Bacillus* spp. in kuruma shrimp (*Penaeus japonicus*) (Zhang *et al.* 2011). However, this study did not investigate the efficacy of the individual supplementation of IMO.

15.2.4 Xylooligosaccharides

Xylooligosaccharides (XOS) are sugar oligomers consisting of xylose units which occur naturally in fruits, vegetables, bamboo, honey and milk. Commercial scale XOS are produced

from xylan-rich materials (Vázquez *et al.* 2000). There remains a paucity of research on the use of XOS in shellfish species, with only one study on *L. vannamei* published (Wang *et al.* 2010). Wang and colleagues (2010) observed that XOS provided enhancements in feed efficiency and immune parameters (increased peroxidase activity, superoxide dismutase activity, lysozyme activity, phenoloxidase activity and serum albumin levels) when fed at 0.3 g kg⁻¹. Enhanced feed conversion was also reported at varying dietary levels of 0.1–0.5 g kg⁻¹ and shrimp total body protein was increased with supplementation at 0.1 g kg⁻¹ XOS.

15.2.5 Inulins

Inulins are naturally occurring fructans with a degree of polymerization of 2 to 60. Inulin is mainly extracted from chicory roots; however it can be found in a variety of common foods such as garlic, onion, artichoke and asparagus (Van Loo *et al.* 1995). As with XOS, the use of inulin appears to be limited in shellfish. A study by Hoseinifar *et al.* (2010) supplied a commercial source of inulin (Raftiline ST) via *Artemia* to Indian white shrimp (*Fenneropenaeus indicus*) larvae and post larvae. No significant effect of inulin was recorded on shrimp growth for either larvae or post larvae stages, nor was survival affected during larval stages. However, significant increases in survival were observed in post larvae at 22 days post hatching.

15.2.6 Other prebiotics

The application of the commercial prebiotic GroBiotic-A[®], a mixture of partially autolysed brewer's yeast, dairy components and fermentation products including oligosaccharides (Gatlin *et al.* 2008), has been reported to have no effect on the growth or survival of Pacific white shrimp under non-challenged conditions (Li P. *et al.* 2009). However, the study also reported elevated survivability of *L. vannamei* fed 2% GroBiotic-A[®] after a salinity challenge.

Other established prebiotics such as galactooligosaccharides (GOS) and arabinoxylooligosaccharides (AXOS) have shown potential in teleost species (Chapter 14) but no reports of their use in crustacean species are available at present.

15.3 PREBIOTIC BENEFITS

Documented prebiotic studies in shellfish often focus on growth, survival and disease resistance, though fewer studies have sought to elucidate the causative actions behind these apparent benefits. Causative actions highlighted to date include enhanced immune status (Li *et al.* 2007; Hai and Fotedar 2009; Sang *et al.* 2009; 2011a; 2011b; Sang and Fotedar 2010; Zhang *et al.* 2011), improved GI morphology (Daniels *et al.* 2010; Sang and Fotedar 2010), modulated GI microecology (Li *et al.* 2007; Zhou *et al.* 2007; Daniels *et al.* 2010; 2013; Sang and Fotedar 2010; Zhang *et al.* 2011) and enhanced digestive enzyme activity within the GI tract (Wang *et al.* 2010). It is evident that there is likely to be a complicated suite of interactive effects, which work concomitantly to bring about the observed host benefits. Despite a lack of comprehensive studies which focus on the mechanistic actions which underpin the prebiotic success commonly reported in shellfish, it is documented that prebiotics can improve shellfish survival, immune status, disease resistance, growth performance and feed utilization (Table 15.1).

15.3.1 Growth and survival

One of the main objectives of prebiotic studies is to induce direct improvements in culture success. Improvements in growth and/or survival have previously been reported in shellfish species with the use of prebiotics (Genc *et al.* 2007; Zhou *et al.* 2007; Hai and Fotedar 2009; Sang *et al.* 2009; Sang and Fotedar 2010; Sang *et al.* 2011a; 2011b). The extent of growth and/or survival enhancements is dependent on the developmental stage, the prebiotic type, the dosage, the duration of application and the culture conditions (Table 15.1). MOS applied singularly, and in combination with *Bacillus* probiotics, has been reported to enhance culture success in larval European lobster (Daniels *et al.* 2010). Greater improvements in growth performance were achieved with the combined dietary application of MOS and *Bacillus* spp.; however, this trend was not apparent in respect to survival where both MOS and MOS + *Bacillus* spp. fed larvae showed equally elevated levels of survivability compared to the control (Daniels *et al.* 2010). Comparatively using a different culture technique (green water), during European lobster culture, provided markedly different results in growth and survival (Daniels *et al.* 2013) to those seen in the earlier study. In this study survival and growth were similar in larvae fed prebiotics and probiotics both individually and in combination, though these results were significantly greater than non biotic fed larvae. Similar varying enhancements in survival have been shown with the combined use of other prebiotics and probiotics (Li P. *et al.* 2009). Positive effects of MOS on growth performance have also been documented in western king prawn (Hai and Fotedar 2009), tiger shrimp (Genc *et al.* 2007), *C. destructor* (Sang *et al.* 2011b) and tropical spiny lobster (*Panulirus ornatus*) (Sang and Fotedar 2010). However, in a study on freshwater crayfish (*Astacus leptodactylus*), Mazlum *et al.* (2011) reported that varying levels (1.5, 3.0 and 4.5 g kg⁻¹) of dietary MOS had no significant effect on final weight or FCR. MOS has been demonstrated to enhance survival in Marron *Cherax tenuimanus* when provided at 2 and 4 g kg⁻¹ (Sang *et al.* 2009; Sang *et al.* 2011a), Pacific white shrimp (*Litopenaeus vannamei*) at 2, 4, 6 and 8 g kg⁻¹ feed (Zhang *et al.* 2012), tiger shrimp at 3 and 4.5 g kg⁻¹ feed (Genc *et al.* 2007) and tropical spiny lobster at 4 g kg⁻¹ feed (Sang and Fotedar 2010). These studies however demonstrated no effect, in comparison to control individuals, on survival or growth at lower MOS supplementation levels. Contrary to these studies, survival was unaffected in western king prawn fed MOS at 5 g kg⁻¹ (Hai and Fotedar 2009). Enhancements in survival with alternative prebiotic dietary supplements have also been documented (Table 15.1), for example in white shrimp (*Fenneropenaeus indicus*) fed inulin supplemented diets (Hoseinifar *et al.* 2010). The enhancements in growth and survival documented with prebiotics can in part be ascribed to a suite of interrelated developments in GI morphology, GI microbiology, GI enzyme activity and immune status, as described in the following sections.

15.3.2 Immunomodulation and disease resistance

It has been suggested that the innate immune response is biologically linked to gut health (Gómez and Balcázar 2008; Perez *et al.* 2010). As the establishment of a commensal and/or defensive microbiota is a key factor in the exclusion of potential opportunistic pathogens and sustaining health, there is clearly a delicate balance between the immune system and the GI microbiota (Gómez and Balcázar 2008). Furthermore it is becoming increasingly clear that the host–microbiota interactions at the mucosal interface play key roles in regulating both localized and systemic immunological status. In shellfish the innate/non-specific immune system is the primary defence mechanism against pathogenic microorganisms in which haemocytes (coelomocytes in lower invertebrates) play key functions (Lee and Söderhäll

2002). An improved immune status can thus be inferred by an increase in the number of circulating haemocytes/coelomocytes as well as an increase in the level (or proportion) of granulocytes due to their phagocytic and respiratory burst functions in shellfish (De Guise *et al.* 2008).

The application of dietary scFOS can enhance total haemocyte levels in Pacific white shrimp (Li *et al.* 2007) and changes in haemocyte counts and/or granulocyte proportions have also been reported with dietary MOS supplementation in *C. tenuimanus* (Sang *et al.* 2009; 2011a) and *C. destructor* (Sang *et al.* 2011b), tropical spiny lobster (Sang and Fotedar 2010) and western king prawn (Hai and Fotedar 2009). The increase in total haemocyte counts reported in freshwater crayfish *C. tenuimanus* increased immune capability and improved the host's defence against *V. mimicus* infection and abiotic stressors such as air and NH₃ exposure (Sang *et al.* 2009). While no such changes at the cellular level of the immune system have been reported with other prebiotic supplements in shellfish, alternative indicators of immune status have also been assessed. These immune parameters include haemocyte respiratory burst activity, phenoloxidase activity, lysozyme activity, peroxidase activity, superoxide dismutase activity, nitric oxide synthase activity, serum albumin and protein levels, clotting time, haemolymph bacterial loading and neutral red retention time (Li *et al.* 2007; Hai and Fotedar 2009; Li J. *et al.* 2009; Li P. *et al.* 2009; Sang *et al.* 2009; 2011a; Sang and Fotedar 2010; Wang *et al.* 2010; Zhang *et al.* 2011). For example, enhanced humoral immune defences including phenoloxidase, lysozyme, nitric oxide synthase, and superoxide dismutase activities have been observed with the dietary addition of IMO in the shrimp *Penaeus japonicus* (Zhang *et al.* 2011). It has been suggested that prebiotic may enhance the immunity in host species by stimulating the growth of selected bacteria such as lactic acid bacteria and *Bacillus* spp. at the expense of potentially pathogenic and opportunistic species (Shan *et al.* 2006). Shifts in the GI microbial communities may therefore affect the host immunological status as microbes possess cell wall components, such as lipopolysaccharides, peptidoglycan and β -glucans, that have immunomodulatory properties in shellfish (Sritunyaluksana *et al.* 1999; Chang *et al.* 2000; Sung *et al.* 2000; Hai and Fotedar 2009; Xian *et al.* 2009; Bai *et al.* 2010; Chang *et al.* 2011). For example, the bacterial cell wall components peptidoglycan and lipopolysaccharides have been shown to increase phenoloxidase (PO) activity in black tiger prawn (*Penaeus monodon*) (Sritunyaluksana *et al.* 1999). Another major component of probiotic Gram-positive bacterial cell walls is lipoteichoic acid (LTA) which has reported anti-inflammatory and immunostimulatory effects (Thomas and Versalovic 2010).

Not all studies have reported enhanced immunity with the dietary application of prebiotics in shellfish; for instance, Li P. *et al.* (2009) reported no change in total haemocyte counts, haemolymph protein, phenoloxidase activity, haemocyte respiratory burst or bacterial clearance from haemolymph with the dietary application of Grobiotic-A[®] fed to white shrimp. Studies have also shown varying effects on immune response dependent on the parameters studied; for example, haemocyte respiratory burst activity was improved in Pacific white shrimp fed scFOS, though haemocyte phenoloxidase was unaffected (Li *et al.* 2007). Other studies show varying degrees of efficacy dependent on the prebiotic dosage; Sang *et al.* (2011a) observed positive effects of MOS on haemolymph clotting time in marron (*C. tenuimanus*) at dietary levels of 2 and 4 g kg⁻¹ in comparison to control fed individuals, although this was not observed at 0.5, 1 and 8 g kg⁻¹. Li J. *et al.* (2009) reported positive effects of IMO in combination with *Bacillus* sp. on THC in *L. vannamei*, although at higher concentrations of dietary IMO (in combination with *Bacillus*) results similar to those of control fed individuals were observed. Indeed, immunosuppression has previously

been proposed in the presence of high concentrations of immunostimulatory feed additives (Bricknell and Dalmo 2005) which may be a result of an overstimulation of the immune system (Sakai 1999). This was hypothesized to be the case where higher concentrations of MOS supplementation (7.2 g l^{-1} live feed culture water) reduced European lobster survival, compared to the control and lower MOS concentrations (0.072 and 0.72 g l^{-1}) (Daniels *et al.* 2006).

Despite the potential for immunosuppression at high dosage levels, the ability of prebiotics to positively modulate the immune response has shown great potential to enhance disease resistance in shellfish culture (Li J. *et al.* 2009; Sang *et al.* 2009; Sang and Fotedar 2010; Zhang *et al.* 2011). For instance dietary application of IMO has been reported to increase resistance against *Vibrio alginolyticus* in shrimp (*Penaeus japonicus*) (Zhang *et al.* 2011) and against the white spot syndrome virus (WSSV) in Pacific white shrimp (Li J. *et al.* 2009). Similar enhancements in disease resistance have also been demonstrated when supplementing MOS to the diet of freshwater crayfish, as indicated by a reduction of *Vibrio* levels within the haemolymph of individuals fed MOS in comparison to control fed individuals, following *V. mimicus* infection (Sang *et al.* 2009). A later study by Sang and Fotedar (2010) also showed enhanced disease resistance as indicated by increased survival in the tropical spiny lobster fed with MOS at 0.4 g kg^{-1} when challenged with *Vibrio* spp. This study also showed reduced haemolymph bacteraemia and reduced THC and granular haemocyte counts associated with enhanced survival post *Vibrio* challenge (Sang and Fotedar 2010). A later study also reported reductions in bacterial concentrations within the haemolymph, similar to those recorded with enhanced disease resistance, showed in freshwater crayfish with the dietary use of MOS (Sang *et al.* 2011a). However, alternative studies supplementing Grobiotic-A[®] (Li P. *et al.* 2009) and MOS (Zhang *et al.* 2012) to the diets of *L. vannamei* showed no effect on the clearance of bacteria from haemolymph, although it should be noted that both of these studies used alternative stressors to disease.

15.3.3 GI microbiota

Despite the fact that prebiotic benefits are driven by enhanced growth or activity of beneficial microbes, there remains a scarcity of information available on the effect of prebiotics on shellfish GI microecology with the use of prebiotics (Li *et al.* 2007; Zhou *et al.* 2007; Daniels *et al.* 2010; 2013; Sang and Fotedar 2010). Applications of MOS have been reported to increase the stability of bacterial populations in the GI tract of larval European lobster by increasing the similarity of microbial profiles within treatment replicates (Daniels *et al.* 2010). In this study culture-dependent techniques revealed that dietary MOS did not affect *Vibrio* levels but did reduce the total number of aerobic heterotrophic bacteria at certain larval stages. In tropical spiny lobster juveniles fed MOS, however, elevated cultivable GI total aerobic bacteria and *Vibrio* spp. levels have been reported (Sang and Fotedar 2010).

A study by Li *et al.* (2007) also assessed whole bacterial community changes in GI microbiota using denaturing gradient gel electrophoresis (DGGE), which showed microbial community changes with the application of scFOS in *L. vannamei*. scFOS decreased the abundance of a *Roseobacter* sp. and increased abundance of a number of unidentified uncultured microbes (most similar to microbes from aquatic environments, sediments and the GI tract of mammals) and *Alkalibacillus* sp. Overall, the microbial communities from the two scFOS treatments (0.1% and 0.8% dietary inclusion) displayed 74.9% similarity to the control community; in contrast, the communities of the two scFOS treatments were highly similar to each other

(92.3%). Using selective agar Zhou *et al.* (2007) reported that dietary scFOS increased the proliferation of *Streptococcus faecalis* and *Vibrio parahaemolyticus* in the GI microbiota of Pacific white shrimp *L. vannamei*.

Considering the fact that prebiotic effects are driven, in part at least, by influencing the host microbiota, it is clear that this topic has not been sufficiently explored in shellfish. Future studies should utilize quantitative techniques such as FISH, qPCR and metagenomics to elucidate the impact of prebiotics on the complex microbial communities within the GI tract of shellfish. The host–microbe interactions at the intestinal epithelium are a key determinant of gastric morphology, activity and immunity.

15.3.4 GI morphology and digestion

Prebiotic induced improvements in the length, regularity and density of mucosal folds and/or improvements in microvilli formations have been documented in a number of teleost species (Pryor *et al.* 2003; Yilmaz *et al.* 2007; Salze *et al.* 2008; Sweetman *et al.* 2008; Dimitroglou *et al.* 2009; 2010a; 2010b). It has been suggested that oligosaccharides can promote the build-up of lactic acid (and other short-chain fatty acids (SCFAs)) in the GI tract and that such acids induce mucosal cell proliferation (Lji *et al.* 2001), thus improving mucosal structure. Moreover, SCFAs, mainly acetate, propionate and butyrate, are produced as a result of prebiotic fermentation and these SCFAs are considered an important energy sources for intestinal enterocytes (Scheppach 1994). It has also been suggested that the acids produced by LAB can cause higher solubility of minerals, increasing the uptake of metal ions (Merrifield *et al.* 2010a).

Despite the plethora of information in finfish, such effects in shellfish have only been investigated with the use of MOS in lobsters and shrimp (Genc *et al.* 2007; Daniels *et al.* 2010; 2013; Sang and Fotedar 2010; Sang *et al.* 2014). Genc *et al.* (2007) studied the effects of MOS on the hepatopancreas histology in tiger shrimp and reported no effect on the morphology of the hepatopancreas tissue. In a later study the application of MOS in larval European lobster resulted in elevated intestinal microvilli length and density (Daniels *et al.* 2010; Figure 15.1). The addition of MOS to juvenile spiny lobster diets also improved absorptive surface area, as measured by the internal perimeter to external perimeter ratio of the gut wall using light microscopy (Sang and Fotedar 2010). Increased epithelium thickness and epidermal cell density have also been reported in juvenile brown tiger prawn (*P. monodon*) fed diets supplemented with MOS at lower concentrations (1–2 g kg⁻¹) (Sang *et al.* 2014). These improvements in the development of GI mucosal structure provide a larger surface area for the absorption of nutrients, thus potentially allowing for improved feed conversion and growth (as documented in Table 15.1).

However, the reports of enhanced feed conversion could also be due to enhanced digestive enzyme activity. Prebiotics provide a nutrient source for bacteria and thus prebiotics can enhance the presence of certain bacteria which may aid digestion or provide vitamins (Gibson 2004; De Vrese and Schrezenmeir 2008). For example, FOS can be fermented by bacteria including lactobacilli and bifidobacteria (although bifidobacteria are not commonly reported in fish) which express the enzyme β -fructosidase (Sghir *et al.* 1998; Manning and Gibson 2004). *Bacillus* spp., which can ferment oligosaccharides (Mahious *et al.* 2006), are also known to generate enzymes that aid the digestion of proteins and starch via the production of protease and amylase, respectively (Ellouz *et al.* 2001; Ben Messaoud *et al.* 2004; Konsula and Liakopoulou-Kyriakides 2004; Liu *et al.* 2009). Stimulation of such exogenous enzymes

may also stimulate the production of endogenous enzymes by the host, further enhancing enzyme activity (Zhou *et al.* 2009). Few studies have addressed this topic in shellfish; however, the dietary application of MOS to freshwater crayfish has been reported to improve amylase activity in the mid gut and protease activity in the hepatopancreas which subsequently improved digestion capabilities and thus improved growth of crayfish (Sang *et al.* 2011b).

15.4 CONCLUSION

This chapter highlights the great potential shown for the use of dietary prebiotics in shellfish culture. However, compared to the reported applications of prebiotics in finfish species there is a scarcity of accessible studies at present. This discrepancy in the level of research between shellfish and finfish is almost certainly related to the size of the aquaculture industry of the associated species. Regardless of this, the benefits of using dietary prebiotics in shellfish culture are evident with reported improvements in growth, feed conversion, survival, immune status, GI morphology, GI microbiota and GI enzyme activity. However, it must be noted that the success of applying dietary prebiotic supplements appears to depend on many factors and is thus governed by specific circumstances: the prebiotic under consideration, length and dosage of application, diet, temperature, salinity, oxygenation and reproductive status (Hauton and Smith 2004). One such example, life stage, was highlighted by Hoseinifar *et al.* (2010) where inulin was shown to elevate the survival of Indian white shrimp (*Fenneropenaeus indicus*) post larvae but not larvae. Dose-dependent effects of prebiotics have also been highlighted in European lobster where an increased concentration of MOS caused negative effects on larval culture success compared to lower doses of MOS (Daniels *et al.* 2006). Dose-dependent effects were also reported in shrimp fed scFOS (Li *et al.* 2007), XOS (Wang *et al.* 2010) and MOS (Genc *et al.* 2007; Zhang *et al.* 2012) and freshwater crayfish fed MOS (Sang *et al.* 2009; 2011a).

The use of prebiotics in shellfish culture warrants greater attention and particular consideration should be paid to understanding the causative actions behind the prebiotic host benefits in order to improve the overall efficacy of applications. This is particularly important due to the ever increasing need to improve aquaculture productivity to support the growing demand for shellfish.

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16 Live Feeds: Microbial Assemblages, Probiotics and Prebiotics

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ABSTRACT

Rearing of most species of marine fish larvae relies on the use of live feeds. Bacteria in and associated with live feeds have a major influence on the microbiota of larvae. Live feed can be a vector of entry of specific pathogens or non-specific opportunistic bacteria to the larvae. Basic knowledge on bacterial communities associated with live feeds and the development of methods to control bacterial microbiota in live feed production are crucial to enhance larval survival. Environmental and health concerns have resulted in a progressive reduction of the use of chemotherapeutics and in a search for alternatives, based on considerations of bacteria as an active part of rearing ecosystems. The present chapter discusses bacterial aspects of live feed, reviews the use of probiotics and prebiotics as a strategy to improve live feed cultures, and also considers the use of live feed as a vector to supply prebiotics and probiotics to fish larvae cultures.

16.1 INTRODUCTION

Live feeds are necessary in the rearing of most species of marine fish larvae as the digestive system is not yet fully developed during the early developmental stages and the larvae are unable to capture and ingest artificial diets (Conceição *et al.* 2010). Initially, larvae are fed on rotifers and *Artemia*. Due to their small size and slow swimming velocity, rotifers, such as *Brachionus plicatilis*, are a suitable and widely used prey in first feeding of fish larvae (Dhert *et al.* 2001). *Artemia* are able to form cysts, which facilitates storage and culture, and different life stages of *Artemia* (nauplii, metanauplii and adults), with sizes adapted to the different steps of developing fish larvae, can be offered to fish larvae (Dhont and Lavens 1996). Copepods are the natural prey in most fish species in nature, being considered more adequate than rotifers and *Artemia* because of their high nutritional value, with higher contents in unsaturated fatty

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acids such as eicosapentaenoic (EPA) and docosahexaenoic (DHA) (Støttrup 2003; Drillet *et al.* 2006; Ajiboye *et al.* 2011) and a longer passage time through the fish gut (Pedersen 1984). Copepods can also be administered under different forms (nauplii, copepodites, adults) adapted to different larvae sizes. Copepods with potential use in aquaculture belong to three orders: Harpacticoids, such as *Tisbe* spp., are epibenthic copepods, with in many cases pelagic nauplii; and Cyclopoids and Calanoids, such as *Acartia* sp., are pelagic copepods suitable for feeding a wide range of species.

Fish larvae have a requirement of specific highly unsaturated fatty acids (HUFAs), such as DHA and EPA, in their diet (Watanabe *et al.* 1983; Rainuzzo *et al.* 1997; Conceição *et al.* 2010). Cultured rotifers and *Artemia* do not contain these fatty acids in the amounts required for the rearing of most fish larvae and need to be enriched using emulsions of marine oils, commercial products or microalgae. Several microalgae species, including flagellates (e.g. *Isochrysis*, *Tetraselmis* and *Rhodomonas*), diatoms (e.g. *Skeletonema*, *Chaetoceros* and *Phaeodactylum*) or chlorococcalean (e.g. *Chlorella* spp.) are cultivated as food sources for zooplanktonic live feed. Microalgae can be also delivered directly to the rearing tanks ('green water technique') to improve water quality, bacterial control and growth/survival of fish larvae.

The bacterial colonization of the gastrointestinal (GI) tract of fish larvae starts after the opening of the mouth, which occurs some hours/days after hatching, depending on the fish species and culture conditions. The establishment of the GI microbiota is influenced by bacteria present in eggs, water and live feed (Nicolas *et al.* 1989; Munro *et al.* 1994; Reitan *et al.* 1998; Ringø and Birkbeck 1999; Olafsen 2001). The different stages of evolvement of gut microbiota in the larvae can be summarized as follows:

- (1) In the yolk-sac stage, the larvae represent a pristine environment practically devoid of bacteria. After opening of the mouth, the pioneer colonizing species originate mainly from the microbiota associated with the water, though the origin of bacteria associated with fish larvae at this stage are multiple (fish eggs, inflow water, bacteria associated with tank walls and other parts of the rearing system, and microalgae cultures). Marine fish larvae drink seawater to osmoregulate, and accumulate bacteria from the water (Mangor-Jensen and Adoff 1987). In fact, the numbers of bacteria in the gut are much higher than those in the rearing water, indicating that there is a selective uptake of bacteria and/or bacterial multiplication in the GI tract of the larvae (Reitan *et al.* 1998).
- (2) From the onset of the exogenous feeding, the numbers of bacteria increase exponentially and the diversity of the microbiota decreases. At this stage, opportunistic species, mainly derived from the ingested live feed organisms, increase in number by several log scales (Bergh *et al.* 1994; Skjermo and Vadstein 1993).

Bacteria colonizing the intestinal mucosa in first-feeding larvae can provide protection against potential pathogens (e.g. competition for attachment sites or nutrients, production of inhibitory compounds, etc.) and interact with the host (e.g. enhancing the immune response, improving GI morphology and digestive function) (Hansen and Olafsen 1999; Bergh 1995; Gómez and Balcázar 2008). However, live feeds are also potential carriers of pathogenic or opportunistic bacteria (Verdonck *et al.* 1997), which can increase larval mortality.

Basic knowledge of the bacterial communities associated with live feeds and the development of methods to control bacterial microbiota in live feed production are crucial to enhance larval survival. The development of strategies other than the use of antibiotics and disinfectants,

such as the application of probiotics, prebiotics, phytobiotics and immunostimulants, would lead to a more environmentally friendly aquaculture sector, in accordance with the Ecosystem Approach for the sustainable growth and expansion of aquaculture, promoted by FAO in *The State of World Fisheries and Aquaculture* (FAO 2007).

The present chapter discusses bacterial aspects of live feed and reviews the use of probiotics and prebiotics as a strategy to improve live feed cultures, and also considers how live feed can be used as a vector for prebiotics and probiotics to fish larvae cultures.

16.2 BACTERIAL ASPECTS OF LIVE FEED

16.2.1 Microalgae

Extracellular compounds resulting from the photosynthetic activity of algae constitute the carbon source of heterotrophic bacteria, forming an algae–bacteria interaction region known as the phycosphere. These interactions range from mutualism to parasitism (Cole 1982). Algae can produce compounds that promote or inhibit bacterial growth and bacteria can have a negative or positive effect in the algae culture (Austin *et al.* 1992; 1990; Fukami *et al.* 1997; Naviner *et al.* 1999). In mass cultivation systems, it is almost impossible to cultivate microalgae under axenic conditions. Taking advantage of the high organic load accumulated in the system, high numbers of bacteria attach to microalgae cells or proliferate in the water. The abundance and diversity of bacterial microbiota in microalgae are determined by the method of culture, the species cultured, the growth media used, the quality of seawater and the growth phase of the culture (Salvesen *et al.* 2000). Culturable bacteria in microalgae cultures range from 10^3 to 10^8 colony forming units (CFU) ml^{-1} (Nicolas *et al.* 1989; 2004; Salvesen *et al.* 2000). Analysis by culture-independent methods showed that different algae species (*Pavlova lutheri*, *Isochrysis galbana*, *Chaetoceros calcitrans*, *Skeletonema costatum*, *Chaetoceros gracilis* and *Chaetoceros muelleri*) are associated with a large spectrum of bacteria with bacteria belonging to the α -Proteobacteria, β -Proteobacteria, γ -Proteobacteria, Cytophaga–Flavobacterium–Bacteroides (CFB) group, *Actinobacteria* and *Bacillus* (Nicolas *et al.* 2004). Members of the *Roseobacter* clade and the CFB group are dominant in microalgae cultures (Nicolas *et al.* 2004; Porsby *et al.* 2008) and *Vibrionaceae* are generally absent or in very low numbers (Salvesen *et al.* 2000).

16.2.2 Rotifers

Rotifers are filter feeders with a high bacterial load and a variable bacterial microbiota on their external surface and in digestive tract. The microbiota of rotifers is similar to the microbiota of the water in the rearing system, indicating a non-selective uptake of bacteria (Muroga and Yasunobu 1987; Skjermo and Vadstein 1993; Vadstein *et al.* 1993; Makridis *et al.* 2000a). The most usual approach for cultivation of rotifers involves the method of batch culture, where a tank is inoculated and harvested in its entirety after some days. Another less common approach is the continuous culture, where after an initial stage with increasing population density the culture is partially harvested and replaced with water at regular time intervals (Dhert *et al.* 2001). Continuous cultures may result in lower microbial load and more predictable microbiota, which is associated with the rotifers rather than with batch cultures (Rombaut *et al.* 2001). In addition to the method of cultivation, the diet of rotifers has a strong influence on their

associated microbiota (Qi *et al.* 2009). Rotifers grown on baker's yeast show 10^3 – 10^4 bacteria per rotifer (Skjermo and Vadstein 1993; Pintado *et al.* 2010), while the values for rotifers grown on microalgae are generally lower, around 10^2 CFU rotifer⁻¹ (Øie *et al.* 1994). Culturable bacteria in rotifers fed with baker's yeast with added capelin oil were reported to be dominated by members of the *Pseudomonas/Alcaligenes* group, *Cytophaga/Flavobacterium* group, *Alteromonas* and *Vibrio* genera (Skjermo and Vadstein 1993). Nicolas *et al.* (1989) observed that the bacteria associated with rotifer cultures, identified by biochemical assays, were mainly *Pseudomonas*, *Vibrio* and *Aeromonas*, and to a lesser extent *Alteromonas* and *Acinetobacter*. By denaturing gradient gel electrophoresis (DGGE) analysis, Rombaut *et al.* (2001) demonstrated that bacteria in the culture water of rotifers were different depending on the culture mode (batch or continuous culture). In recirculation systems, γ -Proteobacteria were predominant in the water, revealing the presence of species belonging to the genera *Marinomonas* and *Pseudoalteromonas*. However, the bacterial communities of rotifers were not analysed. In a two-year survey, using both culture-dependent methods and DGGE, McIntosh *et al.* (2008) observed a stable gut microbiota of rotifers grown on commercial diets, with predominance of putative *Arcobacter* spp., and with unclassified *Rhodobacteraceae* and other genera such as *Roseobacter*, *Alteromonas* and *Vibrio* being detected at some times during both years.

Most bacteria in rotifers are not pathogenic, but detrimental effects on fish larvae can be caused by the accumulation of bacteria in prey (Dhert *et al.* 2001). Bacteria associated with rotifer cultures have been related to unexpected mortalities or to suppressed growth in rotifers (Harzevili *et al.* 1997), as well as low survival and growth in fish larvae (Pérez-Benavente and Gatesoupe 1988; Gatesoupe 1989; Nicolas *et al.* 1989). In a survey in two different hatcheries in Greece and in Spain, Verdonck *et al.* (1997) sampled routinely produced rotifers (*Brachionus plicatilis*) and found *Vibrio* (*Listonella*) *anguillarum* and *Vibrio alginolyticus* as predominant bacteria, although none of the *V. anguillarum* strains were identified as pathogenic to fish. However, it has been demonstrated that *V. anguillarum* causes crashes in rotifer cultures (Harzevili *et al.* 1997) and that rotifers can be a vector for pathogenic *Vibrio* to fish larvae, directly by ingestion or by transference from the surrounding seawater (Prol-García *et al.* 2010).

16.2.3 Artemia

Artemia are also filter feeders that ingest and digest microalgae, yeasts, bacteria, protozoans and abiotic particles (Intriago and Jones 1993; Dhont and Lavens 1996; Makridis and Vadstein 1999). Bacteria can also adhere to their external chitinous surface of *Artemia*.

Dry *Artemia* cysts have a very low number of associated bacteria. Austin and Allen (1982) isolated bacteria belonging to the genus *Bacillus*, *Erwinia*, *Micrococcus*, *Staphylococcus*, *Vibrio* and also Gram-negative and Gram-positive rods from dehydrated cysts, and *Aeromonas*, *Bacillus*, *Micrococcus*, *Staphylococcus*, Gram-negative rods and Gram-positive rods from cyst-hatching water. In samples from *Artemia* cysts of 26 different commercial brands, López-Torres and Lizárraga-Partida (2001) reported that from 617 bacterial isolates on TCBS media, 94% were Gram-positives, such as *Staphylococcus* spp. or *Micrococcus* spp., and only 6% were Gram-negative. *Vibrio* spp. were not detected. When cysts were hatched in non-sterile conditions Gram-negative bacteria constituted 100% of the bacteria isolated from TCBS medium, and *V. alginolyticus* became predominant. These results indicate that *Vibrio* are not associated with *Artemia* cysts, but are introduced during *Artemia* culture in hatcheries. Other authors have corroborated that hatching and enrichment involve an increase of organic

mater in the water and the proliferation of bacteria, reaching values up to 10^4 CFU *Artemia*⁻¹, and that *Vibrio* is the predominant group among culturable bacteria, while *V. alginolyticus* was the dominant species (Olsen *et al.* 2000; Villamil *et al.* 2003).

In a recent study, Høj *et al.* (2009) analysed the localization, abundance and community structure of bacteria associated with *Artemia* nauplii. Most bacteria were localized in the nauplii gut, with external surfaces having limited bacterial colonization. Enrichment with microalgae or with commercial lipid emulsions for 24 h generally increased the bacterial load of newly hatched nauplii from 10^1 to 10^2 – 10^6 CFU *Artemia*⁻¹ and in some cases, the enrichment process shifted the bacterial community towards *Vibrio* spp. Strains isolated from newly hatched nauplii belonged to the genera *Sphingomonas*, *Rhizobium*, *Pseudomonas* and *Brevundimonas* and also several *Micrococcus* spp. were isolated. *Pseudomonas* and *Vibrio* spp. were predominant in enriched nauplii. *V. alginolyticus* was the dominant species among the *Vibrio* isolated in all treatments. DGGE profiles confirmed that the bacterial community was composed of populations related to members of γ -Proteobacteria and also members of the Planctomycetales phylum. In nauplii enriched with algae, a phylotype identified as *Antartobacter* sp., from the *Roseobacter* supercluster, was detected.

Strains of *Vibrio proteolyticus*, *Pseudomonas fluorescens* and *V. alginolyticus* have been demonstrated to have a negative effect on *Artemia* cultures (Verschuere *et al.* 1999) and can be potentially detrimental to fish larvae.

16.2.4 Copepods

Copepods continuously ingest particles of organic matter and produce faecal pellets, incorporating and releasing bacteria to the surrounding medium and acting as a ‘microbial hotspot’ in the sea (Tang 2005). Copepods have bacteria attached to their external surface (Carman and Dobbs 1997; Brandt *et al.* 2010), predominantly near the mouth and the anus and between the segments (Carman and Dobbs 1997), and in their intestine and faecal pellets (Hansen and Bech 1996). The numbers of culturable bacteria in cultured copepods are generally lower than in *Artemia*, ranging in values from 10^2 to 10^4 CFU copepod⁻¹ (Hansen and Bech 1996; Verner-Jeffreys *et al.* 2003; Tang *et al.* 2009). The bacterial community composition of copepods is influenced by the food and copepod life history (Tang *et al.* 2009). The main species of culturable bacteria associated with marine copepods belong to the *Vibrio*, *Pseudomonas* and *Cytophaga* genera (Sochard *et al.* 1979). Copepod faecal pellets promote the development of bacteria which differ from the surrounding water, with predominant species belonging to the genera *Bacillus*, *Cytophaga*, *Pseudomonas* and *Vibrio* in *Acartia tonsa* (Delille and Razouls 1994; Hansen and Bech 1996). Recently, culture-independent methods revealed a prevalence of α -Proteobacteria and also δ -Proteobacteria and Bacteroidetes on copepods (Møller *et al.* 2007; Tang *et al.* 2009). Using different grinding procedures, Brandt *et al.* (2010) compared surface-attached bacteria and total (internal and external) bacteria in wild copepods (*Acartia* spp. and *Temora* spp.) collected from the German Bight (the south-eastern bight of the North Sea) and also in laboratory cultured *Acartia tonsa*. DGGE analysis showed distinct banding patterns for each of the three copepods species analysed. Differences in banding patterns between ground and intact copepods were also observed, suggesting distinct internal and external bacterial communities. Band sequencing revealed the presence of α -Proteobacteria related to the *Roseobacter* clade in all types of copepods. Similarly, in *Calanus* spp. sampled in the North Sea, DGGE and 16S rRNA gene sequencing revealed a predominance of bacteria belonging to the *Roseobacter* clade (Møller *et al.* 2007). The fact that bacteria from the

Roseobacter clade are predominant in marine plankton and that some species are able to form biofilms and colonize surfaces (Slightom and Buchan 2009) would explain the predominance of those phylotypes.

Pathogenic strains of *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *V. alginolyticus* and *Aeromonas hydrophila* have been isolated from copepods, which present a risk of introducing harmful bacteria to larvae, as well as humans (Rawlings *et al.* 2007; Gugliandolo *et al.* 2008).

16.3 BACTERIAL CONTROL OF LIVE FEED CULTURES

The reduction of the bacterial load in zooplanktonic organisms is essential in the successful rearing of marine fish larvae for two main reasons:

- (1) Live feed organisms are added daily in large amounts to the rearing tanks, promoting the accumulation of associated bacteria. This fact may result in serious deterioration of the environmental culture conditions due to respiration of bacterial population and accumulation of their excretory products.
- (2) In most cases, the microbiota associated with live feed includes opportunistic bacteria, which may be harmful to the larvae under certain conditions, causing increased mortalities.

Surface-attached bacteria in prey can be removed by washing procedures but bacteria could still remain in the GI tract. Freshwater, ozone, sodium hypochlorite, hydrogen peroxide, formaldehyde, sodium nifurstyrenate, and various antibiotics have been tested for the reduction of the bacterial load in rotifers and *Artemia* (Tanasomwang and Muroga 1992; Tolomei *et al.* 2004; Giménez *et al.* 2006). Most of these treatments are very effective in the reduction of the bacterial load of live prey, though the effect of residues of some compounds, such as hypochlorite and formaldehyde, on the viability of fish larvae is often unknown. The use of antibiotics should be avoided due to the high risk of increasing antibiotic resistance in bacterial communities.

UV light has been tested at experimental scale for rotifer disinfection, showing a quite high efficiency and eliminating more than 90% of viable bacterial counts (Munro *et al.* 1999). However, the efficiency of UV light is highly influenced by the presence of particles in the water. Therefore, upscaling could be problematic in routine operations at industrial scale as rotifer cultures should be particle-free.

Ozone is applied to disinfection areas, inflow waters, eggs or live feed (Suantika *et al.* 2001). The advantage of ozone is that it counteracts viral infections as well as bacterial pathogens. However, ozonation conditions must be carefully controlled to avoid problems with excessive production of oxygen radicals. In addition, ozonation must take place in well-aerated areas of the hatcheries as ozone inhalation by personnel can cause health problems.

The application of natural products, such as essential oils of various plants with antimicrobial properties (phytobiotics), is another new and interesting approach (Stefanakis *et al.* 2011). The advantages of such products are that the appearance of resistant strains is less probable, and that their application will not have a negative effect on the public image of the aquaculture industry.

Although the reduction of bacterial load has advantages, several studies have demonstrated that the microbial community present in live feed also contributes to the success of live feed

cultures. The elimination of bacteria from live prey may favour a more rapid colonization by opportunistic bacteria with high growth rates (*r*-strategists) when the live feed is introduced into the larval rearing system. Moreover, the use of disinfectants or antibiotics may enhance the growth and proliferation of *Vibrio* spp. (Høj *et al.* 2009), which could indicate a higher resistance of *Vibrio* to some treatments.

16.4 ENRICHMENT OF LIVE FEED AND MICROBIAL IMPLICATIONS

Before delivery to fish larvae, live prey are usually enriched with essential fatty acids (PUFAs) by the addition of microalgae (e.g. *Tetraselmis* sp., *Isochrysis* sp., *Rhinomonas* sp. and *Rhodomonas* sp.) or emulsions rich in HUFAs such as EPA and DHA. Enrichment greatly improves the viability of first feeding larvae (Reitan *et al.* 1993; 1998; Bergh *et al.* 1994).

Addition of microalgae into the rearing tanks (green water technique) results in reduced proliferation of opportunistic bacteria (Salvesen *et al.* 1999). When the microalgae are added together with their growth medium, large amounts of bacteria associated with the microalgae cultures are also added (Makridis *et al.* 2009). As described in Section 16.2.1, algal cultures have been associated with specific bacterial populations (Nicolas *et al.* 1989; 2004; Sandaa *et al.* 2003; Schulze *et al.* 2006), which might influence bacterial numbers and the microbiota in live feeds and fish larvae (Olsen *et al.* 2000). Algae can also influence the grazing of bacteria by live feeds (Nicolas *et al.* 1989).

There are several mechanisms by which microalgae influence the microbiota associated with live prey: (1) the effect of the microbiota associated with microalgae, (2) the effect of oxygen radicals produced during the process of photosynthesis, and (3) the effect of the microalgal cells. As mentioned earlier in this chapter, bacteria isolated from microalgae cultures have shown inhibitory abilities against fish pathogens (Makridis *et al.* 2006), indicating that these bacteria may play a significant role in the modulation of bacterial communities present in a rearing system. During photosynthesis, oxygen radicals with a general antibacterial activity (Marshall *et al.* 2005) are produced. Oxygen radicals may have a modulatory effect on the species composition of bacterial communities in the system. Under certain conditions, some microalgae cells show antibacterial activity against specific groups such as *Vibrio* spp. by production of antibacterial compounds such as superoxide (Marshall *et al.* 2005). This has been demonstrated in microalgae cultures, thereby excluding the effect of associated microbiotas (Kokou *et al.* 2012). The presence of axenic algae (*Tetraselmis chui*, *Chlorella minutissima*, *Nannochloropsis* sp., *Arthrospira platensis* sp., and *Isochrysis* sp.) inhibited the growth of all *Vibrio* species (*V. parahaemolyticus*, *V. anguillarum*, *V. splendidus*, *V. scophthalmi*, *V. alginolyticus* and *V. lentus*) tested compared with the control treatments, in which all *Vibrio* bacteria tested were able to utilize the microalgae medium to a high degree.

16.5 PROBIOTICS IN LIVE FEED PRODUCTION

Probiotics constitute a potential tool in the reduction of mortalities in the rearing of aquatic organisms (Gatesoupe 1999; Verschuere *et al.* 2000a; Gram and Ringø 2005; Vine *et al.* 2006; Kesarcodi-Watson *et al.* 2008; Dimitroglou *et al.* 2011). In the rearing systems, where host and

microorganisms share the same aquatic environment, the concept of probiotics includes also microorganisms which exert a beneficial effect not only by colonizing the host but also by being present in the water (see *Chapters 7 and 8*). The different modes of action of probiotics include: production of inhibitory compounds (e.g. antibiotics, bacteriocins, organic acids, oxygen peroxide), competition for limiting nutrients (e.g. siderophores for iron), enzymatic contribution to digestibility of feed, competence in adhesion to mucus, stimulation of the host immune system or improvement of water quality (Verschuere *et al.* 2000a; Gram and Ringø 2005; Vine *et al.* 2006; Kesarcodi-Watson *et al.* 2008, Dimitroglou *et al.* 2011). In some cases, the beneficial effect requires the combination of several mechanisms. Replacement of the opportunistic bacteria in live feeds by a preventive colonization with probiotic bacteria, with persistence in the water or in live feed, has therefore been proposed as a strategy to improve live feed cultures and to provide protection to the larvae.

16.5.1 Probiotics in microalgae

Antibacterial activity of some microalgae species, such as *Tetraselmis suecica* (Austin *et al.* 1992) or *Skeletonema costatum* (Naviner *et al.* 1999), against fish pathogens has been demonstrated. For example, *S. costatum* extracts inhibited the growth of *V. anguillarum*, *V. mytili*, and pathogenic *Vibrio* spp. S322 and VRP (Naviner *et al.* 1999). Microalgae-associated bacteria can also have antagonistic effect. This effect has been reported in *Tetraselmis chuii* and *Chlorella minutissima* towards the fish pathogens *Photobacterium damsela* subsp. *piscida* and *V. anguillarum* (Makridis *et al.* 2006). Furthermore, bacteria associated with microalgae cultures can have a probiotic effect due to nutritional properties (e.g. essential amino acids, PUFAs) or enzymatic activities (proteolytic or lipolytic), which improve larval feeding and feed utilization (Riquelme and Avendaño-Herrera 2003).

Suminto and Hirayama (1997) demonstrated a positive effect of a *Flavobacterium* strain on the growth of axenic cultures of the diatom *Chaetoceros gracilis* but not on the growth of the phytoflagellates *Isochrysis galbana* and *Pavlova lutheri*. However, the addition of the bacteria extended the maintenance of high cell densities in the stationary growth phases for both microalgae. These results suggest specific algae–bacteria interactions, and therefore beneficial strains could be selectively isolated from microalgae in non-axenic cultures. This approach was recently used by Rivas *et al.* (2010), who isolated a biofilm-forming *Rhizobium* sp. from *Botryococcus braunii* cultures. The bacteria, re-inoculated into microalgae cultures, acted as a probiotic and significantly enhanced microalgae growth, indicating a possible use of the bacteria as inoculum for microalgae mass cultures.

16.5.2 Probiotics in rotifers

The first approaches to the use of probiotics to improve the growth of rotifers were conducted using commercial lactic acid bacteria (LAB) additives or selected LAB (*Lactobacillus plantarum* and *Lactobacillus helveticus*) (Gatesoupe *et al.* 1989). Although the application of these bacteria did not improve rotifer production rates, in some cases they improved the rotifer nutritional value, reduced the bacterial load or inhibited specifically the growth of fish pathogens such as *Aeromonas salmonicida* (Gatesoupe 1991). Similarly, Douillet (2000a) reported enhancement in the growth of rotifers by adding selected commercial bacterial products (including *Bacillus* and *Pseudomonas* strains), though repeated trials were not consistent. Planas *et al.* (2004) obtained rotifer growth rates 8–13 times higher than control cultures,

using terrestrial LAB strains of *Lactococcus casei*, *Pediococcus acidilactici* or *Lactobacillus lactis*, individually or jointly. As rotifers use bacteria as feed, and may utilize bacterial metabolites, it has been hypothesized that enhanced rotifer growth by bacteria can be due to inorganic nutrients such as phosphorus (Hessen and Andersen 1990), or micronutrients such as vitamin B₁₂ (Yu *et al.* 1989), produced by the bacteria. The supplementation of LAB can also have a regulating effect on the microbiota of rotifers. It has been reported that feeding rotifers with *Lb. plantarum* is an effective way to decrease the bacterial counts in rotifers, especially of the dominant members of the *Vibrionaceae* (Gatesoupe 1991).

The inconvenience of using telluric LAB, such as *Lc. casei*, *P. acidilactici* or *Lb. lactis*, as probiotics in rotifer cultures is that survival in seawater may be limited and their use would imply periodical additions to rotifer cultures (Planas *et al.* 2004). Autochthonous LAB strains could avoid this problem. Harzevili *et al.* (1997) used a probiotic *Lactococcus lactis* strain AR21, with *in vitro* antagonism against *V. anguillarum*, isolated from a rotifer mass culture in a hatchery. Antagonistic activity of *Lc. lactis* AR21, due to the production of the bacteriocin diplococcin, counteracted the negative effect of *V. anguillarum* in *in vitro* trials with rotifers. Other isolated non-LAB strains (an *Alteromonas* strain and unidentified Gram negative strains) enhanced rotifer growth rates under different feeding regimes in comparison with axenic cultures or control cultures inoculated with microbial communities present in seawater (Douillet 2000b). In a similar approach, Rombaut *et al.* (1999) isolated bacterial strains from rotifers in well-performing cultures and determined the effect of the isolates under monoaxenic conditions. The addition of some of those strains (which were not identified) enhanced rotifer growth rate and egg ratio, as compared with the axenic control treatment. This finding was also observed by Hagiwara *et al.* (1994) when bacterial strains previously isolated from cultures of actively reproducing *B. plicatilis* were delivered to axenic cultures of rotifer. Selected *Pseudomonas*, *Moraxella* or *Micrococcus* strains increased rotifer reproduction by 4–10 fold. Martínez-Díaz *et al.* (2003) also reported an increase in egg production and growth of axenic rotifers in the presence of *V. proteolyticus* and *Aeromonas media* strains.

Bacteria isolated from rotifers may also have a positive effect in fish larvae due to antagonism towards pathogens. For example, Makridis *et al.* (2005) isolated bacterial strains (*Cytophaga* sp., *Roseobacter* sp., *Ruegeria* sp., *Paracoccus* sp., *Aeromonas* sp. and *Shewanella* sp.) from well-performing rotifer cultures, which significantly improved the survival of unfed sea bream (*Sparus aurata*) larvae compared to control treatment in filtered seawater.

16.5.3 Probiotics in *Artemia*

Artemia can use bacteria as food (Yasuda and Taga 1980; Intriago and Jones 1993) and although a diet consisting solely of bacteria has not been successfully established (D'Agostino 1980), it has been demonstrated that selected bacteria improve axenic cultures of *Artemia* fed with other foods (Douillet 1987). The culture of *Artemia* under non-axenic conditions results in higher biomass production than under axenic conditions, and colonization of bacteria can be essential to fulfil nutritional requirements when some foods such as rice-bran are used (D'Agostino 1980; Douillet 1987). *Artemia* require lipids and proteins in early development, proteins and carbohydrates in juvenile and adult stages, and fatty acids and vitamins for reproduction and growth. Bacteria can be a source of proteins, vitamins (such as B₁₂), essential amino acids, fatty acids, polyamines, enzymes and inorganic nutrients (Gorospe *et al.* 1996; Hessen and Andersen 1990). It has also been hypothesized that bacteria may also remove toxic metabolic substances from *Artemia* cultures (Verschuere *et al.* 1999).

Focusing on the contribution of bacteria to nutritional quality, Rico-Mora *et al.* (1995) challenged *Artemia* cultures with bacterial strains isolated from the diatom *Skeletonema costatum* and observed a positive effect of two strains of *Flavobacterium* and *Aeromonas* on *Artemia* survival. Gorospe *et al.* (1996) also demonstrated that *Pseudomonas* sp. can be a good source of proteins and amino acids required for growth and survival of *Artemia* in rice-bran culture. Bacteria can also improve *Artemia* diets based on microalgae. A *Flexibacter* strain isolated from shrimp ponds added together with the microalgae *Rhodomonas* sp. improved growth and biomass of *Artemia*, as compared with the culture only with microalgae (Intriago and Jones 1993). This finding suggests the use of bacteria as food as well as a probable probiotic effect in the digestion of the microalgae.

Orozco-Medina *et al.* (2002) isolated Gram-positive heterotrophic bacteria strains belonging to the genera *Microbacterium* and *Exiguobacterium* from well-performing commercial cysts. Gnotobiotic (axenic) *Artemia* nauplii fed on autoclaved baker's yeast and challenged with a mixture of those bacteria showed significantly enhanced growth and development. Both strains also promoted growth and survival in agnotobiotic (xenic) conditions (Hipólito-Morales *et al.* 2009). Light and scanning electron microscopy demonstrated the ingestion of both bacteria by *Artemia* nauplii, and fluorescence microscopy allowed the detection of the introduced bacteria, live and dead, in the gut lumen (Orozco-Medina *et al.* 2009a). However, no evidence was found that those bacteria adhered or colonized the intestinal epithelium and the authors hypothesized that the probiotic effect can be due to the enzymatic contribution provided by bacterial proteases to the digestion of baker's yeast (Orozco-Medina *et al.* 2009b).

To elucidate whether the effect of bacteria is nutritional or probiotic, Marques *et al.* (2005) used gnotobiotic *Artemia* challenged with the dead or live bacteria, combined with different axenic live feeds (yeasts or microalgae) differing in their nutritional values. Dead bacteria exerted a significant effect on survival when *Artemia* were fed with poor feeds, but a weak or no effect when good-quality feeds were used. With some strains, such as *Cytophaga* sp. GR8, isolated from well-performing rotifer cultures (Rombaut *et al.* 1999), and *Bacillus* sp. LVS2, isolated from well-performing *Artemia* cultures (Verschuere *et al.* 1999), the addition of live bacteria improved the performance of *Artemia* cultures compared with the addition of the same dead bacteria, in all of the feeds supplied. This would indicate a probiotic effect of those strains, independently of the nutritional effect. This study highlights the importance of considering the interactions with feeds in the evaluation of the probiotic effect.

The positive effects of selected bacteria in *Artemia* cultures are based not only on their nutritional or enzymatic contribution but also on their role as bacterial control agent against pathogenic or opportunistic bacteria, through antagonism or by competition for available resources (nutrients, space, adhesion sites etc.). Verschuere *et al.* (1999) tested different bacteria isolated from well-performing *Artemia* cultures, and selected nine (unidentified) strains based on the positive effect on survival and growth of *Artemia* in monoxenic cultures. Those strains were used for a pre-emptive colonization of xenic cultures of *Artemia* juveniles, adding 10^6 bacteria ml^{-1} to the culture medium. A positive effect on the survival and/or the growth rate was observed with all selected strains at 36 h and only the cultures pre-emptively colonized contained living *Artemia* at the end of the trial (132 h). No significant differences in total CFU per *Artemia* were observed between treatments but a clear influence of the pre-emptive colonization was observed in the microbial communities associated with the *Artemia*, as determined with Biolog GN community-level physiological profiles. Although chemo-taxonomical results were not sufficient to confirm the identity, isolates recovered

from *Artemia* showed similarity with five of the nine introduced bacteria, indicating that those strains may be able to colonize or populate the *Artemia* GI tract and to minimize the levels of other opportunistic bacteria. The protection of the selected strains was confirmed in *Artemia* cultures challenged with a *V. proteolyticus* shown to cause mortality in monoxenic *Artemia* cultures (Verschuere *et al.* 2000b). Pre-emptive colonization by some strains, such as LVS8 (identified as *Vibrio* sp.), gave total protection against infection (with survival similar to that of non-infected controls) while other strains such as LVS2, identified as *Bacillus* sp., gave only a partial protection and intermediate survival levels (Marques *et al.* 2005). The *in vitro* antagonism tests were negative and extracellular bacterial compounds did not protect *Artemia*. Living cells were required to protect *Artemia* against *V. proteolyticus*, suggesting that pre-emptive colonization allows the selected strains to compete effectively for nutrients or adhesion sites with the pathogen and suppress its development (Verschuere *et al.* 2000b).

As the probiotic effect can be strongly influenced by the nutritional status of *Artemia*, the protective effect of two selected bacterial strains, *Bacillus* sp. LVS2 and *A. hydrophyla* LVS3, were tested by Marques *et al.* (2006) on gnotobiotic *Artemia* nauplii fed with different *Saccharomyces cerevisiae* mutant strains (linked to nutritional factors such as a lower concentration of mannose or higher concentration of chitin and glucans in the yeast). Subsequently the *Artemia* were challenged with two bacterial pathogens, the opportunistic *V. proteolyticus* CW8T2 (Verschuere *et al.* 2000b) and the virulent pathogenic *Vibrio campbellii* LMG21363 (Soto-Rodríguez *et al.* 2003). This study demonstrated that the quality of the feed had a stronger protective effect than the added bacteria. Good quality yeasts counteracted the detrimental effect of both pathogens, and adding probiotic bacteria with them did not give additional protection. Only when *Artemia* were supplied with lower quality yeast feeds did probiotics show an effect, counteracting opportunistic *V. proteolyticus* but not the virulent *V. campbellii*. The authors suggest that a higher presence of β -glucans and chitin in the cell wall of mutant yeasts would provide a possible stimulation of the innate immune system of *Artemia*. The study of the development of the digestive tract showed an influence of live *A. hydrophyla* LVS3 on cell proliferation of the gut (Gunasekara *et al.* 2010) and a slight increase in the growth of the digestive tract was confirmed by stereology and computer assisted three-dimensional image reconstruction. The authors hypothesize an increased cell proliferation in GI epithelia and underlying muscle in *Artemia* which received the live probiotic bacteria (Gunasekara *et al.* 2011).

16.5.4 Probiotics in copepods

It is presumed that bacteria play an important role in the nutrition of copepods in nature and under cultured conditions. Harpacticoid copepods have been successfully cultured by feeding only on dried bacteria and on bacterial biofilms, showing a clear preference for certain bacterial strains (Rieper 1978; Dahms *et al.* 2007).

Research on the use of probiotics in copepods is still very scarce, limited to studies on the beneficial effect of the addition of bacteria, probably due to nutritional factors (Guérin and Rieper-Kirchner 1992; Guérin *et al.* 2001). In a recent study, Drillet *et al.* (2011) tested the effectiveness of a commercial probiotic preparation (PSI: Sorbial A/S DANISCO, Allonnes, France) based on heat inactivated *Lactobacillus farciminis* and *Lactobacillus rhamnosus* strains in the calanoid copepod *Acartia tonsa* fed with *Rhodomonas salina*. The addition of the probiotic to the algal food significantly increased egg production (by 50%), egg hatching success (by 17%) and female growth (by 10%) in comparison to controls. These effects

were observed despite the biochemical analysis revealing that the probiotic preparation was a nutritionally poor food, lacking essential fatty acids. The authors suggested the activation of digestive enzymes or a supply of specific nutrients (e.g. vitamins, proteins, amino acids or other metabolites) as possible mechanisms.

16.6 BIOENCAPSULATION OF PROBIOTICS IN LIVE FOOD AND DELIVERY TO LARVAE

As discussed, probiotics have been used in live feed to enhance growth and to control opportunistic bacteria in live feed, but they are also applied to live feeds as a way to efficiently deliver probiotic bacteria to larvae. The addition of probiotics to the water of the rearing system may not be an effective way to incorporate probiotics (Suzer *et al.* 2008). Bioencapsulation has been demonstrated to be a more effective way to introduce the probiotic bacteria to the larval gut, and in the case of some allochthonous bacteria (e.g. LAB of tellurian origin, with a low survival in seawater) it can be the only efficient route (Gatesoupe 1994). Studies of probiotic bacteria bioencapsulated in live feed are summarized in Table 16.1.

16.6.1 Bioencapsulation of probiotics in microalgae

The feasibility of co-culturing microalgae and beneficial bacteria has been demonstrated by several authors (Suminto and Hirayama 1997; Fukami *et al.* 1997; Rico-Mora *et al.* 1998), but assays on the use of microalgae as a vector to deliver probiotics to larvae are still scarce. Avendaño and Riquelme (1999) studied the incorporation of bacteria, isolated from scallop *Argopecten purpuratus* cultures and with inhibitory activity against the pathogen *V. anguillarum*, into axenic cultures of *Isochrysis galbana*. The microalgae cultures were subsequently used as a vector of the probiotic into cultures of scallop larvae. The results demonstrated a stimulation of microalgae growth and a significant ingestion by scallop larvae of the strain delivered in conjunction with the microalgae. Moreover, the addition of the bacteria bioencapsulated in the microalgae promoted a higher maintenance of the probiotic in the larvae (10^2 CFU larva⁻¹) than when the bacteria were added to the water of the rearing tanks (10^1 CFU larva⁻¹). Although the effect of the probiotic on growth, survival or bacterial microbiota of scallop was not reported, the effectiveness of the bioencapsulation in the microalgae as a way to introduce the probiotic to larvae was demonstrated.

16.6.2 Bioencapsulation of probiotics in rotifers and *Artemia*

The first studies, conducted by Gatesoupe (1990), reported that LAB resembling *Lactobacillus* or *Carnobacterium* and isolated from rotifers can be effectively bioencapsulated in rotifers. Feeding turbot (*Scophthalmus maximus* L.) larvae with those rotifers subsequently improved growth rate and significantly reduced mortality rate in first-feeding larvae challenged with pathogenic *Vibrio* sp. (Gatesoupe 1994).

Strains previously isolated from healthy fish or larvae have also been bioencapsulated in rotifers. Olsson *et al.* (1992) screened bacteria from the turbot intestine (gut content and mucus) or skin mucus, and selected isolates based on inhibition of the growth of *V. anguillarum* and of adhesion to turbot intestinal mucus. The incorporation into rotifers of a bacterial strain (strain 4:44) isolated in the study, along with others isolated from turbot larvae,

Table 16.1 Bioencapsulation of probiotics in live feeds.

Live feed	Probiotic strain	Isolation/source	Targeted host species	Mechanism	Reference
Microalgae <i>Isochrysis galbana</i>	<i>Vibrio</i> strains 11, 334, 113, 127, 142 and C33 and unidentified strain 77	Scallop veliger larvae (<i>Argopecten purpuratus</i>) and microalgae (<i>Chaeotoceros calcitrans</i>)	Scallop (<i>Argopecten purpuratus</i>)	Antagonistic activity to <i>V. anguillarum</i>	Avenidaño and Riquelme (1999)
	<i>Lactobacillus delbrueckii</i>	Gut of adult European sea bass	Gilthead sea bream	Stimulation of immune system	Abelli <i>et al.</i> (2009)
Rotifer <i>Brachionus plicatilis</i>	<i>Lactobacillus fructivorans</i> + <i>Lactobacillus plantarum</i>	Human faeces	European sea bass and gilthead sea bream	Stimulation of immune system	Abelli <i>et al.</i> (2009)
	<i>Lactobacillus helveticus</i> + <i>Streptococcus thermophilus</i>	Commercial probiotic mixture (Adjulact 1000, Lallemand SAS)	Turbot	Enhancement of dietary value of live food	Gatesoupe (1991)
	<i>Lactobacillus plantarum</i>	Commercial probiotic (Bel Industries)	Turbot	Enhancement of dietary value of live food	Gatesoupe (1991)
	<i>Carnobacterium</i> sp.	Live food	Turbot	Enhancement of dietary value of live food	Gatesoupe (1994)
	<i>Lactobacillus plantarum</i>	Rotifers	Turbot	Enhancement of dietary value of live food	Gatesoupe (1994)
	<i>Vibrio</i> sp. Strain PB52	Turbot larvae	Turbot	Antagonism	Gatesoupe (1997)
	Strain 4:44	Turbot larvae	Turbot	Colonization of larval gut	Makridis <i>et al.</i> (2000a; 2000b)
	<i>Phaeobacter</i> 27-4	Turbot rearing tank walls	Turbot	Colonization of larval gut	Makridis <i>et al.</i> (2000a; 2000b)
				Antagonism	Planas <i>et al.</i> (2006)

(continued overleaf)

Table 16.1 (continued)

Live feed	Probiotic strain	Isolation/source	Targeted host species	Mechanism	Reference
Rotifer and <i>Artemia</i> nauplii	<i>Bacillus subtilis</i> , <i>B. licheniformis</i> , <i>B. plumilis</i>	Commercial probiotic mixture	Gilthead sea bream	Increase of growth and higher expression of genes involved in muscle growth	Avella <i>et al.</i> (2010)
	<i>Bacillus subtilis</i> , + <i>B. licheniformis</i> + <i>B. megaterium</i>	Commercial probiotic mixture (EcoPro™, Eco Microbials)	Cobia	Unknown	Benetti <i>et al.</i> (2008)
	<i>Lactobacillus fructivorans</i>	Gut of gilthead sea bream	Gilthead sea bream	Antagonism	Carnevali <i>et al.</i> (2004)
	<i>Lactobacillus plantarum</i>	Human faeces	Gilthead sea bream	Antagonism	Carnevali <i>et al.</i> (2004)
	<i>Lactobacillus delbrueckii</i>	Gut of adult European sea bass	European sea bass	Stimulation of immune system	Picchiatti <i>et al.</i> (2007)
	<i>Lactobacillus fructivorans</i> + <i>Lactobacillus plantarum</i>	Sea bream gut Human faeces	Gilthead sea bream	Increased stress tolerance	Rollo <i>et al.</i> (2006)
	<i>Lactobacillus</i> spp.	Commercial probiotic mixture (Biotexin and Protexin, Novartis)	Gilthead sea bream	Enhancement of enzymatic activity	Suzer <i>et al.</i> (2008)
	<i>Lactobacillus delbrueckii</i>	Gut of adult European sea bass	Gilthead sea bream	Stimulation of immune system	Abelli <i>et al.</i> (2009)
	<i>Lactobacillus fructivorans</i> + <i>Lactobacillus plantarum</i>	Human faeces	European sea bass and gilthead sea bream	Stimulation of immune system	Abelli <i>et al.</i> (2009)
	<i>Lactobacillus</i> spp.	Commercial probiotic mixture	Cutthroat trout (<i>Oncorhynchus clarkia pleuriticus</i>)	Unknown	Arndt and Wagner (2007)
<i>Pediococcus acidilactici</i>		Commercial probiotic mixture (Bactocel®)	Pollock (<i>Pollachius pollachius</i>)	Antagonism	Gatesoupe (2002)

was subsequently quantified by Makridis *et al.* (2000b) using immunocolony blot (ICB) and enzyme-linked immunosorbent assay (ELISA). The addition of 10^7 bacteria ml^{-1} in the rotifer rearing tanks for a short period (20–30 min) led to a total dominance (100% of total CFU) of the added bacteria and a load of 10^3 – 10^4 bacteria rotifer $^{-1}$. Once transferred to seawater, the probiotic persisted in the prey for 4 to 24 h. This study demonstrated that the bioencapsulation is highly dependent on several variables such as bacteria concentration, grazing rate and presence of microalgae. Particle size is however the main factor for the initial grazing by the rotifers, so the average cell size and the degree to which bacteria aggregate are the most important factors (Makridis *et al.* 2000a).

In a first feeding trial with turbot larvae, Makridis *et al.* (2000b) reported that the bioencapsulation of strain 4:44 in rotifers resulted in probiotic population of the GI tract (up to 10^4 bacteria larva $^{-1}$), indicating that the bioencapsulation is an effective way to deliver the probiotic into larvae. However, it was also shown that the bacteria bioencapsulated into rotifers were quickly released (in 2–4 h) from rotifers to the culture water and that the bacteria were present in the water of the larval rearing tanks. This finding suggests that larvae may ingest bacteria also by drinking. The presence of the probiotic did not modify total CFU levels in larvae or improve larval survival and growth, but it could potentially improve resistance of larvae against pathogens due to antagonistic activities (Makridis *et al.* 2000a).

The bioencapsulation of the probiotic *Phaeobacter* 27-4 in the rotifer *Brachionus plicatilis* by three different protocols was monitored by Pintado *et al.* (2010) using culture-dependent methods and DGGE. The results demonstrated a rapid (3 h) incorporation of the probiotic by rotifers (10^2 CFU rotifer $^{-1}$) and subsequent maintenance for 48 h, allowing the larvae to graze on the rotifers and to incorporate the probiotic. DGGE fingerprints showed a shift of bacterial communities of the rotifers due to temporal colonization by the probiotic. Planas *et al.* (2006) fed turbot larvae on rotifers enriched with *Phaeobacter* 27-4 in parallel to induced infections with the fish pathogen *V. anguillarum*, following model challenge trials (Planas *et al.* 2005). These authors demonstrated a clear *in vivo* probiotic effect, reducing the accumulated mortality of the larvae to the level of the uninfected control larvae. The probiotic bacteria was detected by immunohistochemistry in the intestine of turbot larva fed rotifers enriched with *Phaeobacter* 27-4 (Figure 16.1). However, *Phaeobacter* 27-4 did not colonize turbot larvae intestinal epithelia and continuous additions of the probiont were necessary to maintain an effective concentration of the probiotic in seawater and rotifers.

Encouraging results in the reduction of mortality were also achieved in sea bream larvae when *Lactobacillus fructivorans* (isolated from the sea bream gut) and *Lb. plantarum* (from human faeces) were bioencapsulated in rotifers and *Artemia* (Carnevali *et al.* 2004). Both strains were administered concomitantly (4:1 ratio) and specifically identified in larvae by culture in selective medium (MRS) and biochemical tests (API 50 CHL). It was observed that the non-autochthonous *Lb. plantarum* was prevalent during early stages (35 days post hatching (dph)), indicating that the establishment of the gut microbiota occurs in a non-selective way. The autochthonous *Lb. fructivorans* became predominant at the end of the experiment (90 dph), probably due to the more favourable condition of the GI tract for colonization by that strain at that developmental stage. These results were related to an immunostimulatory effect and the development of gut-associated lymphoid tissue (GALT) (Picchiatti *et al.* 2007). At 90 dph probiotic-fed larvae showed a higher density of B lymphocytes and Ig $^{+}$ cells and acidophilic granulocytes in the intestinal epithelium than the control fish. These results showed the importance of live feed supplementation with probiotics in early stages of larvae development. On

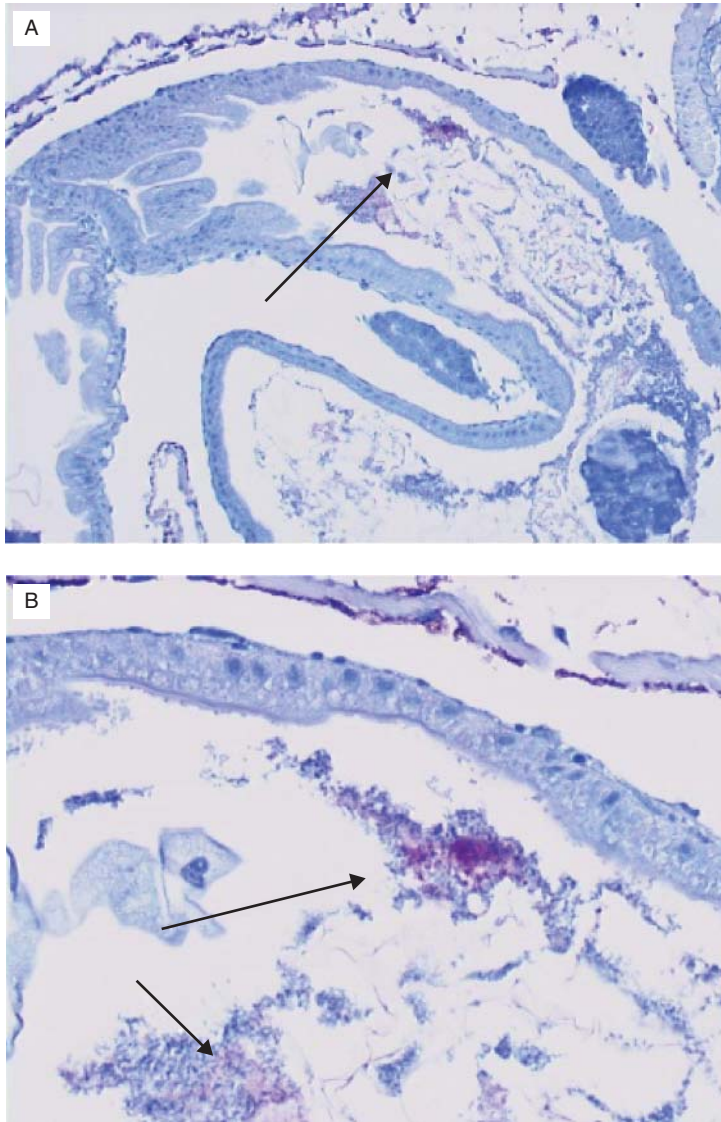


Fig. 16.1 (A) Intestine of turbot larva fed with rotifers with bioencapsulated *Phaeobacter* 27-4. Counterstaining with haematoxyline (blue) and immunohistochemistry staining with primary antiserum against *Phaeobacter* 27-4 (red). The arrows point to partially digested rotifers with positive immunostaining, enlarged in (B). (Source: Planas *et al.* 2006. Reproduced with permission of Elsevier.) For colour detail see Plate 18.

a molecular basis, using pH as a stressor and cortisol and 70 kDa heat shock protein (HSP70) gene expression as indicators, Rollo *et al.* (2006) demonstrated that the addition of the probiotics *Lb. fructivorans* and *Lb. plantarum* could also improve larval stress tolerance. These results showed the importance of live feed supplementation with probiotics in early stages of larval development.

In an interesting multiple application approach, Avella *et al.* (2010) tested a commercial probiotic mixture (*Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus pumilus*) delivered to sea bream larvae via water exclusively or via both water (10^4 bacteria ml^{-1}) and live prey (rotifers and *Artemia*). The probiotic effect was assessed based on survival and on microbial, morphometric and molecular analyses. The expression of the genes involved in muscle growth, insulin-like growth factor I (IGFI – muscle and cartilage growth promoter), and myostatin (MSTN – myoblast proliferation inhibitor), and in stress (HSP70 and glucocorticoid receptor (GR)) were quantified by real-time PCR. The mixture of probiotics increased growth (length, weight, higher expression of IGFI and lower of MSTN) and improved fish welfare (lower expression of HSP70 and GR). The probiotic effect was higher when the bacteria were delivered exclusively to live feed. The probiotic bioencapsulation reduced total presumptive *Vibrio* (cultures on TCBS) in *Artemia* but no significant differences were found in total heterotrophic bacteria (cultures on TSAS) or in total presumptive *Vibrio* in the larval gut. However, the presence of the introduced bacteria in larvae was not monitored specifically and bacteria composition of the gut of larvae and juveniles was not studied.

16.7 PREBIOTICS AND SYNBIOTICS IN LIVE FEED

Prebiotics are non-digestible dietary compounds which cause a beneficial effect on the host by selecting growth or activating the metabolism of one or a limited number of health-promoting bacteria (Gibson and Roberfroid 1995). The application of prebiotics in aquaculture has been recently reviewed (Merrifield *et al.* 2010; Dimitroglou *et al.* 2011; Ringø *et al.* 2010; Chapter 14), and includes the use of inulin, fructooligosaccharides (FOS), short-chain fructooligosaccharides (scFOS), mannanoligosaccharides (MOS), galactooligosaccharides (GOS), xylooligosaccharides (XOS), arabinoxylooligosaccharides (AXOS) and isomaltooligosaccharides (IMO). Prebiotics are usually included in dry diets, in the form of extruded dry pellets with different diameters according to fish size, limiting their use to weaned larvae and adults.

To our knowledge, no studies have been published on the use of prebiotics to increase live feed production or to modulate their microbial communities, or in controlling detrimental bacteria development. Only a recent publication by Daniels *et al.* (2010) described the use of prebiotics in the enrichment of live prey. Those authors used *Artemia* as a way of bioencapsulation and delivery of MOS (12 mg l^{-1}), alone or jointly with a commercial probiotic preparation (containing *Bacillus* spp., 100 mg l^{-1}), to European lobster (*Homarus gammarus* L.) larvae. The study demonstrated the viability of prebiotic bioencapsulation in live feed and the beneficial effect of MOS in the growth and survival of lobster larvae. The positive effect was enhanced by the simultaneous use of the probiotic, although the statistical analysis showed that the enhancement could be explained by the cumulative (additional) outcome and not by a synbiotic (synergistic) effect of both treatments. Electron microscopy revealed significant increases in intestinal microvilli length and density in lobster larvae and post-larvae fed with MOS-supplemented *Artemia*. The addition of MOS did not have a significant effect

on the number (CFU) of total viable bacteria or culturable *Vibrio* levels in lobster larvae. However, PCR-DGGE profiles of MOS-treated larvae were dissimilar to control, even though richness (Margalef species richness) and diversity (Shannon index) showed similar values. Therefore, the addition of the prebiotic alone exerted an effect, modifying the indigenous bacterial community. When MOS and *Bacillus* spp. were added together, more dissimilar profiles and reductions in richness and diversity were observed.

16.8 CONCLUSIONS AND FUTURE PERSPECTIVES

Bacteria in live feeds have a major influence on the microbiota of marine fish larvae, and live feed can be the mode of entry of specific pathogens or non-specific opportunistic bacteria to the larvae. Environmental and health concerns have resulted in a progressive reduction of the use of chemotherapeutics and to the search for alternatives, which should be based on considering bacteria as an active part of the rearing ecosystems.

The development of strategies for microbial control in live feeds will rely on knowledge of their microbiotas and the microbial interactions with fish larvae and rearing systems, as well as host–bacterial interactions. Tailored solutions should be developed for each case. Probiotics and prebiotics (or combined as synbiotics) are suitable tools to enhance particular bacterial groups and to prevent infection by identified pathogens. When the aim is to improve feed cultures or to control the bacterial microbiota in feeds, isolation of autochthonous probiotic bacteria in live feeds will favour co-culture and colonization of the feed. Bioencapsulation of probiotics in live feeds, prior to their delivery to fish larvae, would be a more useful tool when probiotics are isolated from the natural microbiota of the gut of the target larval species.

Studies of probiotics, prebiotics or immunostimulants should involve a multidisciplinary approach including the study of: (1) the influence in host survival and growth parameters, (2) the presence of the probiotic and the modification of the microbial community of the host or of the surrounding water, and (3) the impact on host immunological parameters. The use of live feed gnotobiotic cultures, combined with new molecular techniques, is giving new insight, improving our knowledge of microbial ecology and host–microbe interactions, and helping to elucidate underlying mechanisms.

The use of probiotics and prebiotics will constitute a strategy to improve the fitness of new live feeds, such as copepods, helping to upscale to industrial levels. Bioencapsulation of probiotics in copepods must also be explored. Particular research should be conducted on stable co-cultures of probiotics with microalgae, both for the feeding or enrichment of live feeds and for direct addition to larvae.

The application of prebiotics in aquaculture may have advantages over probiotics in terms of production and preserving costs, and in the context of regulatory issues as a consequence of avoiding the use of live microorganisms (Ringø *et al.* 2010). The possibility of incorporating prebiotics to extruded dry feeds has great potential to increase live feed production, by modulating microbial communities and controlling the development of detrimental bacteria. However, research is necessary to document and understand the effects of prebiotics on the microbiota of live prey. Also, as described above, live prey have been demonstrated to be an effective way of delivering prebiotics to larvae (Daniels *et al.* 2010) and more research should be conducted on this topic.

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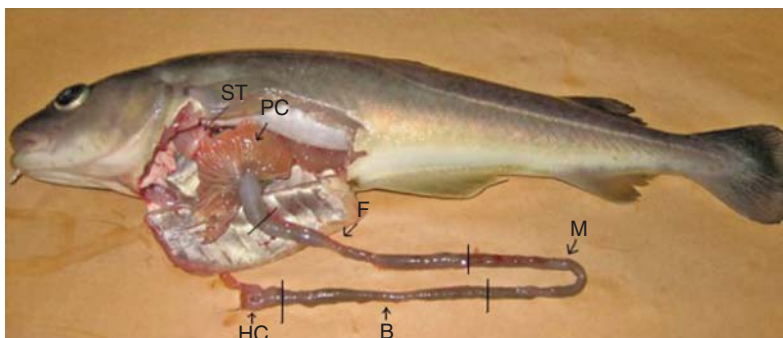


Plate 1 The alimentary tract of Atlantic cod (*Gadus morhua* L.). ST, stomach; PC, pyloric caeca; F, proximal intestine; M, mid intestine; B, distal intestine; HC, fermentation chamber. (Source: Lisbeth Løvmo Martinsen.) For further information see text near Figure 1.1.

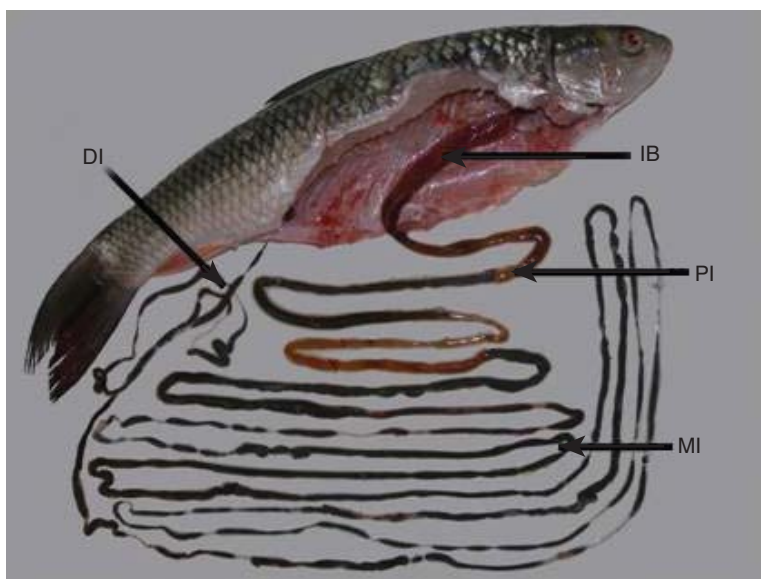


Plate 2 Alimentary tract of the mrigal (*Cirrhinus mrigala*). IB, intestinal bulb; PI, proximal intestine; MI, mid intestine; DI, distal intestine. Relative intestinal length (RIL) is 14–15. (Source: Arun K. Ray.) For further information see text near Figure 1.2.

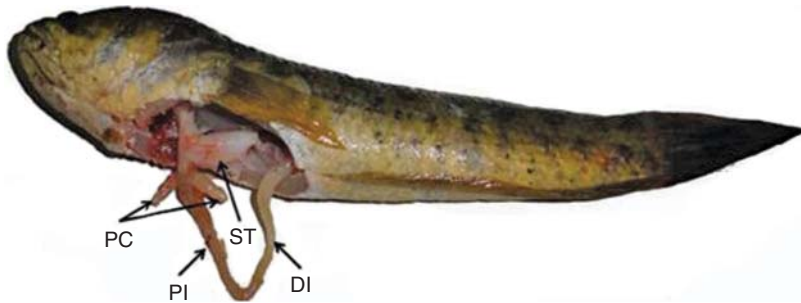


Plate 3 Alimentary tract of murrelet (*Channa punctatus*). ST, stomach; PC, pyloric caeca; PI, proximal intestine; DI, distal intestine. Relative intestinal length (RIL) is 0.5. (Source: Arun K. Ray.) For further information see text near Figure 1.4.



Plate 4 Alimentary tract of detritivorous mrigal (*Cirrhinus mrigala*) showing extremely coiled intestine. (Source: Arun K. Ray.) For further information see text near Figure 1.5.

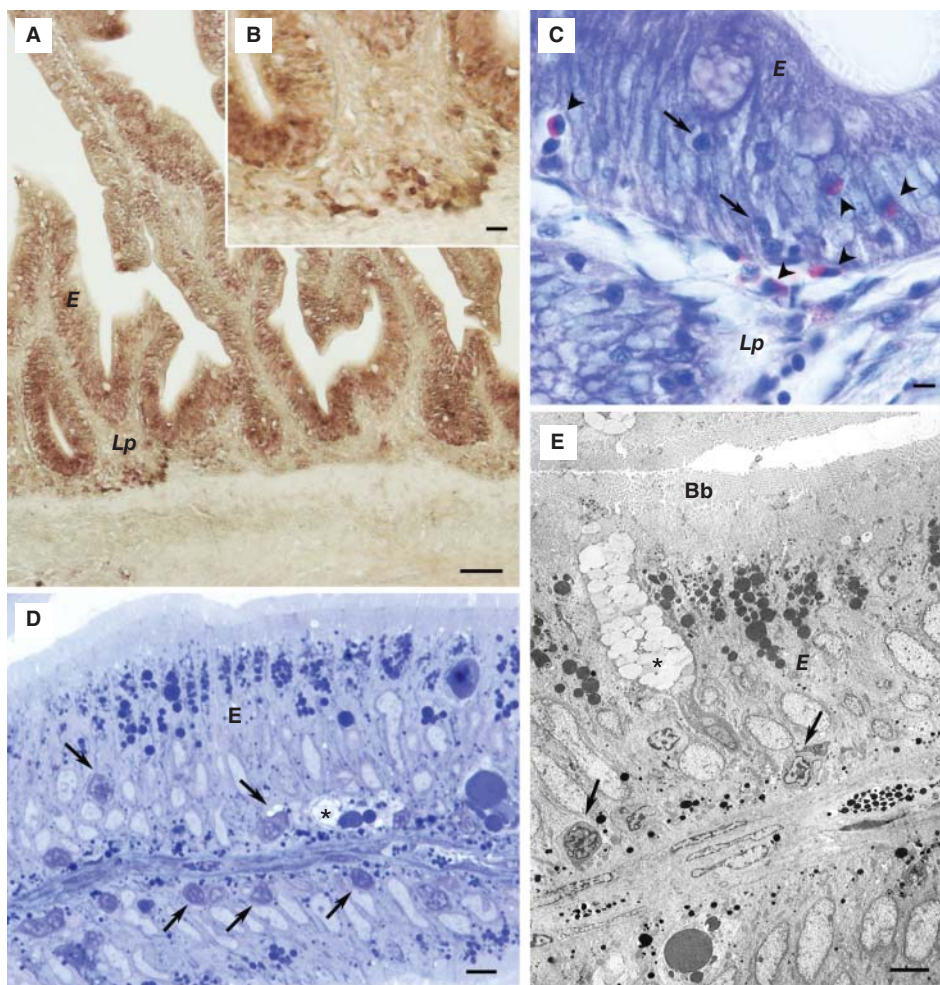


Plate 5 Histological characterization of intestinal mucosal immune cells. Immune cells of the teleost intestinal mucosa are less well organized and more diffusely arranged than in mammals. This figure shows the distribution of some of these immune cells in the mucosa and the lamina propria of bony fish in a series of five images. (A/B) Predominance of CD8 α -positive cells in both the epithelium and the lamina propria of the European sea bass intestine (CD8 α RNA *in situ* hybridization). Scale bar = 50 μ m. The CD8 α ⁺ cells aggregated in the lamina propria are shown at higher resolution in the insert (B), where the scale bar = 10 μ m. (C) May-Grünwald-Giemsa staining of the gilthead sea bream (*Sparus aurata*) intestinal mucosa, showing lymphoid cells (arrows) and acidophilic granulocytes (arrowheads). Scale bar = 4 μ m. (D) Semi-thin section of the gilthead sea bream intestinal mucosa housing numerous basolateral lymphocytes (arrows) and an intraepithelial macrophage (star). Scale bar = 5 μ m. (E) Transmission electron microscopy of the gilthead sea bream intestinal mucosa showing a goblet cell (star) amongst enterocytes and basolateral lymphocytes (arrows). Scale bar = 3 μ m. Key: Ep, epithelium; Lp, lamina propria; Bb, brush border. For further information see text near Figure 2.1.

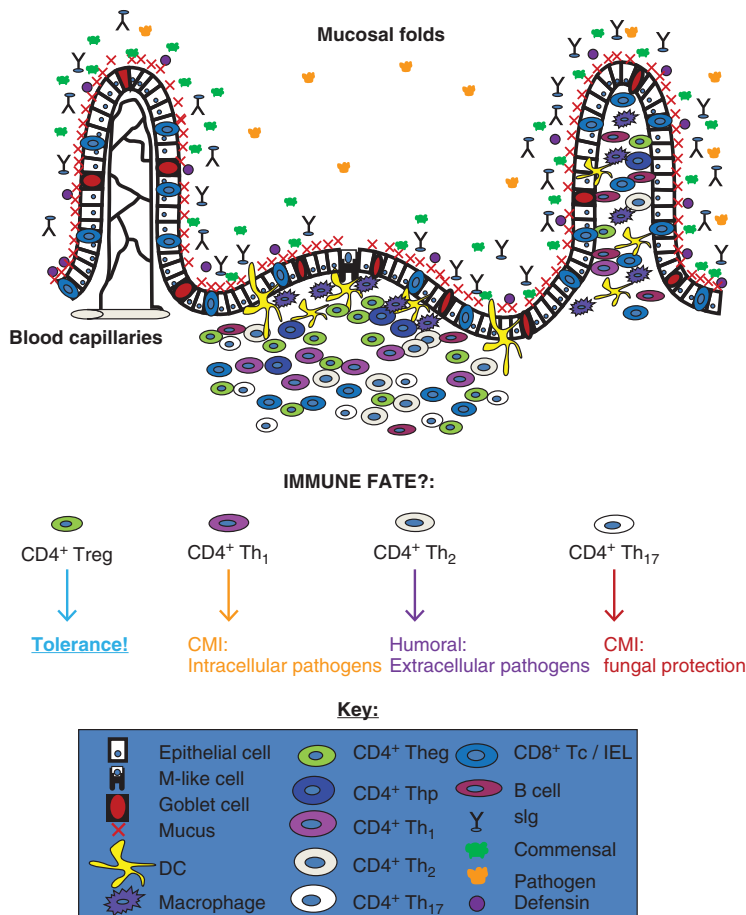


Plate 6 Mucosal immune system of the gut. This figure shows the current understanding that exists for the gut mucosal immune system of the teleost fish. This tissue exists in folds, but cannot be described as villi due to the lack of lacteal/lymphatics. The immune defences exist at many levels of the layers of the mucosal tissue, namely commensal organisms, mucus, epithelial cell layer and sub-epithelial mucosa. Unlike the mammalian GALT, teleosts do not exhibit Payer's patches or lymphoid follicles. Instead, their lymphoid tissue is rather diffuse but contains all the immune cells which give the teleost GALT the capability to either tolerate immune mechanisms (T_{reg}) or initiate immune mechanisms directed at the clearance of intracellular-resident (CD4⁺ Th₁, CD4⁺ Th₁₇ and CD8⁺ T_c) and extracellular-resident pathogens (CD4⁺ Th₂ and B cells). One area of controversy in this diagram is the existence of dendritic cells (DCs); it is not understood whether DCs, specialized macrophages or specialized M-cell-like epithelial cells capable of antigen transcytosis act as APCs to contextualize MHC-restricted T-cell-driven adaptive immune responses. Broad-spectrum pathogen defence is associated with barrier function (commensal organisms, mucus secreted by goblet cells and trapping of slg-bound pathogens, intact tight junctions and epithelial cell secretion of anti-microbial products such as defensins) and innate immune responses (macrophage and granulocyte activity mediated by cytokines and anti-microbial compounds). Through the induction of expression of teleost cytokine homologues, immune responses can be directed towards the desired effector response to pathogens and non-pathogens. Such teleost cytokines include TGFβ and IL-10 (T_{reg}), IL-12 (Th₁), IL-4 (Th₂) and IL-23 (Th₁₇), thus directing mechanisms of mucosal tolerance, cell-mediated immunity, humoral immunity and anti-fungal responses, respectively. For further information see text near Figure 2.2.

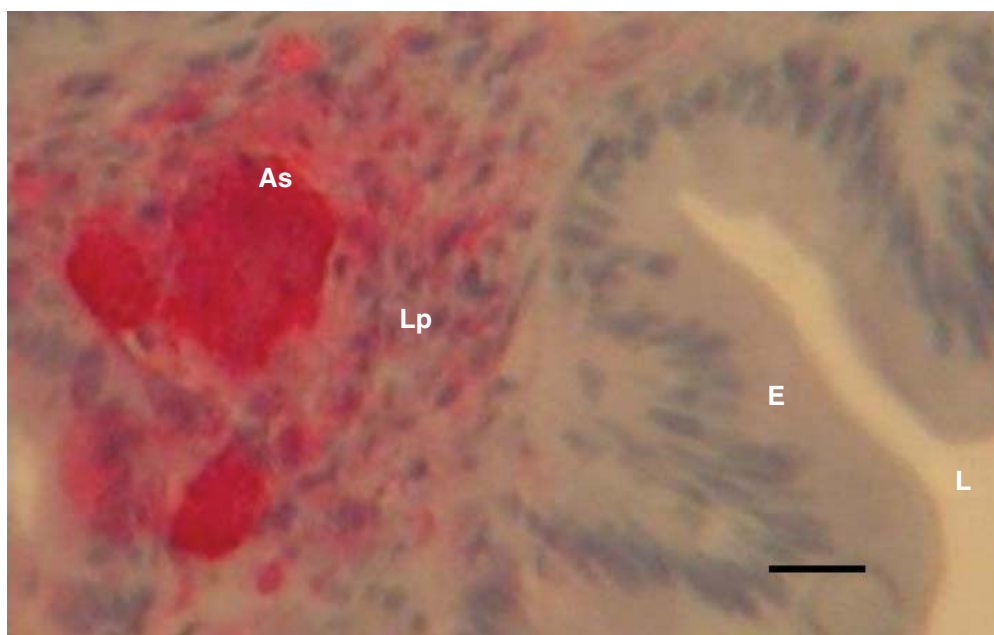


Plate 7 Immunohistochemical staining, using rabbit anti-*A. salmonicida* LPS antiserum diluted 1:1000, biotinylated goat anti-rabbit Ig and streptavidin biotinylated alkaline phosphatase, and Fast red/Naphthol AS-Mx as the substrate, in analysis of the spotted wolffish intestine from fish infected with waterborne atypical *A. salmonicida*. Large numbers of bacteria (As) were observed in the lamina propria (Lp). Sections obtained from non-infected controls were negative for bacteria. Key: E, epithelium; L, lumen. Scale bar = 10 μ m. (Source: Dag Vollstad.) For further information see text near Figure 3.5.

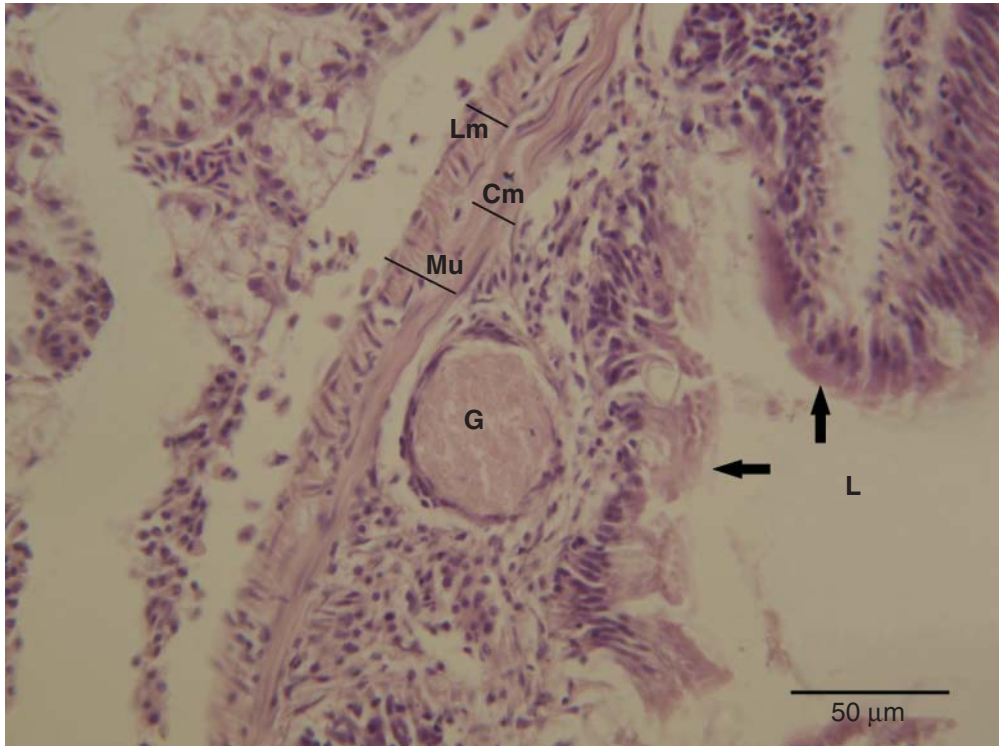


Plate 8 Light microscopy (H&E staining) transverse section of the intestine of zebrafish with clinical signs of chronic mycobacteriosis. A lesion (granuloma, G) in the submucosa is indicated by the formation of fibrous connective tissue around a small (~50 µm) focus of acellular material. Key: arrow, epithelium; Cm, circular muscle; L, lumen; Lm, longitudinal muscle; Mu, muscularis. Scale bar = 50 µm. (Source: Kuehlwein *et al.*, unpublished.) For further information see text near Figure 3.6.

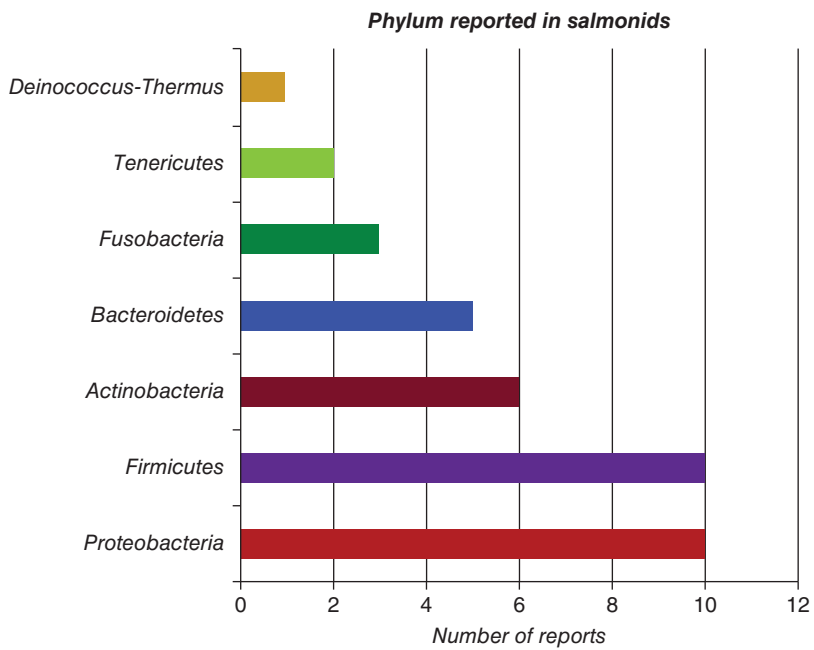


Plate 9 Bacterial phyla observed in the gut microbiota of salmonids. (Source: Nayak 2010. Reproduced with permission of John Wiley & Sons.) For further information see text near Figure 4.2.

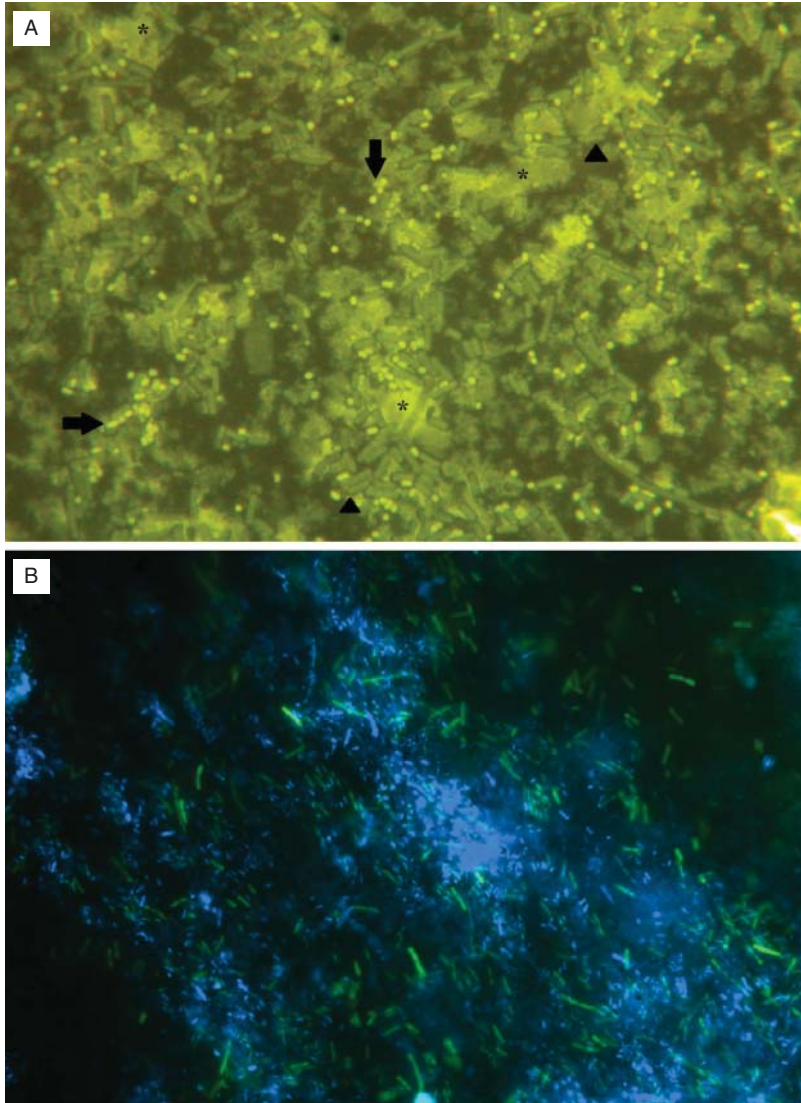


Plate 10 Epifluorescence micrographs of the indigenous gut microbiota of fish. (A) *In situ* hybridization of rainbow trout gut microbiota with green fluorescein labelled probe EUB338-mix (detecting all bacteria) (Waines and Merrifield unpublished data). Despite the clearly identifiable cocci (arrows) and rod-shaped bacteria (arrowheads) present within the sample, reliable enumeration is difficult due to the dense nature of the gut contents which leads to poor differentiation between individual cells in some cases. In addition, non-specific hybridization and fluorescence of background material (digesta and/or mucus) is present (five-pointed stars). (B) DAPI staining (blue) and *in situ* hybridization of common carp gut microbiota with green fluorescein (FITC) labelled probe VA (detecting *Aeromonas* and *Vibrio* spp.). (Source: Mark Rawling.) For further information see text near Figure 5.1.

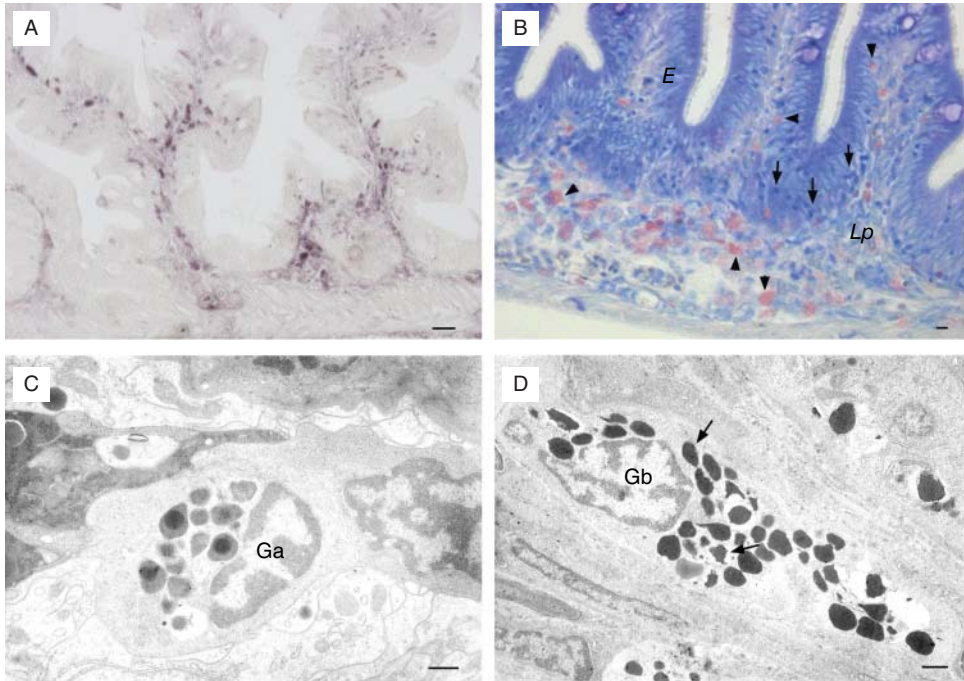


Plate 11 (A) Immunohistochemistry of Ig⁺ intestinal lymphocytes in 99 dph sea bream fry. The polyclonal antiserum ORa immunostained Ig⁺ cells seed both the mucosal epithelium and the lamina propria (probiotic group A). (B) May-Grünwald-Giemsa staining of posterior intestine in 99 dph sea bream fry showing lymphoid cells (arrows) and acidophilic granulocytes (arrowheads) in the mucosal epithelium (E) and lamina propria (Lp) (probiotic group A). (C) Electron microscopy of sea bream granulocytes housed in the intestinal mucosa. This subpopulation (Ga) is characterized by a rounded shape and cytoplasmic granules. The granules have electron dense paracrystalline cores. (D) Electron microscopy of intestinal sea bream granulocytes housed in the lamina propria (Gb). Gb granulocytes have an elongated shape, a distinct granule type and autolysosomes (arrows). Scale bars: A = 20 μ m; B = 5 μ m; C = 500 nm; D = 1 μ m. (Source: Simona Picchietti.) For further information see text near Figure 10.1.

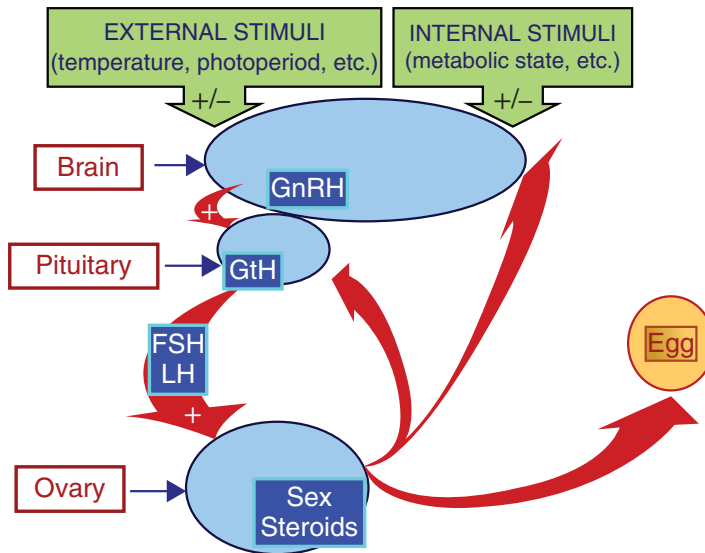


Plate 12 Diagrammatic presentation of the actions of the HPG axis in the control of reproductive function. The hypothalamus, integrating internal and external stimuli, releases the gonadotrophin releasing hormone (GnRH). Under the influence of the GnRH, the pituitary secretes follicle stimulating hormone (FSH) and luteinizing hormone (LH) which act upon the ovary, regulating oogenesis by activating steroidogenesis. For further information see text near Figure 12.1.

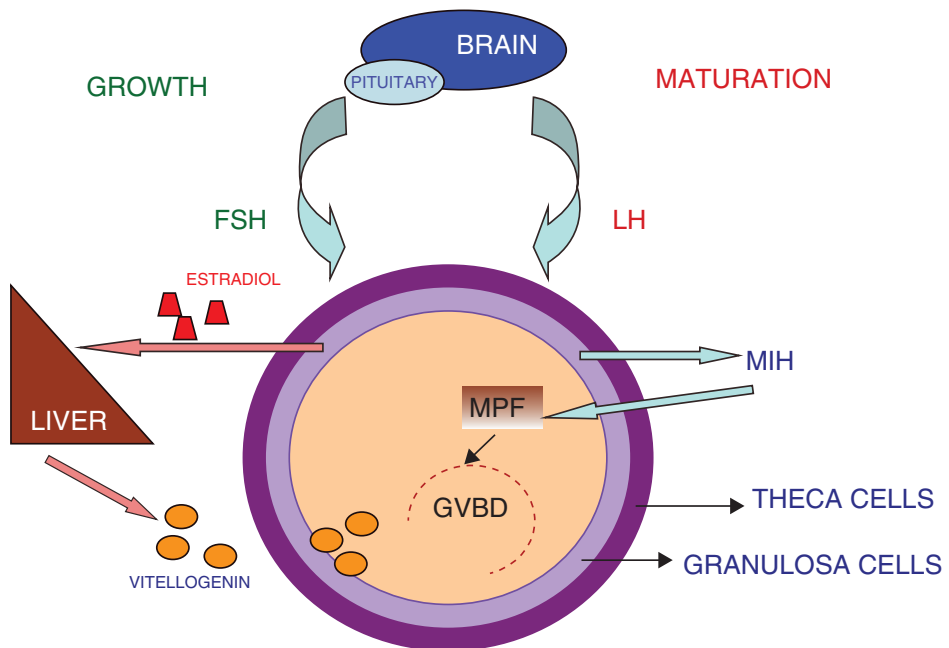


Plate 13 Control of follicle growth and maturation in teleosts. The action of follicle stimulating hormone (FSH) and luteinizing hormone (LH) is depicted in teleost oocytes during growth and maturation phases. A teleost follicle contains an oocyte, one layer of granulosa cells and one layer of theca cells. The basement membrane separating the granulosa and thecal cells and the zona radiata are omitted in the Plate. Under the influence of FSH, granulosa cells aromatize testosterone to estradiol, which initiates vitellogenin production in the liver, leading to follicle growth. Under the influence of LH, granulosa cells produce MIH, leading to the oocyte maturation. (Source: The model is based primarily on the work of Clelland and Peng 2009.) For further information see text near Figure 12.2.

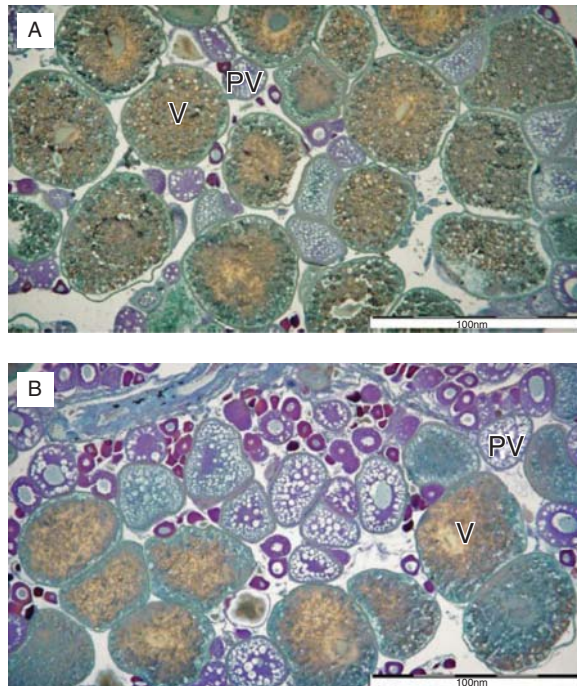


Plate 14 Representative micrographs of ovaries from (A) zebrafish females fed on an *Lb. rhamnosus* supplemented diet and (B) control fed females, revealing an increase in vitellogenic follicles in ovary sections from fish treated with the probiotic. PV = pre-vitellogenic follicles; V = vitellogenic follicles. Scale bars = 100 μm. (Source: Gioacchini *et al.* 2011.) For further information see text near Figure 12.3.

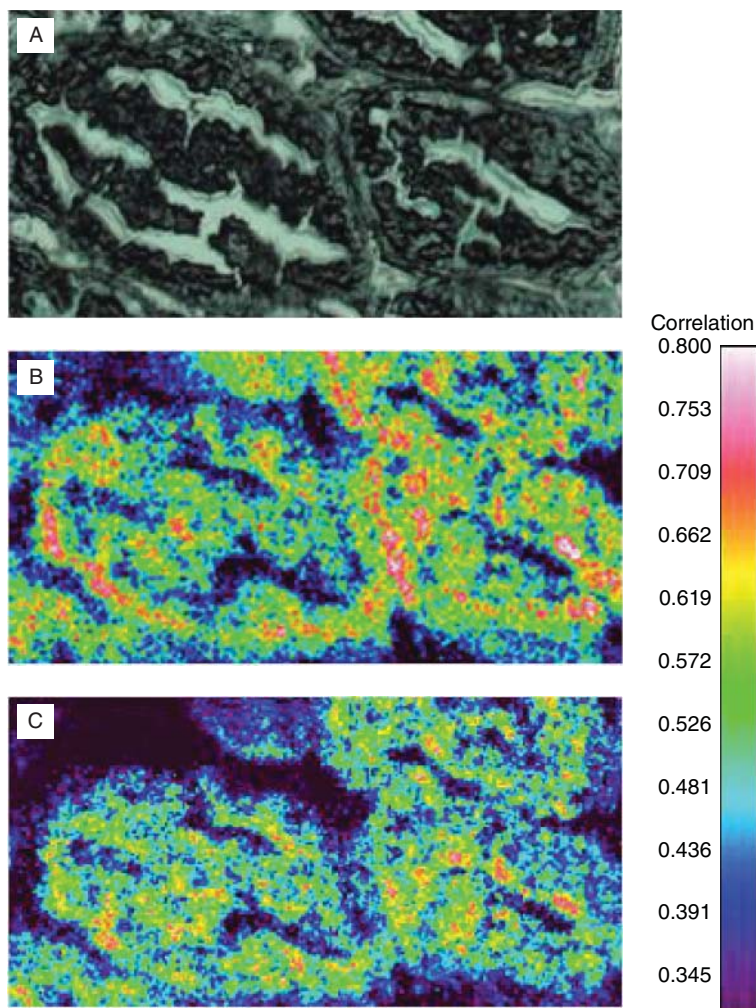


Plate 15 Compare (A) correlation map in the range 1800–1480 cm⁻¹ on an ovary section from zebrafish with (B) VTG and (C) LV representative spectra. The correlation maps obtained suggest that vitellogenin is present mainly in the outer zone of IV class oocytes, with lipovitellin homogeneously distributed in the inner. (Source: Carnevali *et al.* 2009.) For further information see text near Figure 12.5.

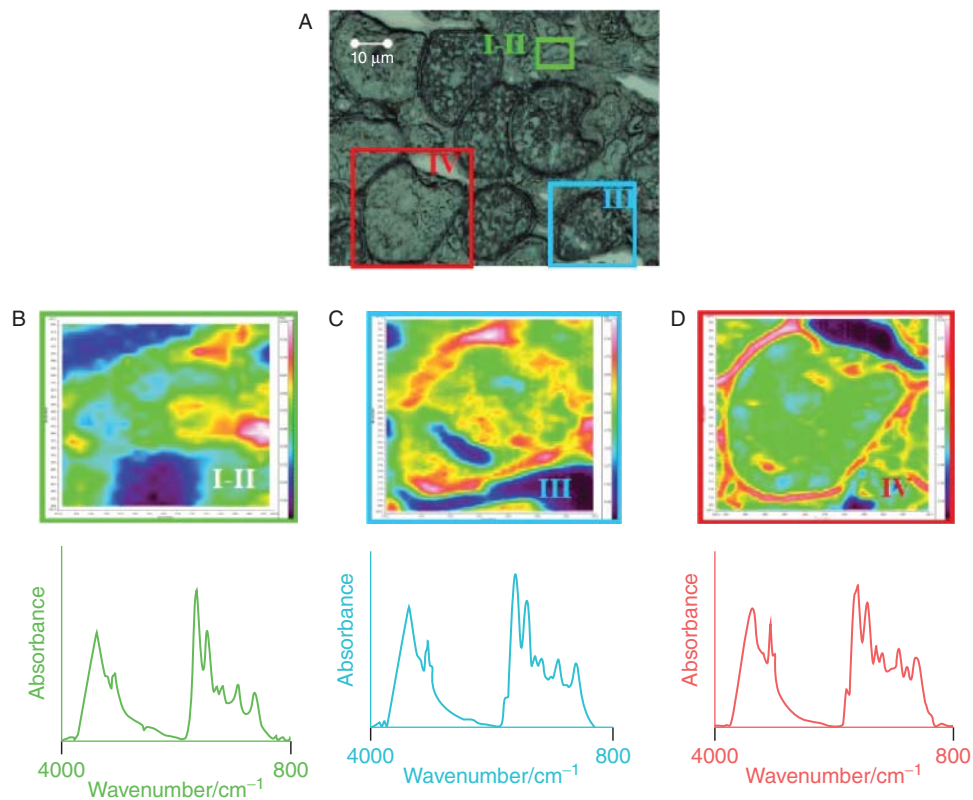


Plate 16 Compare (A) correlation map in the range $1800\text{--}1480\text{ cm}^{-1}$ on an ovary section from zebrafish with representative spectra from (B) class I-II, (C) class III and (D) class IV oocytes. (Source: Carnevali *et al.* 2009.) For further information see text near Figure 12.6.

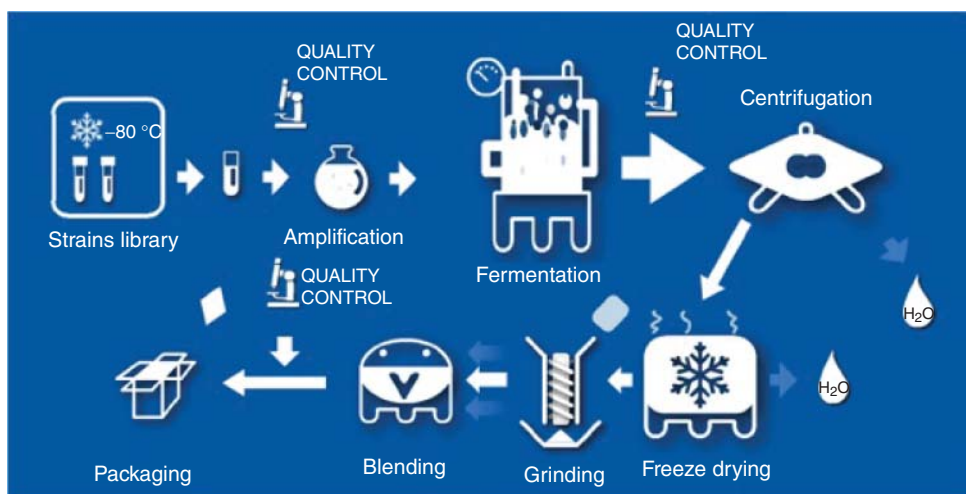


Plate 17 Schematic representation of a probiotic bacteria production process. For further information see text near Figure 13.1.

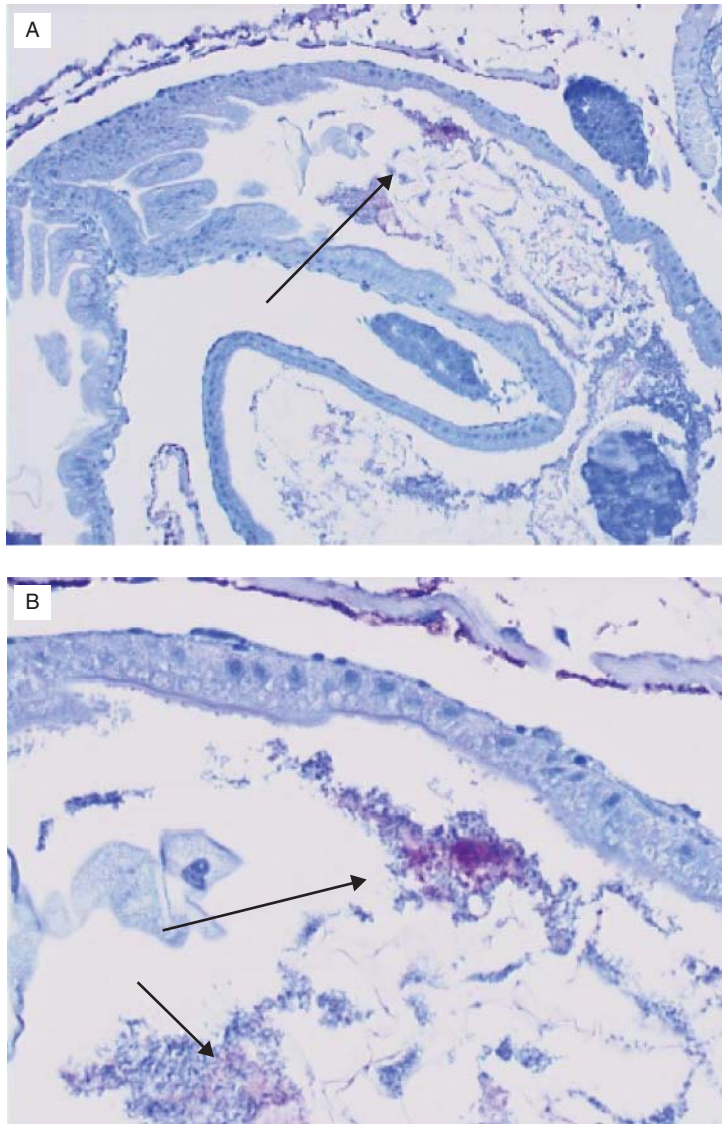


Plate 18 (A) Intestine of turbot larva fed with rotifers with bioencapsulated *Phaeobacter* 27-4. Counterstaining with haematoxyline (blue) and immunohistochemistry staining with primary antiserum against *Phaeobacter* 27-4 (red). The arrows point to partially digested rotifers with positive immunostaining, enlarged in (B). (Source: Planas *et al.* 2006. Reproduced with permission of Elsevier.) For further information see text near Figure 16.1.

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