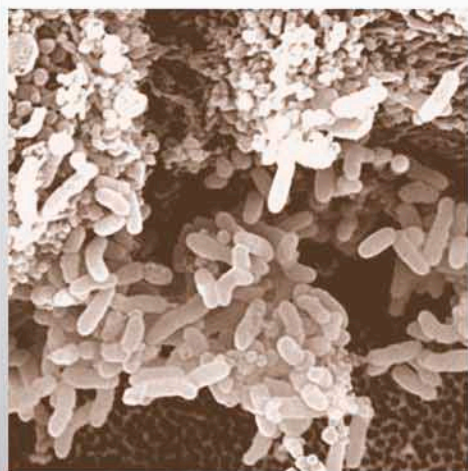




Functional dairy products

Volume 2

Edited by Maria Saarela

Functional dairy products

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Preface

According to the FUFOS (concerted action ‘Functional Food Science in Europe’) consensus document ‘a food can be regarded as “functional” if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease. Functional foods must remain foods, and they must demonstrate their effects in amounts that can normally be expected to be consumed in the diet. They are not pills or capsules, but part of a normal food pattern’ (see, e.g., Diplock *et al.*, *British Journal of Nutrition*, 81 (Suppl.):S1–S27). Thus a functional food can be a natural food in which one of the components has been enhanced, a food to which a component has been added to provide benefits, or a food from which a component has been removed to minimise the adverse health effects.

Dairy products have so far been in the front line in the development of functional foods. Fermented dairy products have traditionally been considered to have health benefits and thus broadening the product range to other types of health-promoting products is quite natural for the dairy industry. Probiotic dairy products, which contain health-promoting lactic acid bacteria (LAB) and/or bifidobacteria in addition to traditionally used starter LAB, are one very successful example of a functional dairy product type. Others include products supplemented with prebiotics, fibres, calcium, omega-3, plant stanols, and bioactive peptides produced by LAB. Functional dairy products are increasingly available in the daily-dose product format which has gained increasing popularity in the past few years. Consumers’ concerns about personal health are key drivers in creating markets for functional food products. In the future we will probably see more products targeted for special consumer groups. However, well-being trends as such are not gender or age specific: the consumer group of functional foods comprises all those whose concerns include physical and mental health. (see, e.g., Business Insights: the health food and drinks outlook to 2006). Provided that functional dairy foods taste good, are convenient to use and offer clear health benefits to the consumers,

this market segment will in all likelihood continue to grow also in the future.

The book *Functional Dairy Products, Volume 2* provides the reader with a concise overview of the field. It consists of three parts giving state-of-the-art information about the following topics: the health benefits of functional dairy products (e.g., weight management, infant health, gut health), functional dairy ingredients (pro- and prebiotics, hypoallergenic hydrolysates, and plant sterols and stanols), and product development (e.g., biomarkers, experimental models, probiotic genomics, technology, safety and products). This book should be of special benefit to individuals involved with nutrition and health, research on functional foods, and food product development.

Maria Saarela

Part I

The health benefits of functional dairy products

2 Functional dairy products

1

Dairy components in weight management: a broad perspective

L. S. Ward and E. D. Bastian, Glanbia Research Center, USA

1.1 Introduction

Milk as a nutritional food has recently been the topic of research and discussion related to weight management. In fact, consumption of dairy products has been linked to several health benefits that are the direct antitheses of diseases and complications that arise from overweight and obesity. For example, individuals that consume low-fat dairy products are more likely to have lower weight (Zemel, 2004), lower blood pressure (Moore *et al.*, 2005, Vollmer *et al.*, 2001), and decreased risk of stroke (Abbott *et al.*, 1996), colon cancer (Kampman *et al.*, 2000, Holt, 1999) and osteoporosis (McCabe *et al.*, 2004, Savaiano, 2003).

This review is an attempt to put into perspective the advances that have been made connecting milk components to weight management and to show some of our own data that support the concept that milk-derived components can positively impact weight management. For more information about the regulation of food intake and the effects of dairy products on satiety see Chapter 2.

1.2 Components from skimmed milk and weight loss

1.2.1 Calcium and weight loss

It may not seem obvious to discuss blood pressure in relation to weight management, but the link between dairy components and weight management was initially derived from blood pressure studies. Zemel (2002) reported a 4.9 kg reduction in body fat in an African American population that had

4 Functional dairy products

elevated blood pressure and were being 'treated' with dairy products to reduce blood pressure. When the dairy connection to weight management was proposed, Heaney *et al.* (2002) re-examined calcium-related blood pressure and bone studies and reported a strong relationship between dairy consumption and weight reduction.

Two mechanisms have been proposed to explain calcium's impact on weight and fat loss in the body: (1) reduced absorption of fatty acids and (2) metabolic shifts in adipocytes that reduce lipogenesis and increase lipolysis.

Dietary calcium and magnesium have a modest impact on overall energy balance through inhibition of fatty acid absorption via formation of calcium and magnesium soaps (Vaskonen, 2003). A randomized crossover study (Jacobsen *et al.*, 2005) evaluated the short-term effect of dietary calcium on fat absorption. A total of 10 subjects consumed a low calcium and normal protein diet, a high calcium normal protein diet or a high calcium and high protein diet. The high calcium normal protein showed a 2.5-fold increase in fecal fat excretion compared to the other two diets. Another study (Shahkhalili *et al.*, 2001) compared the absorption of cocoa butter with calcium (900 mg/day) or without calcium in a chocolate supplement. In this randomized, double blind, crossover study 10 men were fed control diets with or without the calcium supplemented chocolate. The results of the study showed a 2-fold increase in fecal fat excretion and a 9% decrease in absorbable energy. These two studies (Jacobsen *et al.*, 2005, Shahkhalili *et al.*, 2001) both obtained similar fecal fat values (8.4 g/day and 8.2 g/day) with an increase of calcium per day of 900 mg/day and 1261 mg/day respectively. Decreasing fat absorption results in less available energy to the body. In theory, obese individuals with a stable weight and low calcium consumption could lose 3.5 kg/y by increasing calcium consumption provided they have a consistent weight to begin with and maintain the same energy intake (Jacobsen *et al.*, 2005).

The second proposed mechanism by which calcium enhances fat loss is an indirect influence on fat lipolysis or lipogenesis through calcitrophic hormone regulation. Calcitrophic hormones respond to dietary levels of calcium. Particularly, 1, 25 dihydroxy-vitamin D is up-regulated when dietary calcium is low and down-regulated when dietary calcium is high. Increased levels of dihydroxy-vitamin D, in response to low calcium intake, cause calcium to be channeled into adipocytes. Conversely, when dietary calcium is high, calcium levels in adipocytes decline. Intracellular adipocyte calcium levels have a regulatory role on lipogenesis and lipolysis. When intracellular calcium levels increase, lipogenesis is up-regulated and lipolysis is down-regulated. When intracellular calcium levels are low, the situation is reversed. Thus, low dietary calcium intake results in high intracellular calcium, lipogenesis and reduced lipolysis; but high dietary calcium decreases lipogenesis and increases lipolysis (Zemel, 2002, 2003b, 2004, Zemel *et al.*, 2004, 2005). A recent study (Zemel, 2002) showed that increasing dietary calcium, via a serving of

yogurt, resulted in a larger decrease in body fat, body weight, waist circumference and trunk fat when compared to a non-dairy control. Trunk fat loss was 81% greater than the control samples and also resulted in a significant decrease in waist circumference (−0.58 versus −3.99 cm). Other researchers have specifically focused on calcium and implications in weight loss (Zemel, 2001, 2003a, 2003c, 2004, Zemel and Miller, 2004, Schrager, 2005).

1.2.2 Protein and weight loss

Protein plays a satiety, thermogenic and lean muscle preservation role during weight loss. Skov *et al.* (1999) compared a control group to two treatment groups that consumed either a high carbohydrate and low protein (12% energy) diet or a high protein (25% energy) and low carbohydrate diet. Both groups were on reduced fat diets and obtained 30% of their energy from fat. Foods that had the desirable protein, carbohydrate and fat levels were designated for each group. Each group consumed the various food products ‘ad libitum’. After a six-month time period the high protein group lost more (8.7 kg) than the high carbohydrate group (5.0 kg). Part of the difference was attributed to a protein satiety effect. The high protein group consumed less food energy (9.3 MJ/day) compared to the high carbohydrate group (11.2 MJ/day). It was also pointed out that the thermogenic effect of protein is much higher than carbohydrate (30% versus 4–8%) resulting in more calories being ‘burned’ when protein is consumed in place of carbohydrates. Weight loss also was partitioned differently between the two groups. The intra-abdominal adipose tissue decreased by 16.8 cm² in the high carbohydrate group compared to 33.0 cm² in the high protein group.

Layman *et al.* (2003) conducted a study looking at protein to carbohydrate ratios. A high protein group (carbohydrate to protein ratio of 1.4) was compared to a high carbohydrate group (carbohydrate to protein ratio of 3.5). This study controlled for caloric intake and both groups received isoenergetic diets over a 10-week period of time. Results showed that both groups lost significant weight. The high protein group lost 7.53 kg compared to 6.96 kg for the high carbohydrate group. This difference was not significant. However, when looking at body composition, the high protein diet showed a greater sparing of lean muscle tissue loss compared to the high carbohydrate diet. The high carbohydrate diet resulted in a lean muscle loss of 1.21 kg compared to the high protein group that lost 0.88 kg.

Some dairy proteins may offer added benefit for muscle sparing because of a high content of the branch chain amino acids, leucine, isoleucine and valine. The role of leucine in weight management has recently been published (Layman, 2003, Layman and Baum, 2004a). Leucine plays a regulatory role in signaling protein synthesis. Increasing intracellular levels of leucine by dietary consumption of protein promotes protein synthesis. When intracellular leucine concentration increases, it stimulates the activity of a kinase (mTOR). mTOR increases protein synthesis via phosphorylation of the 4E-BP1 binding

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protein or activation of p70^{S6} kinase. Both reactions stimulate subsequent reactions that lead to protein synthesis.

Leucine content in milk proteins is higher than other food protein sources. Skim milk contains both casein (~80% of the total protein) and whey proteins (20% of the total protein). Caseins are divided in the four main classes of α_{s1} -casein, α_{s2} -casein, β -casein and κ -casein. The occurrence of branch chain amino acids (number of BCAA/total number of AA $\times$ 100) found in each casein is approximately 20%, 13%, 19% and 24%. The major whey proteins include β -lactoglobulin and α -lactalbumin. The occurrence of branch chain amino acids for these proteins is 26% and 22% respectively. In comparison many other food proteins only contain 15–18% BCAA.

Though BCAA content is important, another aspect of milk proteins is the release (during digestion of skim milk) or the occurrence in many whey products of glycomacropeptide (GMP). GMP is a small (64 amino acid bioactive peptide) that stimulates the release of cholecystokinin. Cholecystokinin (CCK) is a peptide/hormone that is released from intestinal cells into the blood stream following the consumption of food. CCK acts on the stomach to slow gastric emptying and help maintain a feeling of satiety. CCK also binds to brain cell receptors indicating to the brain that the body is in a state of satiety. In humans, CCK levels in the blood normally reach a peak within 20 minutes following a meal and return to baseline within one hour (Portman, 2001).

Initial research linking milk proteins to release of CCK was done by Russian scientists who found that peptic hydrolysis of casein released physiologically active peptides that were capable of inhibiting gastric emptying (Stan *et al.*, 1983). In an effort to find which proteins and peptides were involved, they showed that a fragment of κ -casein (fragment 106–169) called glycomacropeptide (GMP) was the peptide responsible, in dogs, for inhibition of gastric emptying. Further research confirmed that GMP stimulates secretion of CCK in rat intestinal cells (Beucher *et al.*, 1994) and human clinical work done on a small number of volunteers showed that GMP increased blood levels of CCK up to 270% within 20 minutes after consumption (Yvon *et al.*, 1994). This work suggests that GMP, or fragment 106–169 of κ -casein, stimulates CCK release and thereby slows gastric emptying and creates a feeling of satiety. For further information on GMP please see a recent review (Brody, 2000). Recently, GMP was included in a weight loss product that was aimed at promoting satiety (Portman, 2001). Hall *et al.* (2003) compared the satiety impact of a casein-based beverage versus a whey-based beverage. Subjects consumed the casein- or whey-based beverage 90 minutes before a buffet meal. Results showed a statistically significant ($p < 0.05$) difference. When the whey beverage was consumed an average of 3676 kJ was consumed compared to 4537 kJ for the casein-based beverage. Specific circulating hormones, cholecystokinin and glucagons-like peptide, that are known to play an important role in satiety were measured. Results showed that the whey-based beverage produced overall higher levels of CCK and GLP-1.

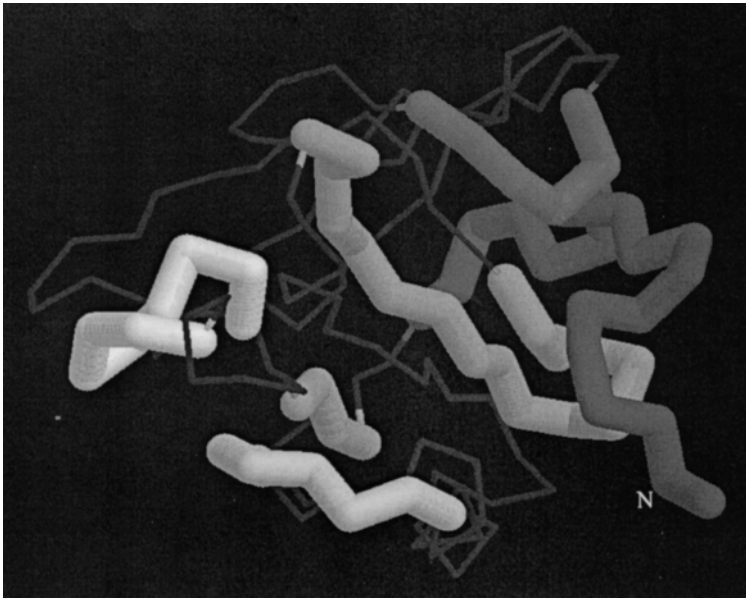


Fig. 1.1 Beta-lactoglobulin showing specific regions that are the primary source of bioactive peptides released by enzymatic hydrolysis.

Part of the physiological impact of whey proteins may be a result of rapid digestion that may also be related to enhanced satiety. Whey proteins are digested quickly and result in a rapid increase in serum amino acids (Dangin *et al.*, 2001).

Another aspect that has been studied is bioactive peptides produced as a result of enzymatic hydrolysis of intact milk proteins. *In vitro* research has shown that bioactive peptides derived from milk proteins influence lipogenesis in the adipocyte tissue. Adipocytes have a renin-angiotensin system with angiotensin II receptors on the cell membrane. When angiotensin II binds to its receptor, it triggers a cellular response that up-regulates expression of fatty acid synthase and glycerol-3-phosphate dehydrogenase. These two enzymes are, in part, responsible for lipogenesis in the adipocyte. Whey-protein derived, ACE-inhibitory peptides potentially reduce the level of angiotensin II binding to adipocyte receptors by 1) inhibiting ACE itself and 2) competitively binding the receptor, thus reducing the up-regulation of fat deposition enzymes (Jones *et al.*, 1997a, 1997b). Figure 1.1 shows ACE peptide regions of β -lactoglobulin, the major protein in bovine whey.

1.3 Obesity-induced diseases, weight loss and low-fat dairy products

The etiology of several diseases and physical ailments find root in obesity. In many cases obesity contributes, directly or indirectly, to the development

of heart disease (Buchholz and Bugaresti, 2005, Stefan *et al.*, 2005, Stamler, 1959), hypertension (Sharma *et al.*, 2001), diabetes (Sullivan *et al.*, 2005, Weinstein *et al.*, 2004), osteoarthritis (Felson, 2005, Powell *et al.*, 2005, Lieverse *et al.*, 2002, 2003, Gelber, 2003, Nevitt, 2002, Christensen *et al.*, 2005), dislipidemia, body inflammation (King *et al.*, 2005, Aronson *et al.*, 2004, Hiura *et al.*, 2003, Trayhurn and Wood, 2004), steatohepatitis (Yang *et al.*, 2004, Adams *et al.*, 2005), renal failure (Soto *et al.*, 2005, Agnani *et al.*, 2005), and various types of cancer (Batty *et al.*, 2005, McTiernan, 2005). Some of the adverse effects can be linked to adipokines. White adipose tissue secretes a class of molecules termed adipokines (Trayhurn and Wood, 2004). Adipokines include a wide variety of pro-inflammatory cytokines and other regulatory proteins that have a pleiotropic and adverse influence on functions such as inflammation, insulin sensitivity, immunity, blood pressure, lipid metabolism, appetite and angiogenesis (You *et al.*, 2005, Park *et al.*, 2005, Hung *et al.*, 2005, Marette, 2002).

1.3.1 Weight management and blood pressure

Obese and overweight individuals can have higher blood pressure which increases the risk for stroke and heart disease. Milk contains an abundance of potassium (140 mg/100 g), calcium (118 mg/100 g) and magnesium (12 mg/100 g). These minerals have been shown to lower blood pressure. Massey (2001) reviewed the available information relating to dairy food consumption and stroke. Two studies were highlighted that linked lower rates of heart disease to milk consumption. The first study was the Honolulu Heart Program that reported Japanese men (aged 55–68) who did not consume milk experience thromboembolic stroke at twice the rate of men who consumed two glasses or more of milk each day. The other study referenced found that the intake of Ca, K, and Mg reduced the relative risk of ischemic stroke. A recent observational study (Alonso *et al.*, 2005) assessed the relationship between hypertension and dairy consumption in 5880 individuals. A statistically significant effect against hypertension was found for skim milk, but not whole milk.

He *et al.* (2005) studied the impact of potassium in 14 individuals that had an initial high blood pressure (systolic ≥ 140 mm Hg and/or diastolic ≥ 90 mm Hg) in a randomized crossover study where individuals took potassium chloride or potassium citrate. There was a one-week treatment period followed by a one-week washout period before the crossover. Both potassium supplements showed that supplementing potassium chloride or potassium citrate resulted in an 11–13 mm Hg drop in systolic blood pressure and a 5 mm Hg drop in diastolic blood pressure.

Another mineral that has been shown to impact blood pressure is magnesium. A study (Rylander and Arnaud, 2004) compared three different waters that were consumed regularly with different mineral profiles. One water contained calcium 67.6 mg/L, another contained magnesium 82.3 mg/L and another

contained calcium 486 mg/L and magnesium 84 gm/L. The isolated calcium and magnesium groups did not show a significant reduction in blood pressure but the group that received both calcium and magnesium showed a significant decrease in systolic blood pressure of 6 mm of Hg and a diastolic drop of 2.5 mm Hg.

Calcium, as mentioned earlier in this chapter, has been linked to lower blood pressure. McCarron (1998) pointed out that the DASH diet (rich in dairy products, fruits and vegetables) results in a significant decrease (5–6 mm Hg) in systolic blood pressure in normotensive individuals and an even greater effect (11–12 mm Hg) in mildly hypertensive individuals. A similar diet without dairy did not produce the same results. Meta analysis of 42 published calcium blood pressure studies has confirmed a strong relationship between dairy, calcium, and decreased blood pressure (McCarron, 2000). Some of this effect is thought to be a result of the high level of potassium, calcium and magnesium in low-fat dairy products.

Bioactive peptides isolated from intact milk proteins also influence blood through inhibition of the angiotensin converting enzyme (ACE) in a dose-dependent manner. Encrypted in the primary sequence of whey proteins and caseins are bioactive peptides that can be activated by enzymatic release. Many peptides have been isolated and identified from casein and whey protein (Clare and Swaisgood, 2000, Abubakar *et al.*, 1998, FitzGerald, 2000, Pihlanto-Leppala *et al.*, 2000). For example, Fig. 1.1 shows the three dimensional structure of β -lactoglobulin. Many different peptides with ACE inhibition have been characterized from β -lactoglobulin. The highlighted areas identify the regions from which bioactive peptides originate. Specific regions of the protein serve as a source of these bioactive peptides. Thus, enzyme selection is critical for the release and preservation of these bioactive peptides. A recent study (Mizuno *et al.*, 2005) using bioactive peptides from casein showed a dose-dependent response and a decrease in systolic blood pressure from 1.7 to 10.1 mm Hg over a six-week period of time. Another study (Pins *et al.*, 2004) showed that a hydrolyzed whey protein isolate reduced both systolic and diastolic blood pressure by 11 mm Hg and 7 mm Hg during a six-week study.

1.3.2 Weight management and bone health

Low-fat dairy products provide an excellent mineral profile to minimize bone loss during weight loss. The elderly and people who have had weight reduction surgery (Hogan, 2005) are at higher risk for bone loss during weight loss periods and bone demineralization during weight loss can lead to subsequent bone fracture. Elderly women (cohort of 6785), examined over a six-year period of time, showed that weight loss was correlated with decreased hip-bone density (−0.92%) and a 1.8-fold greater risk of fracture. A short-term, six-week study (Cifuentes *et al.*, 2004) found that weight loss is associated with elevated requirements for calcium and, if not met, could result in increased

bone loss and put individuals at risk for bone fracture. Thus, low-fat dairy products provide an excellent source of bone minerals to help minimize bone loss during weight loss, particularly for the elderly. It is recognized that calcium supplementation during weight loss will minimize bone loss (Jensen *et al.*, 2001).

Though calcium is recognized as important for bone health, most people do not realize the added benefit of taking calcium with other supporting minerals that dairy products provide such as Mg, Cu, and Zn. For example, it has been shown (Saltman and Strause, 1993) that in postmenopausal women (average age 64.6), over a two-year period of time, a placebo group lost 2.23% bone mineral density compared to 1.66% loss for the group that supplemented with trace minerals (Cu, Mn, Zn) or a 0.5% loss for those taking a calcium supplement (1000 mg/day). When trace minerals and calcium were both consumed, there was an observed increase of 1.28% in bone mineral density. Additional studies support the idea of needing to supplement with both calcium and non-calcium minerals to minimize bone loss and maximize bone strength (Strause *et al.*, 1994, Saito *et al.*, 2004, Hermann *et al.*, 1997).

1.3.3 Weight management and insulin sensitivity

Feeding whey protein to insulin-resistant obese rats has been shown to decrease caloric intake, reduce body fat and result in a significant improvement in insulin sensitivity when compared to a red meat diet (Belobrajdic *et al.*, 2003, 2004). A better insulin response and a reduction in postprandial blood glucose has also been observed in human clinical studies (Frid *et al.*, 2005). The results from the CARDIA study showed that dairy intake was associated with all components of insulin resistance syndrome in a protective manner (Pereira *et al.*, 2002). Research (Layman and Baum, 2004b) has shown that individuals with abnormally high levels of insulin (76 μ U/ml, 2 hours postprandial) returned to normal levels within 10 weeks of being on a moderately high protein calorie-reduced diet (Layman and Baum, 2004b). The control carbohydrate group remained significantly higher over the 10-week period of time. Another feeding study (Nilsson *et al.*, 2004) determined the insulinogenic index for several proteins including milk, cheese, whey, cod and wheat gluten compared to white-wheat bread as a reference. Whey contained a significantly higher insulinogenic index compared to the other protein sources with the exception of milk. The whey component in milk is thought to contain the best insulinogenic component. The whey meal also showed increased secretion of glucose-dependent insulinotropic polypeptide (GIP); a peptide that enhances the secretion of insulin.

1.4 Purified components to enhance weight management

1.4.1 Component enrichment

Based upon the available research, components were purified from skim milk to form a whey protein/peptide-based ingredient (Prolibra®) that contained intact whey proteins (β -lactoglobulin and α -lactalbumin), peptides (glycomacropeptide and ACE inhibiting peptides) and minerals. Intact whey proteins were isolated by passing sweet whey through a microfiltration system followed by ultrafiltration to concentrate the protein. Some of the intact protein was hydrolyzed to produce a whey protein hydrolysate that contained bioactive peptides originating from the highlighted regions shown in Fig. 1.1. Milk minerals were purified from whey using differential solubility followed by ultrafiltration to remove lactose. The final composition of the product was 75% protein, 3% lactose, <1% fat, 14% ash (4% calcium) and 4% moisture.

1.4.2 Study design

This study was a double blind, placebo controlled, randomized study and was approved by an institutional review board and performed according to Good Clinical Practice Guidelines, the Declaration of Helsinki (2000), and US 21 CFR Part 50 (Protection of Human Subjects) and Part 56 (Institutional Review Boards). A total of 63 healthy individuals were recruited and randomly assigned to a control or treatment group. Baseline physical characteristics for each group are found in Table 1.1. The treatment and control groups consumed a dry mix beverage once per day that contained 26.5 grams of whey proteins and peptides or an isocaloric dry mix beverage. This was a free living study and subjects were counseled to reduce caloric intake by 500 calories per day. Weight measurements were made at time 0, 2, 4, 8, 12 and 16 weeks. Body composition was determined using dual energy x-ray absorptiometry (DEXA) at time 0 and 16 weeks. Caloric intake and food consumption was evaluated using a 3-day dietary record intake at the beginning of the study and for each visit during the study.

1.4.3 Results

There were 41 subjects that finished the study. The whey protein group lost a statistically significant amount of weight when compared to the initial

Table 1.1 Physical characteristics of the control and whey protein groups

	Age (y)	Gender (m/f)	Body weight (kg)	Height (cm)	BMI (kg/m ²)
Control	50.94 $\pm$ 2.99	4/19	93.96 $\pm$ 3.35	166.18 $\pm$ 1.60	33.94 $\pm$ 1.00
Whey protein	49.78 $\pm$ 2.73	4/14	91.65 $\pm$ 2.60	164.66 $\pm$ 2.14	33.83 $\pm$ 0.78

baseline weight ($1.76 \text{ kg} \pm 0.46$; $p = 0.00$), however, it was not statistically different from the control group that lost $0.71 \text{ kg} \pm 0.73$. According to dietary record analysis, the whey protein group reduced calories by 20 per day and the control group reduced caloric intake by 264 calories per day. Part of the weight loss may have been a result of increased dietary calcium. Subjects consuming the whey protein product also increased dietary calcium by 1000 mg/day. Using previous values (Jacobsen *et al.*, 2005) to calculate expected weight reduction indicate that the increase in calcium in the treatment group would result in a 1.07 kg loss in weight if caloric intake remained the same and calcium consumption increased.

Comparative analysis between the two groups showed expected trends and some significant differences ($p < 0.05$). Table 1.2 shows the changes in body fat and percent body fat from the start of the study to the end of the study. The whey protein group lost more body fat ($1.68 \pm 0.27 \text{ kg}$) than the control group (0.87 ± 0.36).

The percent body fat was significantly different between the two different groups ($P < 0.05$). Figure 1.2 shows the difference in percent body fat change

Table 1.2 Anthropometric measurements over time

	Total body fat (kg)		% Body fat	
	Start	End	Start	End
Control	35.48 ± 1.73	34.61 ± 1.67	40.03 ± 1.24	39.26 ± 1.25
Whey protein	33.03 ± 1.52	31.36 ± 1.71	38.18 ± 1.68	36.70 ± 1.84

	Total body lean mass (kg)		% Lean mass	
	Start	End	Start	End
Control	50.54 ± 1.88	51.04 ± 1.92	57.15 ± 1.19	57.96 ± 1.21
Whey protein	51.47 ± 2.34	51.86 ± 2.22	58.88 ± 1.60	60.36 ± 1.75

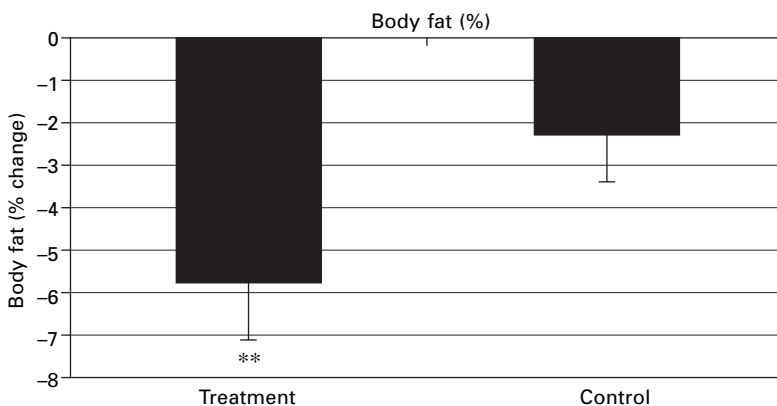


Fig. 1.2 Change in percent body fat over a 16-week period of time. ** $P < 0.05$.

over the 16-week period of time. The whey protein group had a $5.81\% \pm 1.28$ decrease in total fat mass compared to the control group that had a $2.29\% \pm 1.09$ decrease in body fat. The significant decrease in total body fat was attributed to the increased intake of dietary calcium and whey protein. This research suggests that, in an isocaloric state, a shift in body composition can be obtained by increasing whey protein, peptide and mineral consumption.

There was a slight increase in the total lean body mass over the 16-week period of time. The whey protein group increased in lean body mass by 0.39 ± 0.29 kg compared to the control group that gained 0.50 ± 0.34 (Table 1.2). These values were not statistically different from each other. However, the change in percent lean muscle content (Fig. 1.3) showed that the whey protein group had a $1.49\% \pm 0.26$ increase in percent lean muscle compared to the control group had a $0.81\% \pm 0.26$ increase in lean muscle content ($p = 0.08$).

1.5 Conclusions

Low-fat dairy products provide a solid nutritional base for losing weight. Calcium influences adipocyte metabolism indirectly through calcitrophic hormone levels. Dietary calcium and magnesium can form undigestable complexes that decrease the energy available from fat in food products. Milk minerals may play an important role in bone metabolism and help arrest bone loss during weight loss. This is more important in elderly than young. Protein components in milk provide high branch chain amino acid content and help maintain lean muscle tissue. Several components found in skim

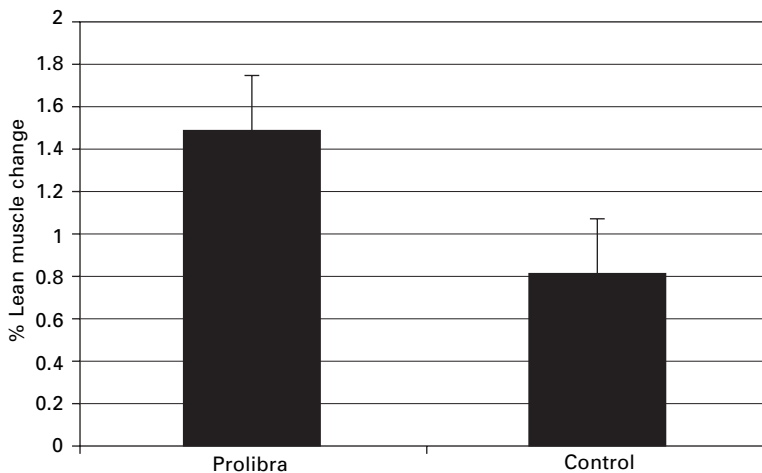


Fig. 1.3 The percent lean muscle change approached significance ($P = 0.08$) over the course of a 16-week study that evaluated enriched components from skim milk.

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milk may have a protective effect against the onset of disease that occurs as a result of obesity. Many of the components found in skim milk can be isolated and used in specific applications for individuals that do not consume dairy or may be lactose intolerant.

1.6 Sources of further information and advice

For further information, please review the references listed. Also, readers may want to review the Proceedings of the 4th International Whey Conference published by the American Dairy Products Institute, 2006.

1.7 Acknowledgement

The professional administrative assistance of Linda Harrison was greatly appreciated.

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2

The effects of dairy components on food intake and satiety

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2.1 Introduction

The prevalence of obesity has been described as a global epidemic (Bray and Bellanger, 2006) and warrants immediate interventions to halt its rampant progression. Recent advances in the field of genetics and molecular biology have greatly improved our understanding of the physiologic mechanisms regulating food intake and body weight. However, more than six hundred genes have been found to contribute to obesity (Perusse *et al.*, 2005), making a genetic solution unlikely. In addition, pharmacological and surgical interventions have either failed or are high-risk procedures that make them unsuitable for treatment, except for a relatively few, or for prevention of obesity (Aziz and Anderson, 2006). Therefore, dietary interventions remain the safest and most practical approach to controlling the obesity epidemic.

Among the wide and rich variety of foods in the diet, milk and dairy products have a unique potential to exert beneficial effects on determinants of energy balance. Many components of dairy, including protein, fat, carbohydrate, and minerals act independently, and possibly synergistically, to impart positive effects on body weight through their effects on intake regulation (Aziz and Anderson, 2006). Therefore, the objective of this chapter is to review the effects of dairy components on the regulation of food intake and satiety. The evidence associating dairy consumption with energy intake and body weight is reviewed first, followed by a brief review of the physiologic mechanisms of food intake regulation. The effects and putative mechanisms of action of dairy components, particularly protein and fat, on food intake and satiety are discussed. Finally, potential for developing dietary strategies

involving dairy and the development of dairy-based functional foods and infant formulas aimed at preventing and/or treating obesity is evaluated.

2.2 Dairy consumption, energy intake and body weight

Dairy consumption has been associated with both increased and decreased energy intake. A greater consumption of dairy products was associated with a higher energy intake of adults in Bogalusa, LA (Ranganathan *et al.*, 2005). Because an association was also found between the number of servings of dairy products and saturated fat intake, these results suggest that the higher energy intake results from the consumption of high fat dairy products. Surprisingly, the association between dairy consumption and body weight of the study group was not reported. However, Rajpathak and colleagues (2006) found that American men (Health Professionals Follow-up Study) who increased dairy consumption over 12 years gained slightly more weight than those who reduced intake the most. This association was largely due to the intake of high-fat dairy products because low-fat dairy intake was not associated with body weight change. Nevertheless, weight gain with increased dairy consumption was found to be less than predicted by changes in energy intake in healthy older adults (Barr *et al.*, 2000), suggesting greater energy efficiency with dairy consumption. This might be particularly true during periods of rapid growth, such as childhood and adolescence. Phillips and colleagues (2003) found no relationship between higher dairy intake and BMI z-scores or percent body fat in adolescent girls.

In contrast, a link between increased dairy product consumption and healthier body weight has been suggested in several reports. An inverse association has been reported between ready-to-eat breakfast cereal consumption and the BMI of 4–12-year-old children (Albertson *et al.*, 2003) and between the number of servings of dairy products and body fat in pre-school children (Carruth and Skinner, 2001). Similarly, in a large multi-center, population-based, prospective observational study, the number of dairy servings consumed was inversely related to the ten-year cumulative incidence of obesity and to the insulin resistance syndrome in adults (Pereira *et al.*, 2002).

The association between increased dairy product consumption and healthier body weights has been attributed to several milk components, including conjugated linoleic acid (Wang and Jones, 2004), medium-chain triglycerides (St-Onge and Jones, 2003), and particularly calcium (Heaney *et al.*, 2002; Zemel, 2004). Surprisingly, the role of milk proteins has received little attention, despite the fact that protein makes up a major fraction of milk and is the most satiating among the three macronutrients (Anderson and Moore, 2004).

In the following, evidence is presented to show that the consumption of dairy products or their components has a positive effect on satiety and leads to a reduction in energy intake. In order to provide background for establishing plausibility for the associations between dairy products and reduced food intake, a brief review of food intake regulation is provided.

2.3 The regulation of food intake

Food intake is regulated in both the short and long term by a complex and redundant physiological system that involves a cross-talk between the periphery and the central nervous system (CNS) (Aziz and Anderson, 2006). Although distinct, the pathways controlling short- and long-term food intake overlap in the CNS where they are integrated, orchestrated, and translated into either the suppression or the stimulation of food intake (Schwartz *et al.*, 2000).

2.3.1 The long-term regulation of food intake

Over the long term, the hypothalamus regulates food intake in response to hormones that enter the brain from the peripheral circulation and whose plasma concentrations are related to adipose tissue mass (Schwartz *et al.*, 2000). The two major hormones that have been implicated in the long-term regulation of food intake are leptin and insulin (Badman and Flier, 2005). However, it has recently been shown that ghrelin, a hormone secreted by specialized cells in the stomach, also meets the criteria for a long-term regulator of food intake (Cummings, 2006). Leptin is a hormone arising from adipose tissue and its plasma concentrations are directly proportional to the adipose tissue mass. Although insulin does not arise from adipose tissue, its concentration in blood at fasting and after food ingestion is influenced by adipose tissue mass. It has been proposed that both insulin (Biddinger and Kahn, 2006) and leptin (Munzberg and Myers, 2005) resistance in the brain lead to increased food intake. In contrast to leptin and insulin, ghrelin concentrations in plasma are inversely related to adipose tissue mass (Cummings, 2006).

The major site of action of the adiposity hormones in the brain is a discrete hypothalamic region called the arcuate nucleus (ARC) (Schwartz *et al.*, 2000; Cummings, 2006). Functional receptors for these hormones are expressed in the ARC. Despite the differences in leptin's and insulin's signalling pathways, both hormones elicit an increase in the expression of anorexigenic (appetite-suppressing) neuropeptides and a decrease in that of orexigenic (appetite-stimulating) neuropeptides (Schwartz *et al.*, 2000). On the other hand, the actions of ghrelin in the ARC are opposite to those of leptin and insulin (Cummings, 2006). The major anorexigenic neuropeptides are the pro-opiomelanocortin/ α -melanocyte stimulating hormone (POMC/ α -MSH) and the cocaine-amphetamine related transcript (CART), whereas the major orexigenic neuropeptides are Neuropeptide Y (NPY) and Agouti related protein (AgRP) (Schwartz *et al.*, 2000). Neurons expressing these neuropeptides project to neighbouring hypothalamic nuclei, particularly the paraventricular nucleus (PVN), ventromedial (VMH), dorsomedial (DMH), and lateral hypothalamus (LH) where they modulate the expression of other sets of anorexigenic (corticotropin releasing hormone (CRH) and thyrotropin stimulating hormone (TSH)) and orexigenic (melanin concentrating hormone

(MCH) and the orexins (ORX A and B)) neuropeptides (Schwartz *et al.*, 2000). Ultimately, the activity of these neurons is translated into the modulation of energy intake and expenditure.

2.3.2 The short-term regulation of food intake

The short-term regulation of food intake encompasses both satiation and satiety. Satiation describes the physiological factors that terminate a meal, whereas satiety refers to the time interval between two meals (de Graaf *et al.*, 2004). Although the energy content of the meal ingested is a major determinant of short-term food intake, it has been increasingly acknowledged that both meal size and meal frequency differ according to the macronutrient composition of the meal (Anderson, 1994).

Short-term food intake is controlled primarily by extra-hypothalamic regions of the CNS, specifically, by the brainstem that receives and integrates neural (vagal) and endocrine signals from the gastrointestinal (GI) tract (Badman and Flier, 2005). The absence of the blood-brain barrier from certain circumventricular organs of the brainstem, such as the nucleus of the tractus solitarius, allows circulating hormones to directly exert their actions on short-term food intake independent of the vagus nerve (Ganong, 2000).

The GI tract is the major organ that generates satiety signals in response to the ingestion of food (Badman and Flier, 2005). The complex and partly autonomous enteric nervous system, along with the plethora of specialized entero-endocrine cells, allows the gut to play a major role in the regulation of food intake. Digestive processes have evolved from the simple role of degradation of macronutrients into their absorbable units to the more complex interactions between macronutrients and the gut in the generation of satiety signals and the regulation of gastric kinetics. Therefore, the GI tract is at the interface between the food and the internal milieu and regulates the generation of satiety signals arising from food, both pre- and post-absorptively.

Pre-absorptive satiety signals

Pre-absorptive satiety signals are generated by the actions of food in the gut lumen and can be classified into endogenous or exogenous gut signals. Endogenous gut signals include mechano-, osmo- and chemo-receptors, but most importantly peptide hormones that are released by specialized endocrine cells in response to food ingestion (Anderson, 1994). Many gut hormones have been identified to date, including cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), peptide YY (PYY), and bombesin (Strader and Woods, 2005). Among the gut hormones involved in food intake regulation, only ghrelin has been shown to stimulate appetite (Badman and Flier, 2005). Interestingly, most of these hormones and their receptors are also expressed in brain areas such as the hypothalamus in addition to the hindbrain, emphasizing their importance in the control of food intake (Strader and Woods, 2005).

The regulation of the synthesis and secretion of gut hormones is complex, varies across species, and depends on both the macronutrient composition of the diet and other neuroendocrine factors. For example, fat is the major stimulant of CCK release in humans, probably due to the role of CCK in bile acid secretion (Liddle *et al.*, 1985), whereas protein is its most potent stimulant in rats (Liddle *et al.*, 1986). Furthermore, carbohydrates and fats are more powerful GLP-1 secretagogues than protein (Brubaker and Anini, 2003), although protein might enhance the GLP-1 secreting actions of the other macronutrients (Blom *et al.*, 2006; Lejeune *et al.*, 2006).

Exogenous gut satiety signals arise from dietary proteins. These have a hormone-like effect and are able to activate, at the level of the gut, satiety signalling pathways. Commonly referred to as bioactive peptides (BAP) (Kitts and Weiler, 2003), these intermediate products of protein hydrolysis can bind to and activate hormone receptors in the lumen of the gut and induce satiety. For example, BAP that have been shown to have a direct effect on food intake suppression via receptors are opioid-like peptides released during the digestion of casein (casomorphins), soy and wheat gluten proteins (Froetschel *et al.*, 2001; Pupovac and Anderson, 2002).

In addition to their direct effect on hormone receptors, BAP directly or indirectly stimulate the secretion of endogenous gut hormones. Indirect stimulation occurs when dietary peptides act as substrates for intestinal peptidases, thereby sparing hormone-releasing luminal factor from digestion (Liddle, 1995; Wang *et al.*, 2002). Also, BAP can directly stimulate the release of gut hormones from entero-endocrine cells. For example, glycomacropeptide (GMP), a glycosylated form of caseinomacropeptide (CMP), the first product of digestion of casein, and hydrolysates of β -conglycinin, a major protein in soy, have been shown to be potent CCK secretagogues (Brody, 2000; Nishi *et al.*, 2003a and b). Because BAP are inherent to dietary proteins and can suppress food intake by either stimulating satiety gut hormone release or acting as satiety hormones themselves, they could explain, at least in part, why protein is at the top of the satiety hierarchy of macronutrients.

Table 2.1 shows the major peptide hormones involved in food intake regulation.

Post-absorptive satiety signals

Absorbed nutrients, their metabolites, and some of the hormones involved in their metabolic processes constitute the post-absorptive satiety signals. Theories of feeding have been proposed based on each class of macronutrient: the glucostatic, the aminostatic, and the lipostatic theory of feeding (Anderson, 1994). However the role of changes in blood concentrations of glucose, amino acids, or lipids after a meal in determining either satiation or satiety has been difficult to define.

The fat and carbohydrate metabolites, ketones, lactate, and pyruvate have been proposed to play a role in food intake regulation. Only very high

Table 2.1 Major peptide hormones involved in food intake regulation

	Long-term (origin)	Short-term (origin)
Anorexigenic	Leptin (adipose tissue)	CCK (GI tract)
	Insulin (pancreas)	GLP-1 (GI tract)
	POMC/ α -MSH (hypothalamus)	PYY (GI tract)
	CRH (hypothalamus)	Opioid (dietary BAP)
	TRH (hypothalamus)	Insulin (pancreas)
		Leptin (adipose tissue)
Orexigenic	Ghrelin (GI tract)	Ghrelin (GI tract)
	NPY (hypothalamus)	
	AgRP (hypothalamus)	
	MCH (hypothalamus)	
	ORX (hypothalamus)	

Note: abbreviations are the following: AgRP: agouti related protein; CCK: cholecystokinin; CRH: corticotrophin releasing hormone; GI: gastrointestinal; GLP-1: glucagon-like peptide-1; MCH: melanin concentrating hormone; MSH: melanocyte stimulating hormone; NPY: neuropeptide Y; ORX: orexins; POMC: pro-opiomelanocortin; PYY: peptide YY; TRH: thyrotropin releasing hormone

concentrations of ketones, such as those observed during starvation or high fat, low carbohydrate diets, are associated with suppression of hunger and appetite (Havel *et al.*, 1999; Freedman *et al.*, 2001). In the fed state, lactate concentrations are increased in proportion to the carbohydrate content of the meal and thus could contribute to the suppression of food intake occurring after carbohydrate ingestion (Havel *et al.*, 1999). Pyruvate, an intermediate metabolite of glucose, glycerol and glucogenic amino acids, is at the center of the hepatic hypothesis of feeding that suggests that hepatic receptor discharges, which cause hyperphagia, are inversely related to the concentration of some key metabolite in the liver (Racotta *et al.*, 1984). In addition, because the liver is the first organ that processes nutrients after their absorption from the gut, it has been postulated that this organ plays an important role in the regulation of the satiety response, either through the presence of receptors or by interacting with the vagus nerve (Novin *et al.*, 1985).

A direct role for absorbed nutrients and their metabolites in intake regulation is difficult to define because their utilization is tightly linked to the release and function of many hormones involved in food intake regulation. For example, insulin and glucagon are released in response to carbohydrate and amino acid ingestion and play a role in short-term regulation of food intake (Anderson, 1994). Although insulin is involved in the long-term control of feeding, it is also directly and indirectly involved in short-term regulation of food intake. Insulin plays a role in the availability of glucose and amino acids to cells, including those in the brain, and in directing the interaction between nutrient ingestion and the action of satiety peptides such as CCK and food intake (Figlewicz *et al.*, 1986).

2.4 Dairy components and food intake/satiety

The effects of milk and dairy products on reducing energy intake and increasing satiety have been attributed to several of its components. These include milk proteins, casein and whey, and their products of digestion, milk fat, possibly MCT and CLA, and lactose.

2.4.1 Milk proteins

Milk proteins and food intake

Of the milk components, the proteins have the greatest potential to contribute to satiety for three reasons. First, it has generally been shown that protein results in greater satiety and lower ad libitum energy intake than either carbohydrate or fat in animals and humans (Anderson and Moore, 2004). Second, the magnitude of the effect depends on the source of protein, and milk proteins are more satiating than other sources (Anderson and Moore, 2004). Finally, mechanisms of protein-induced satiety have been described and are well documented (Anderson and Moore, 2004).

Of the milk proteins, whey protein has been studied the most, probably because it is readily available as a by-product of cheese making. It suppressed food intake more than sucrose and egg albumen at a pizza meal one hour later in young men (Anderson *et al.*, 2004). Similarly, intake of a buffet meal at 3 hours (Bowen *et al.*, 2006a; Bowen *et al.*, 2006b) after a 50 g preload of whey was lower than after a glucose preload. However, no differences were found between whey and casein (Bowen *et al.*, 2006a) or among whey, gluten and soy protein preloads (Bowen *et al.*, 2006b). In other reports, however, casein and whey had different effects on food intake. In a study by Hall *et al.* (2003), a preload containing 48 g of whey resulted in lower ad libitum intake of a buffet meal 90 min later than a preload containing the same amount of casein. In contrast, no differences were found in the intake of a pizza meal 90 min after 50 g pure preloads of casein and whey, but casein suppressed energy intake more than whey when the meal was offered 150 min later (Moore, 2004).

These discrepancies in the effect of casein and whey on food intake are possibly attributed to methodological differences. In the studies by Anderson and colleagues (2004) and by Moore (2004) the proteins preloads were essentially fat and carbohydrate free. In contrast, proteins provided only half of the energy found in the preloads whereas carbohydrate and fat provided the other half in the study by Hall (2003). Similarly Bowen and colleagues (2006a and b) used protein preloads that contained 17–30% of the calories from carbohydrate and fat. It is therefore possible that the interactions among the macronutrients were responsible for some of the discrepancies among the studies. Second, all studies except those by Anderson and colleagues (2004) and Moore (2004) did not have an energy-free control against which food intake suppression could be determined. Third, the commercial whey

formulation used in Hall's study (2003) was produced by ultrafiltration, a process that results in a relatively high content of GMP (15–20%) (Brody, 2000). GMP has been proposed to induce satiety due to its potent CCK-releasing property (Gustafson *et al.*, 2001). When low-GMP whey (<5%) was compared to casein, food intake was similar after 90 min, but higher after 150 min (Moore, 2004), suggesting that the relatively high GMP might have accounted for the lower energy intake after whey preloads in Hall's study (2003). It is not clear if a whey isolate free of GMP was used in Bowen's studies (2006a and b). Last but not least, the time of administration of the test meal after the preloads account for most of the differences in the effects observed between casein and whey. Whereas preloads of pure casein and whey equally suppressed food intake 90 min later, casein suppressed it more 150 min later. In rats, whey suppressed food intake more than casein 30 min after the administration of the preloads (Peng, 2005), also suggesting that whey affects satiety and food intake faster than casein, but that casein's effects are stronger later. When combined, there is a possible synergy to the consumption of milk proteins with whey leading to a more rapid satiation and termination of intake, while casein may produce greater satiety and therefore a longer inter-meal interval.

The concept of slow and fast protein

The effect of milk proteins on food intake is consistent with the classification of casein and whey as 'slow' and 'fast' protein, respectively. The classification of casein and whey as 'slow' and 'fast' protein is based on their contribution to protein synthesis and their effect on plasma amino acid concentrations (Boirie *et al.*, 1997). In humans, whey (50 g) results in a fast, but short and transient, increase in plasma amino acids that peak in 1 to 2 hours after its ingestion and return to baseline values after 3 to 4 hours. Casein, in contrast, and consistent with its slow gastric emptying, results in plasma amino acid concentrations that rise more slowly and are lower, but sustain a prolonged plateau lasting for at least 7 hours after its consumption (Boirie *et al.*, 1997; Dangin *et al.*, 2001).

Short-term (7 h) protein accretion, as measured by leucine kinetics postprandially, is different between casein and whey. Casein consumption suppressed whole body protein breakdown by 34%, while whey ingestion did not. Furthermore, whole body leucine oxidation over the 7-hour period was lower after casein ingestion. Conversely, whey ingestion stimulated postprandial protein synthesis more than twice as much as casein (68% vs 31%, respectively) (Boirie *et al.*, 1997). Overall, casein ingestion compared with whey resulted in a more positive leucine balance and greater postprandial protein gain in young but not in elderly subjects (Dangin *et al.*, 2002).

Both the rate of digestion and the presence of BAP could account for the different effects of casein and whey on protein synthesis, as the digestive kinetics is an independent factor affecting postprandial protein deposition (Bos *et al.*, 2003; Dangin *et al.*, 2001), and this in turn is modulated by BAP

released from casein. Whey remains soluble in the acidic media of the stomach, as do small peptides from casein (Ledoux *et al.*, 1999), and both are emptied relatively rapidly from the stomach. The remainder of the casein fraction precipitates in the stomach.

Total GI transit time is longer after casein than after whey ingestion (Daniel *et al.*, 1990; Mahe *et al.*, 1996). However, due to casein's prolonged hydrolysis in the stomach, it enters the duodenum mainly in the form of degraded products, including BAP, which are rapidly digested into constituent amino acids and absorbed (Mahe *et al.*, 1996). Both caseinomacropeptide and casomorphins from casein contribute to longer intestinal transit time because they slow the rate of gastric emptying (Brody, 2000; Daniel *et al.*, 1990). On the other hand, whey is hydrolyzed primarily by pancreatic proteases and is therefore absorbed more distally in the intestine, but overall, whey digestion and absorption are relatively more rapid processes than those of casein (Mahe *et al.*, 1996).

Milk proteins and pre-absorptive mechanisms of food intake regulation

The GI tract plays a dominant role in the interplay between dietary proteins and the regulation of many physiologic functions. It is the largest endocrine organ of the body and the primary source of regulatory peptides whose secretion is stimulated by the ingestion of food (Badman and Flier, 2005). The gut recognizes the composition of the food ingested and sends signals, in the form of peptide hormones, to different organs in anticipation of the metabolic requirements for the processing of the nutrients derived from the digestion and absorption of that particular food. Gut hormones are also crucial in the regulation of food intake (Badman and Flier, 2005).

The pre-absorptive mechanisms of action of dietary protein in general involve the release of endogenous satiety gut hormones and BAP that exhibit direct or indirect effects on food intake (Anderson and Moore, 2004). Among proteins, casein and whey have unique inherent properties that lead to the generation of many satiety signals from the gut and account for the reduction in food intake and appetite after milk and dairy consumption. Among the gut hormones known to date to be involved in milk-protein induced satiety are CCK, GLP-1, ghrelin, insulin, and glucagon.

CCK

CCK is a well established satiety hormone (Strader and Woods, 2005). Studies in rats have demonstrated the involvement of CCK and its A subtype receptor in protein-induced food intake suppression (Figlewicz *et al.*, 1992; Miesner *et al.*, 1992; Trigazis *et al.*, 1997 and 1999). In humans, dietary protein and fat are the most important stimulators of CCK secretion (Liddle *et al.*, 1985), and protein digestion is necessary to elicit CCK release (Liddle, 1995).

The protein-induced release of CCK is mediated by the indirect or direct actions of peptides released during digestion. By acting as substrates for protease and peptidase actions in the GI tract, proteins and peptides arising

from their hydrolysis spare the degradation of endogenous CCK-releasing factors, including the monitor peptide secreted from pancreatic acinar cells into the duodenum (Liddle, 1995) and the luminal CCK-releasing factor (Wang *et al.*, 2002). Milk proteins increase CCK concentrations in plasma. Blood CCK concentrations peak initially at 15–20 minutes. After this peak they fall and then increase again to approximately 90 min (Bowen *et al.*, 2006a; Hall *et al.*, 2003). Whey increased CCK more than casein the Hall (2003) but not in the Bowen (2006a) study. This effect of whey was also found to be similar to soy and gluten proteins (Bowen *et al.*, 2006b).

GLP-1

GLP-1 may also play a role in milk protein-induced satiety. Both carbohydrate and fat are potent stimulators of GLP-1 (Brubaker and Anini, 2003) but milk proteins stimulate GLP-1 release independent of carbohydrate and fat. However, whey appears to be the stronger and its secretagogue effect might be enhanced in the presence of other macronutrients. A high protein breakfast (58% of total energy) consisting mainly of dairy products enriched with whey protein isolate resulted in higher GLP-1 concentrations over 3 hours than a high carbohydrate breakfast (19% of total energy from protein) consisting mainly of plain yoghurt (predominantly casein) (Blom *et al.*, 2006). Similarly, when protein preloads of 50 g were given with an additional 200 kcal from fat and carbohydrate, whey protein ingestion resulted in higher plasma concentrations of GLP-1 than casein for up to 3 hours in humans (Hall *et al.*, 2003). However, with time it may be that casein has a stronger effect than whey in analogy with plasma amino acid concentrations that begin to fall three hours after whey ingestion in humans, but remain elevated for at least 7 hours after casein ingestion (Boirie *et al.*, 1997). In support of this, plasma GLP-1 concentrations fell substantially 2 hours after the administration of isoenergetic whey, whey hydrolysate and casein hydrolysate solutions, but continued to increase only after the casein solution (Calbet and Holst, 2004).

Support for an indirect role of GLP-1 in milk-protein-induced satiety is provided by studies in rats showing that Exendin-4 (Ex-4), a GLP-1 receptor agonist, interacted with milk proteins to suppress food intake (Aziz and Anderson, 2002 and 2003). When rats were given an intraperitoneal injection of Ex-4 five min prior to the administration of casein or whey preloads by gavage, the suppression of food intake was infra-additive in relation to that caused by the preloads and drug alone, suggesting that both the milk proteins and Ex-4 activate the GLP-1 satiety signalling pathway. This effect was observed when the proteins were given in intact, partially hydrolyzed, or free amino acid forms (Aziz and Anderson, 2003), suggesting that GLP-1-, unlike CCK-induced satiety (Trigazis *et al.*, 1997) after protein ingestion does not depend solely on peptides released during digestion. Similarly, in humans, the increase in plasma GLP-1 concentrations was independent of the degree of protein fractionation (Calbet and Holst, 2004).

PYY

The role of the anorexigenic gut hormone PYY in protein-induced satiety has received little attention. Casein hydrolysate was shown to stimulate PYY release more than maltose or oleic acid when perfused into the dog ileum (Wen *et al.*, 1995). Similarly Calbet and Holst (2004) showed that milk proteins given by gastric tube to humans increased plasma PYY concentrations, independent of the degree of fractionation and amino acid composition of the proteins. However, when perfused into the cecum or colon of humans, casein hydrolysate fails to increase plasma PYY (Adrian *et al.*, 1993), suggesting that the small intestine is the site of their action for PYY release. Because PYY and GLP-1 are secreted from the same entero-endocrine cells (L-cells) (le Roux and Bloom, 2005), it is highly probable that both hormones will respond similarly to protein ingestion.

Ghrelin

Ghrelin is the only orexigenic gut hormone known to date; its concentrations usually reach a peak just before meal and are suppressed by food ingestion (Badman and Flier, 2005). Like other gut hormones, the plasma ghrelin response depends on the macronutrient composition of the meal. Whereas a decrease in ghrelin concentrations after carbohydrates is consistently observed, such is not the case with fat and protein (Williams and Cummings, 2005). Specifically, high protein meals or diets have been shown to either increase (Erdmann *et al.*, 2003; Vallejo-Cremades *et al.*, 2004), or decrease (Gomez *et al.*, 2004; Al-Awar *et al.*, 2005; Blom *et al.*, 2006; Bowen *et al.*, 2006a, Tannous Dit el Khoury *et al.*, 2006), or have no effect (Greenman *et al.*, 2004) on plasma ghrelin concentrations.

The variations in these reports can be attributed to species differences, background diet, presence or absence of other macronutrients or the time of administration of the preloads or premeals. However, current reports suggest that the milk proteins, given as preloads, are consistent in decreasing plasma ghrelin. Complete milk protein, casein, whey or GMP, given by gavage as preloads to rats all lowered plasma ghrelin concentrations similarly at 30 min compared to the water control (Peng, 2005). In humans, whey protein isolate and calcium caseinate suppressed ghrelin concentrations similar to lactose but more than glucose over 3 hours, an effect that was correlated with the greater suppression in subsequent energy intake (Bowen *et al.*, 2006 a). Whey, soy and gluten proteins also decreased ghrelin similarly over three hours (Bowen *et al.*, 2006 b).

Bioactive peptides

BAP released during digestion from milk proteins play a role in the suppression of food intake. Among these, GMP and opioid-like peptides are of particular interest. The hypothesis that GMP might play a role in satiety after dairy consumption arises from the observations that it stimulates CCK release in humans (Corring and Beaufrère, 1997) and pancreatic secretion (a marker of

CCK release) in rats in a dose-dependent manner (Pedersen *et al.*, 2000), although it remains to be determined whether its actions are direct, through binding to I-cells, or indirect, through sparing of CCK-releasing factors.

To date, there is only one report on the role of GMP on appetite and food intake in humans (Gustafson *et al.*, 2001). Drinks containing either 0.4 to 2% GMP failed to suppress energy intake an hour later, and failed to affect subjective ratings of satiety compared to control drinks. However, because GMP was the only energy source in the drinks, it is not surprising that such small doses did not suppress food intake and appetite. On the other hand, in rats given equal amounts of complete milk protein, casein, low-GMP whey and GMP by gavage 30 min before the onset of eating, the preloads of whey and GMP suppressed food intake more than preloads of casein and complete milk protein but only during the first half-hour of feeding (Peng, 2005). Whey and GMP resulted in higher plasma amino acids, insulin, and GLP-1 concentrations 30 min later. However, CCK concentrations were highest after casein and lowest after GMP (Peng, 2005). Although these results suggest that GMP is not a potent CCK secretagogue, the time of measurement might have been inappropriate. CCK in rat plasma usually peaks within the first 10 min after protein ingestion, and returns to baseline by 30 min (Liddle *et al.*, 1986). Casein, however, elevates CCK concentrations for up to an hour (Liddle *et al.*, 1986), possibly because of the slow passage of its hydrolyzed products into the small intestine (Mahe *et al.*, 1996). Nonetheless, it does not seem that GMP has uniquely potent inhibitory effect on food intake when given alone, but could act synergistically with other components of milk.

Milk protein, particularly casein, is rich in opioid-like peptides (casomorphins) (Meisel, 2004). The effect of these peptides on food intake depends on their primary site of action. Central administration causes hyperphagia (Cooper *et al.*, 1985; Giraudo *et al.*, 1998; Levine *et al.*, 1985), whereas the opposite occurs after peripheral administration (King *et al.*, 1979; Morley and Levine, 1982). Opioids exert their biological actions by interacting with three types of receptors-mu (μ), delta (δ) and kappa (κ)-(Minami and Satoh, 1995), that are expressed in the gut (Kromer, 1988).

Direct evidence for the role of opioid-like peptides in milk-induced satiety arises from studies in rats. The inhibition of eating elicited by intact casein was blocked by naloxone methiodide, an opioid receptor antagonist that does not cross the blood-brain-barrier (Froetschel *et al.*, 2001). Furthermore, the effect of casein and soy proteins and their respective hydrolysates on food intake was partially reversed by naloxone methiodide at a dose that does not increase food intake by itself (Pupovac and Anderson, 2002). The duration and the strength of the interaction between the opioid receptors and the preloads depended on the type and form of the proteins. The strongest interaction occurred with intact casein and its hydrolysate, suggesting higher opioid activity in casein than in soy, whereas the most prolonged interaction occurred with intact soy protein and its hydrolysate, suggesting that the release of opioid peptides from soy is slow. Although whey protein contains

opioid-like peptides, it remains to be determined if they contribute significantly to whey-induced satiety.

Milk proteins and post-absorptive mechanisms of food intake regulation

Plasma and brain amino acids

Amino acids, which are the major absorbed products of protein digestion, have been proposed to suppress food intake by modulating plasma and brain amino acid concentrations or by serving as precursors to or as brain neurotransmitters (Anderson, 1994). The aminostatic hypothesis was proposed when it was first observed that an increase in plasma amino acid concentrations associated with subjective satiety in humans (Mellinkoff *et al.*, 1956). This theory postulates that appetite is regulated through the ability of the brain to monitor fluctuations in plasma amino acid concentrations. However, increases in plasma and brain amino acid concentrations do not appear to explain either the immediate suppression of food intake occurring in rats or the reduction in food intake and appetite in humans given protein preloads. Amino acid concentrations show no temporal association with the feeding response in rats after preloads of either albumin, a complete amino acid mixture formulated after albumin, or the essential or non-essential amino acid components given by gavage (Anderson *et al.*, 1994). Also, by using the *in vivo* microdialysis technique that allows for continuous sampling of the cerebrospinal fluid in the free-moving animal, alterations in the extracellular amino acid concentrations of several brain regions, including the medial preoptic area, the LH and the PVN were found to occur too late to be the primary signal for satiation during food ingestion (Currie *et al.*, 1995; Choi *et al.*, 1999, 2000).

In young men, post-preload changes in amino acid concentrations did not predict either food intake at a pizza meal nor subjective appetite measurements made between the time of consumption by young men of preloads of complete milk protein, casein or whey and the consumption of the test meal (up to 150 min) (Moore, 2004). Similarly, plasma amino acid concentrations after whey and casein preloads did not associate with energy intake at a buffet meal fed 180 min later to overweight men (Bowen *et al.*, 2006a). Thus, changes in brain amino acid concentrations after protein consumption in the range of that consumed in a meal seem unlikely to be a primary determinant of satiation but may determine the food choice at the following meal (Anderson, 1994).

Fluctuations in plasma amino acid concentrations after protein ingestion alter neuronal activity as a result of the role of some as neurotransmitters or as precursors for neurotransmitters in systems known to regulate food intake (Anderson *et al.*, 1968; Anderson, 1981). Tryptophan (Trp), phenylalanine (Phe), tyrosine (Tyr) and histidine (His) have been investigated extensively because they are the precursors of serotonin, the catecholamines and histamine, respectively (Anderson, 1981). The availability of these precursor amino acids for conversion to neurotransmitters in the CNS depends on the selective

competition between them and other large neutral amino acids (LNAA) for uptake by transporters in the blood-brain barrier. For example, an up-regulation of serotonin synthesis is expected to occur when the Trp/LNAA in plasma increases, leading to subsequent reduction in food intake and appetite (Anderson, 1994).

However, increased neurotransmitter synthesis seems to be an unlikely explanation for the effect of milk proteins on food intake. Both casein and whey are rich in branched-chain amino acids (BCAA) relative to Trp, resulting in low postprandial Trp/LNAA ratio; yet, both proteins suppress food intake and appetite (Moore, 2004). It is possible, though, that the decrease in Trp/LNAA observed after milk protein ingestion affects subsequent food choices. Li and Anderson (1984) demonstrated that 5-hydroxytryptamine (serotonin) metabolism, as determined by prior food consumption, controls in part the relative proportion of protein and carbohydrate selected in a meal. An increase in serotonergic activity and turnover will increase preference for protein in a subsequent meal if rats have a choice between low and high protein diets. Because the plasma Trp/LNAA ratio is increased when proteins are consumed with carbohydrate it would be expected that the effect of proteins on intake mechanisms are different if consumed alone or with carbohydrate. For example, in humans when α -lactalbumin (a component of whey protein) was given as a drink (10 g with 10 g glucose) the plasma Trp/LNAA was higher than after carbohydrate alone (Beulens *et al.*, 2004). However, as would be expected from such small doses the preloads did not affect food intake or macronutrient preference 100 min.

Human and bovine milks vary significantly not only in their whey:casein ratio (Lien, 2003), but also in the fractions constituting these proteins (Meisel, 2004). α -lactalbumin constitutes 25% of human milk whey, but only 5% of bovine milk whey and 10% of whey-dominant infant formula (Lien, 2003). On the other hand, β -lactoglobulin is virtually absent in human whey (Lien, 2003). Although it is unknown whether the absence of β -lactoglobulin from human milk is physiologically important, α -lactalbumin is rich in Trp (Lien, 2003). Some studies showed that breast-fed infants have higher plasma Trp concentrations than formula-fed infants, despite the higher protein concentration of formulas achieved to ensure adequate intakes of all essential amino acids (Lien, 2003). Because breast-fed infants and those on α -lactalbumin-rich formula have higher plasma concentrations of Trp relative to other LNAA, and presumably brain Trp and serotonin, this is a possible mechanism by which breastfed infants tend to be smaller than bottle-fed infants.

A more recent theory supporting a role for milk proteins as determinants of energy balance revolves around the amino acid leucine (Leu) (Layman and Walker, 2006; Cota *et al.*, 2006). Leucine modulates energy partitioning by increasing protein synthesis via a pathway downstream of the insulin receptor. This might be of particular benefit during weight loss when preservation of lean body mass is desired and insulin concentrations fall (Layman, 2004). Thus, because protein synthesis is a signal of sufficient

energy availability, increased satiety might occur in response to high intakes of Leu from dairy products. Recently, it was shown that intracerebroventricular administration of Leu to 24-h fasted rats leads to suppression of food intake through increasing mammalian target of rapamycin (mTOR) activity (Cota *et al.*, 2006). However, whether increased intakes of Leu through dairy proteins play a direct role in satiety and energy intake remains to be investigated.

Insulin

There is epidemiologic evidence that overweight subjects consuming a high intake of milk or dairy products have a lower risk of developing diseases associated with the insulin resistance syndrome (Pereira *et al.*, 2002). Although the insulinotropic effect of milk has received little exploration until recently, it is clear that the insulin response to milk does not relate solely to its lactose content. Lactose, consumed alone has a glycemic index of 68 compared with 100 for white bread. When the same amount of lactose is consumed in milk the glycemic index is only 30. However, the insulin index is 90 for whole milk compared with 100 for white bread and 50 for lactose alone (Ostman *et al.*, 2001).

Of the milk proteins, whey leads to higher pre-meal insulin concentrations compared to casein (Dangin *et al.*, 2001). It has been reported to contain the predominant insulin secretagogue because the insulin area under the curve (AUC) after preloads of 25 g carbohydrate with 18.2 g of whey protein was 50% higher than for milk or cheese (Nilsson *et al.*, 2004). Insulin response was strongly correlated with plasma Leu, valine and isoleucine and lysine concentrations, amino acids that are known to stimulate insulin secretion (Nilsson *et al.*, 2004). However, the insulinotropic action of milk proteins could be explained by their actions in the GI tract and the resulting release of gut peptides by BAP present in the protein (e.g., GMP in the whey fraction) or by the release of BAP during digestion.

2.4.2 Milk fats and their mechanisms of action

Fat is another component of milk and dairy that contributes to satiety. Although fat has been generally shown to be the less satiating among macronutrients (Blundell and MacDiarmid, 1997), certain types of milk fat have been suggested to contribute to the strong effect of dairy consumption on satiety. Like proteins and carbohydrates, the effect of fats on food intake, appetite and their determinants varies among different types (Bellissimo and Anderson, 2003; French, 2004). Of these, MCT and CLA have received particular attention.

Medium-chain triglycerides

Interest in the effect of MCT (chain length between 6 and 12) on satiety arises from the fact that they are processed differently from long-chain

triglycerides (LCT). Whereas LCT are packaged into chylomicrons and transported through the lymphatic circulation to peripheral organs, MCT are absorbed directly into the portal circulation and transported to the liver for rapid oxidation (St-Onge and Jones, 2002). It has been suggested that increased consumption of MCT might be an effective strategy to prevent obesity through combined action of increased energy expenditure, decreased fat deposition and increased satiety (St-Onge and Jones, 2002). However, this discussion will focus exclusively on the effects of MCT on food intake and satiety and their mechanisms of action.

The effects of MCT on food intake and satiety are conflicting. Bray and colleagues (1980) fed rats diets containing 60% of energy from either LCT (corn oil), or MCT, or a mixture of both for 80 days and found that rats on the MCT diet had the lowest food intake and body weight. On the other hand, when rats received intragastric infusions of liquid diets containing 16% of energy as fat from either tributyrin (short-chain triglyceride; SCT), tricaprylin (MCT) or triolein (LCT), no effect of chain length on food intake was observed (Maggio and Koopmans, 1982). The discrepancies in these results might be attributed to the contribution of fat to total energy content of the diet. A high-fat diet, as the one in Bray's study is expected to increase ketone body formation through saturation of the β -oxidation pathway. Indeed, the authors proposed that β -hydroxybutyrate accounted for the lower energy intake in MCT-fed rats. However, increased production of ketones is not the only mechanism by which MCT might affect food intake. When rats were fed diets containing either LCT, or MCT or both (1:1 mixture), food intake decreased dose-dependently with increasing MCT content, and this effect was sustained over 24 hours (Furuse *et al.*, 1992). Devazepide, a CCK-A receptor antagonist injected intraperitoneally 40 min prior to feeding, increased food intake after both diets, although the effect was short-lived after MCT. These results are consistent with our observations that devazepide administration reversed the inhibitory effect of a coconut oil (rich in MCT) preload given by gavage on food intake only during the first hour of feeding (Bellissimo and Anderson, 2003). These findings indicate that both LCT and MCT suppress food intake through CCK and suggest that the transient contribution of CCK to MCT-induced satiety might be accounted for by the rapid rate of digestion of MCT.

Studies in humans have also shown that MCT suppress food intake. Rolls and colleagues (1988) gave preloads containing either 30% energy from LCT or predominantly MCT (24% MCT + 6% LCT) and found that the latter resulted in lower energy intake at a self-selected lunch 30 min later in non-dieters. Stubbs and Harbron (1996) investigated the effect of three different energy level of MCT (20, 31 and 40%) incorporated in a high-fat diet (61.5% of total energy) on energy intake and body weight. They found that the highest MCT level caused a decrease in energy intake without a significant change in body weight. Furthermore, the influence of chain length and degree of saturation of fatty acids on energy intake was examined in non-obese men

(Van Wymelbeke *et al.*, 1998). Energy intake at lunch was lower after the MCT- than after olive oil-, lard- or fat-substitute-containing breakfast, but these effects did not persist over dinner. In another study, the same authors compared the effects of four different lunches (sub-lunch containing fat substitute, LCT, MCT, and carbohydrate lunches) varying in MCT content and fat content on subjective appetite scores and food intake at dinner (Van Wymelbeke *et al.*, 2001). Although dinner meal request was delayed only after the carbohydrate lunch, energy intake at dinner was the lowest after the MCT lunch.

The mechanisms of MCT-induced suppression of energy intake in humans are not always consistent with those observed in animals. This is particularly the case with CCK (Maas *et al.*, 1996 and 1998; McLaughlin *et al.*, 1999; Barbera *et al.*, 2000), suggesting that CCK does not play a role in MCT-induced satiety in humans. However, other gut hormones might be involved. For example, Maas and colleagues (1998) showed that both medium-chain fatty acids (MCFA) and long-chain fatty acids (LCFA) increased plasma PYY concentrations, although the increase was higher after LCFA. GLP-1 release depends on the chain length and degree of saturation of fatty acids, but only fatty acids of chain length between 14 and 18 have been examined (Rocca and Brubaker, 1995). It remains to be determined whether ingestion of MCFA would increase GLP-1 concentrations. The effect of MCT on ghrelin secretion in humans has not been investigated. However, it was recently shown that ingestion of MCT or MCFA increased acylation of ghrelin, which is required for its activity, in gastric cells of mice (Nishi *et al.*, 2005a and b). The acyl addition corresponded to the ingested fatty acid moiety. Nevertheless, this increase was not accompanied by a parallel increase in total ghrelin or ghrelin mRNA, indicating that the MCFA is directly used for acylation reactions. In addition, prematurely weaned rats, which were less exposed to MCT in their mother's milk than normally weaned rats, had lower plasma and stomach acylated ghrelin (Nishi *et al.*, 2005b). The implications of these observations on energy balance are currently unknown, but they are contradictory to the beneficial effects on energy intake and expenditure observed with supplementation of MCT. Therefore, further investigation of this matter is warranted.

In addition to pre-absorptive signals, post-absorptive changes in metabolites and hormones could account for the decrease in subsequent energy intake after MCT ingestion. The postprandial peak in plasma free fatty acids after a high-fat breakfast is followed by a second peak just prior to the subsequent meal (Fielding *et al.*, 1996). In the study by Van Wymelbeke and colleagues (2001), a MCT lunch led to higher plasma free fatty acids than a LCT, carbohydrate and sub lunch prior to dinner, which is in accordance with the lipostatic theory of feeding. Plasma insulin concentrations were also highest prior to dinner after the MCT lunch. Because there is a direct relationship between plasma and cerebrospinal insulin (Schwartz *et al.*, 1990), and because insulin suppresses food intake when administered centrally (Badman and

Flier, 2005), the lower energy intake after MCT could arise from the actions of insulin in the brain.

Conjugated linoleic acid

Only one study examined the effect of CLA on food intake and subjective measures of appetite (Kamphuis *et al.*, 2003). After being placed on a very low calorie diet for 3 weeks, subjects were given either 1.8 g or 3.6 g of CLA, or placebo for 13 weeks. CLA dose-dependently lowered subjective appetite after an overnight fast, but failed to suppress energy intake at breakfast.

Table 2.2 shows appetite-suppressive mechanisms of action of dairy constituents.

2.5 Conclusions

It is concluded that the epidemiologic associations found suggesting that increased dairy consumption contributes to healthier body weights are plausible. The associations are supported by literature showing that dairy components and dairy products suppress short-term food intake and increase subjective satiety and stimulate the mechanisms known to signal satiation and satiety. Nonetheless, the favourable effects of dairy components consumed independently on food intake, subjective satiety and intake regulatory mechanisms were usually observed in experiments where the components were consumed in amounts higher than that found in usual serving sizes of dairy products. Thus, it remains unclear if usual consumption of dairy products has any direct effect on satiety beyond the energy that they contain. However, the current data point to many opportunities to consider further exploration of the role of dairy products and their functional components in dietary plans and in functional foods aimed at control of appetite and metabolism. There are several compelling directions to pursue.

At the present time it is not clear if the effect of dairy components on satiety and reduction of energy intake is best achieved by their consumption as independent entities or by their combination. The greatest support for a

Table 2.2 Appetite-suppressive mechanisms of action of dairy constituents

Pre-absorptive	Post-absorptive
CCK (GMP, casein, whey)	Trp/serotonin (α -lactalbumin)
GLP-1 (Whey, GMP, lactose)	Leucine (whey)
PYY (casein, whey, MCT)	Insulin (whey, GMP, MCT)
Ghrelin (whey, casein, lactose)	
Opioid peptides (casein)	

Note: abbreviations are the following: CCK: cholecystokinin; GLP-1: glucagon-like peptide-1; GMP: glycomacropeptide; MCT: medium-chain triglycerides; PYY: peptide YY; Trp: tryptophan

beneficial effect of dairy consumption on satiety and short-term food intake is provided from studies of milk proteins. Nevertheless, the contribution to satiety of milk fat and lactose should not be overlooked, because of their distinct actions on intake regulatory mechanisms. Additive and potentially synergistic effects between these different dairy components require further exploration. It may be that a functional food can be formulated that contains a balance and quantity of these components that enhance their effects on satiety to a greater extent than can be accounted for by their energy content alone. For example, manipulating the ratio of the different proteins, GMP, MCT, CLA and lactose in a functional dairy food matrix could maximize the generation of satiety signals and lead to a substantial decrease in subsequent energy intake.

Dairy proteins suppress short-term food intake and increase subjective satiety. However, the role of dairy protein and dairy products in the regulation of long-term food intake and body weight is less clear, but several lines of evidence suggest that further research to define its role is merited. In this context, questions raised in this review include: (a) What is the optimal dose, composition and time of day for consuming dairy protein? (b) Is whey better than casein for appetite control or is there some combination that optimizes the advantages of both in stimulation physiologic systems? (c) What are the functional peptides contained in milk proteins that are selectively beneficial for appetite control? (e) What are the mechanisms of action of dairy proteins and peptides? (f) Are there long-term benefits on body weight and insulin control to high dairy protein diets? (g) Are milk proteins beneficial in the management of insulin resistance? Is it possible that an isolate consumed prior to carbohydrate has therapeutic value in blood glucose control? (h) Are there stages in the life cycle where knowledge of the physiologic effects of dairy components may provide added benefits? For the elderly and for infants the functional effects desired will be very different. For example, there are many differences in human and bovine milks that might be of significance to the regulation of metabolism and food intake of the infant in later life (Anderson and Aziz, 2006).

It is anticipated that continued research on the milk components contributing to energy intake control will benefit the dairy industry through the development of functional food products and natural health products, novel processing technologies, substantiated health claims on dairy products and increased awareness of functional and health benefits of dairy product consumption. Consumers will benefit because answers to these questions have the potential to lead to new functional foods, food formulations and dietary recommendations for achieving healthy body weights.

In conclusion, the potential role of dairy components in contributing to improved regulation of energy intake, satiety and body weight is compelling and the development of dairy-based functional foods aimed at preventing and managing obesity merits continued exploration.

2.6 Sources of further information and advice

In this chapter, a thorough and comprehensive review of the effects of the macronutrients in dairy products on food intake and satiety was provided. Most of the discussion has focused on the role and mechanisms of action of proteins and their constituents because (1) protein suppresses food intake more than either carbohydrate or fat, and (2) their physico-chemical properties are translated into an array of biological actions that are distinct but often overlapping. In this regard, the functionality of whey protein has received considerable interest not only because of its unique characteristics, but also because its high availability as a by-product of cheese industry. For further information on the physiological actions of whey, '*The Wonders of Whey...Catch the Power*' is an excellent compilation of the proceedings of the 4th International Whey Conference in Chicago, IL, 2005. The book is available at the Federation of Animal Science Societies website: <http://www.fass.org>.

2.7 References and further reading

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2.8 List of abbreviations

AgRp: Agouti related protein
ARC: Arcuate nucleus
AUC: Area under the curve
BAP: Bioactive peptides
CART: Cocaine-amphetamine related transcript
CCK: Cholecystokinin
CLA: Conjugated linoleic acid
CMP: Caseinomacropeptide
CNS: Central nervous system
CRH: Corticotropin releasing hormone
DMH: Dorsomedial hypothalamus
GI: Gastrointestinal tract
GLP-1: Glucagon-like peptide-1
GMP: Glycomacropeptide
His: Histidine
LCFA: Long-chain fatty acids
LCT: Long-chain triglycerides
Leu: Leucine
LH: Lateral hypothalamus
LNAA: Large neutral amino acids
MCFA: Medium-chain fatty acids
MCH: Melanin concentrating hormone
MCT: Medium-chain triglycerides
MSH: Melanocyte stimulating hormone
mTOR: Mammalian target of rapamycin
NPY: Neuropeptide Y
ORX: Orexin
Phe: Phenylalanine
POMC: Pro-opiomelanocortin
PVN: Paraventricular nucleus
PYY: Peptide YY
SCT: Short-chain triglycerides
Trp: Tryptophan
TSH: Thyrotropin stimulating hormone
Tyr: Tyrosine
VMH: Ventromedial hypothalamus

3

Dairy products, probiotics and the health of infants and children

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3.1 Introduction

3.1.1 Intestinal bacterial microbiota in humans

In mammals the commensal intestinal bacterial microbiota reach a density of approximately 10^{11} bacteria per gram faeces with absolute numbers of approximately 10^{14} bacteria in humans (Neish, 2002). This means that 90 to 95% of cells in humans are made up of bacteria in the intestine. These intestinal commensal bacteria can be divided into the indigenous stable and long-term residents (autochthonous) and transient, temporary occupants (allochthonous) (Neish, 2002). Mammals exist with their commensal intestinal microbiota in an overall symbiotic relationship. The host provides a thermo-stable, nutrient rich environment for bacteria and the resident intestinal microbiota has advantageous effects for the host in terms of infection control, metabolism, and even organ development (Hooper and Gordon, 2001; Stappenbeck *et al.*, 2002). The presence of the resident bacterial microbiota reduces the risk of successful colonisation by pathogens, increases the fitness of the host to fight invading bacteria and contributes to the efficiency of the host's energy resource usage and organ development.

The majority of commensal intestinal bacteria have not been characterised in detail, since they are difficult to culture or not cultivable using conventional microbiological techniques. However, using genetic and biochemical methods, it has been possible to estimate the diversity of the microbiota (Heilig *et al.*, 2002; Satokari *et al.*, 2003; Suau *et al.*, 1999). These studies revealed that not only different host species are colonised by different bacteria, but that there are also inter-individual differences within one host species (Suau *et al.*, 1999; Tannock, 2002). It was estimated that the most common genera in

human intestinal bacterial microbiota are *Bacteroides*, *Clostridium*, *Eubacterium*, *Bifidobacterium*, *Peptostreptococcus* and *Peptococcus*, *Ruminococcus*, *Fusobacterium*, and less common *Escherichia*, *Enterobacter*, *Enterococcus*, *Klebsiella*, *Lactobacillus*, *Proteus*. All bacterial microbiota together consists of more than 500 species (Heilig *et al.*, 2002; Neish, 2002; Satokari *et al.*, 2003; Suau *et al.*, 1999). Recent genetic approaches are helpful to provide a better picture of colonising intestinal bacteria. From 13355 bacterial rRNA genes of the human intestine analysed, 395 different bacterial phylotypes were found. Among these there were 244 novel phylotypes and 80% have not been cultivated (Eckburg *et al.*, 2005). Among >200 000 bacterial rRNA entries in the Genbank, 1822 originated from samples of the human gut and 1689 have never been cultivated (Backhed *et al.*, 2005). These findings highlight the extreme variety of the human intestinal microbiota and our restricted knowledge in the microbiota.

3.2 Dairy products and probiotics in childhood disease

3.2.1 Development of the intestinal microbiota in the first years of life – role of breast feeding, prebiotics and infant diet

Current data suggest that the intestinal bacterial microbiota undergoes substantial changes in the first year(s) of life and remains relatively stable thereafter, although factors such as acute or chronic intestinal diseases, antibiotic treatment and the composition of the diet influence the composition of the microbiota (Barbut and Petit 2001; Hooper and Gordon, 2001; Nowrouzian *et al.*, 2003; Tannock, 2001 (see Fig. 3.1).

Endogenous mechanisms for control of bacterial growth in the intestine include gastric acid production, secretion of gastric, intestinal and pancreatic proteases such as pepsin, trypsin, chymotrypsin or lysozyme, bile-derived detergents, secretion of intestinal bactericidal peptides and IgA (Eckmann, 2005; Fahlgren *et al.*, 2003; Mowat, 2003; Sarker and Gyr, 1992).

While the intestine *in utero* is sterile under physiological conditions, a postnatal stepwise colonisation contributes to the development of a normal immune system (Caicedo *et al.*, 2005; Edwards and Parrett, 2002; Fanaro *et al.*, 2003; Guarner and Malagelada, 2003). The maternal vaginal and intestinal microbiota is a source of colonisation of the child (Fanaro *et al.*, 2003). Initially the child's intestine becomes colonised by pioneering enterobacteria. After changing the growing conditions for bacteria due to feeding of breast milk instead of the initial colostrum and due to the metabolic activity of resident pioneer bacteria, Bifidobacteria gain ground. The stool of breast fed infants contains predominately bifidobacteria and less frequently lactobacilli, bacteroides, enterobacteria, enterococci and clostridia (Fanaro *et al.*, 2003). Stepwise colonisation of the gut by defined bacterial strains is important for the development of the intestinal and systemic immune system and mediate protection against nosocomial infections. Bifidobacteria and lactobacilli inhibit

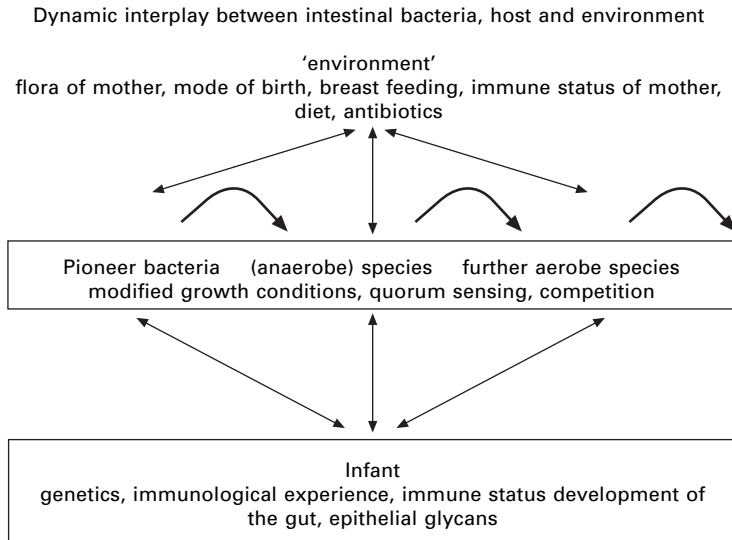


Fig. 3.1 Interactions between mother, developing child and environment contribute to the colonisation of the infant gut.

growth of pathogenic microorganisms through competition for space, production of lactic, acetic and other organic acids as well as H_2O_2 and antimicrobial peptides (Agostoni *et al.*, 2004; Cross, 2002; Howie, 2002; Howie *et al.*, 1990; Vicente *et al.*, 2003).

The specific microbiota of breast-fed children has been explained by the composition of proteins, the complexity of oligosaccharides and by numerous humoral and cellular mediators (Agramonte-Hevia *et al.*, 2002) within the human milk. There are several factors that influence the composition of the bacterial microbiota and contribute to infection control. Beneficial effects of breast milk are partially mediated via different proteins such as immunoglobulins, kappa-casein, lysozyme, lactoferrin, haptocorrin, alpha-lactalbumin, and lactoperoxidase. Those proteins have anti-microbial activity and support the immune defense of breast-fed infants against pathogenic bacteria and viruses (Lonnerdal, 2003). Further proteins such as insulin-like growth factor, epidermal growth factor or lactoferrin are involved in the development of the intestinal mucosa (Lonnerdal, 2003).

Compared to breast-fed infants, in formula-fed infants the microbiota is more diverse, containing *Bacteroides*, *Bifidobacterium*, *Enterococcus*, *Staphylococcus*, *Escherichia coli*, *Lactobacillus* and *Clostridium* as dominant cultivatable species. Children that are fed by breast milk but receive a supplement of formula harbour a microbiota that has overlapping microbiota characteristics of both diets (Fanaro *et al.*, 2003). The mode of delivery is a further important factor affecting the intestinal microbiota (Fanaro *et al.*, 2003). The microbiota of children born by caesarean section has been characterised by low numbers in *Bacteroides* spp. and *Bifidobacterium* and

increased numbers of *Clostridium perfringens* have been found (Fanaro *et al.*, 2003).

Oligosaccharides in human milk belong to the main components influencing the development of the intestinal microbiota (Boehm *et al.*, 2005; Coppa *et al.*, 2004; Newburg, 2000; Newburg *et al.*, 2005). The complexity of human milk oligosaccharides is high and based on the variable combination of glucose, sialic acid, galactose, fucose and N-acetylglucosamine. This complex mixture is further modified by various linkages between the respective sugar residues. Since the breast milk oligosaccharides are only partially digested in the small intestine, they reach the colon and stimulate the development of a bifidogenic microbiota (Boehm *et al.*, 2005; Coppa *et al.*, 2004). The recognition of effects of diet constituents on the promotion of specific probiotic microorganisms has been led to the development of the concept of prebiotics by Gibson and Roberfroid (1995).

Contrary to human breast milk, standard infant formulas are virtually free of prebiotic oligosaccharides (Boehm *et al.*, 2004, 2005). It has been proposed that standard formula-fed infants harbour a modified intestinal microbiota due to a lack of complex oligosaccharides. It was therefore aimed to supplement infant formula with prebiotic ingredients. Recent reviews summarising data of over 400 preterm and term infants, clearly demonstrate that prebiotic mixtures containing short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides stimulated the growth of *Bifidobacteria* and *Lactobacilli*, decreased faecal pH and normalised short-chain fatty acid pattern in infant stool (Boehm *et al.*, 2004, 2005; Fanaro *et al.*, 2005). Furthermore the presence of pathogens can be reduced to levels similar to those of breastfed infants (Boehm *et al.*, 2004, 2005; Fanaro *et al.*, 2005).

Preliminary data are available from clinical trials which indicate a reduced humoral allergic IgE response and reduced episodes of upper airway infection during the first year of life due to prebiotic supplementation (Boehm *et al.*, 2005). Altogether these data show that the use of prebiotic supplements and probiotics is still in its early stages but there is promising evidence that prebiotic oligosaccharides can provide beneficial effects for formula-fed infants.

3.2.2 Prevention of necrotising enterocolitis using probiotics

Necrotising enterocolitis (NEC) is a severe inflammatory reaction of the small and large intestine with a prevalence of one to two percent of all preterm infants (Henry and Moss, 2004). Eighty percent of the affected preterm infants have a birth weight under 2000 g. Major risk factors are prematurity, early enteral feeding and unphysiological bacterial colonisation. Other predisposing factors are absence of breast milk feeding and reduced perfusion of the intestine due to artery hypotension, hypovolaemia or persistence of the ductus arteriosus Botalli.

It has been shown that intestinal microbiota of infants on intensive care

units differs strongly from that of healthy neonates (Harmsen *et al.*, 2000; Millar *et al.*, 2003; Rubaltelli *et al.*, 1998). The microbiota of preterm infants on intensive care units has been shown to contain potentially harmful organisms (Millar *et al.*, 2003; Szajewska *et al.*, 2006). Predominant bacterial species in preterm children were *Enterococcus faecalis*, *E. coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* (Fanaro *et al.*, 2003). It has been proposed that modulating the microbiota of preterm infants on intensive care units into the direction of the one found in healthy breast-fed infants may prevent necrotising enterocolitis.

Several studies attempted to colonise the gut of preterm infants by different probiotic preparations to establish a microbial environment resembling the one by breast-fed infants. Among the strains administered there were *Lactobacillus rhamnosus* GG (ATCC 53103), *Lactobacillus acidophilus* (Chris Hansen Laboratory, Milwaukee) and *Bifidobacterium breve* (YIT 4010). Results of the ability to colonise the intestine were variable (Kitajima *et al.*, 1997; Reuman *et al.*, 1986; Schultz *et al.*, 2004).

In the recent years three high quality randomised controlled trials (RCTs) have evaluated the effect of probiotics on the prevention of necrotising enterocolitis (Bin-Nun *et al.*, 2005; Dani *et al.*, 2002; Lin *et al.*, 2005). Two of them evaluated supplemented formula, one breast feeding plus dissolved probiotics administered directly by spoon. Investigated probiotics strains were *L. rhamnosus* GG and probiotic mixtures, one containing *B. infantis* plus *Streptococcus thermophilus* plus *Bifidobacterium bifidum* (ABC Dophilus, Solgar, Wyeth Consumer Healthcare), the other containing *L. acidophilus* plus *B. infantis* (both ATCC 1973) (Bin-Nun *et al.*, 2005; Dani *et al.*, 2002; Lin *et al.*, 2005). The study performed by Dani *et al.* investigating the effect of *L. rhamnosus* GG on the incidence of necrotising enterocolitis, bacterial sepsis and urinary tract infections included 585 preterm infants (Dani *et al.*, 2002). Although there was a reduction in NEC and urinary tract infections the effect was not statistically significant. The two RCTs using probiotic mixtures revealed a significant decrease in incidence and severity of NEC compared to control group (1.1% versus 5.3% (Lin *et al.*, 2005) and 4% versus 16.4% (Bin-Nun *et al.*, 2005)).

The described RCTs did not reveal any serious side effects by colonisation with probiotic organisms (Szajewska *et al.*, 2006). Probiotics are therefore generally considered as safe. However, it should be taken into consideration that there is a potential and real risk of administering large quantities of viable microorganisms into infants with a highly immature immune system. Indigenous lactobacilli have been shown to cause rare pediatric infections in primarily immunocompromised infants (Thompson *et al.*, 2001).

Although the results on prevention of necrotising enterocolitis are promising, no general recommendation has been given so far for the general application of probiotics in preterm children.

3.2.3 Probiotics are an effective treatment option in viral and antibiotic associated diarrhoea

Enteritis is one of the most common infectious diseases in children. Between one and four years of age most children are affected once or twice a year. Eighty to 90 % of all cases with diarrhoea in developed countries are caused by viruses. Infection with rotavirus is the most frequent, followed by adeno- and norovirus infection. Therapeutic strategies aim to avoid and treat dehydration caused by fluid loss due to watery stools and vomiting. The most effective rehydration strategy is an oral fluid administration as hypoosmolar oral rehydration solution. The World Health Organization recommends oral rehydration in children with mild and moderate dehydration. Intravenous rehydration should be restricted to children with severe dehydration. Other more recently established therapeutic approaches include modulation of intestinal fluid secretion by enkephalinase inhibitors (Salazar-Lindo *et al.*, 2000). Successful efforts have been made to establish a safe and effective vaccination against rotavirus infection (Widdowson *et al.*, 2005).

There is also evidence that administration of probiotic microorganisms is of significant benefit in the treatment of acute diarrhoea in infants and children. Particularly in rotaviral gastroenteritis the beneficial effect of probiotic strains on the shortening of viral diarrhoea and dehydration in children has been well documented (Allen *et al.*, 2004; Huang *et al.*, 2002; Szajewska *et al.*, 2001, 2006; Van Niel *et al.*, 2002). The following probiotic strains were among those investigated: *L. rhamnosus* GG, *Lactobacillus reuteri* (SD 2112), inactivated *Lactobacillus acidophilus* LB (MA 65/4 E), *Bifidobacterium animalis* subsp. *lactis* plus *L. acidophilus* (Infloran berna) and *S. thermophilus* plus *Lactobacillus bulgaricus* (standard starter, International Yoghurt Manufacturers Club, Paris) (Szajewska and Mrukowicz, 2001; Szajewska *et al.*, 2006). Other nonpathogenic strains of different genera, including *Escherichia*, *Enterococcus* (LAB SF68), *Streptococcus faecium* (68) and *Bacillus* as well as the yeast *Saccharomyces boulardii* have also been used (Allen *et al.*, 2004).

L. rhamnosus GG is the probiotic organism studied with the most consistent beneficial effects on acute diarrhoea in childhood when used in doses $> 10^{10}$ CFUs (Szajewska and Mrukowicz, 2001). *L. rhamnosus* GG significantly reduced the number of watery stools in patients affected by rotavirus enteritis. Compared with placebo, the duration of diarrhoea was reduced from 13.6 to 33.6 hours with a normalised mean reduction of 20.1 hours as published in a systematic review of published randomised, double-blind, placebo-controlled trials in the field of probiotics in acute diarrhoea (Szajewska *et al.*, 2006). There were two studies reporting decreased vomiting in the *L. rhamnosus* GG and *L. reuteri* (SD 2112) treated group (Raza *et al.*, 1995; Shornikova *et al.*, 1997). One study showed a reduction in diarrhoea using the non-pathogenic yeast *S. boulardii* (*Saccharomyces cerevisiae* Hansen CBS 5926) (Kurugol and Koturoglu, 2005). Preliminary data suggest that the use of *E. coli* Nissle 1917 may have a significant and robust effect on the duration of acute diarrhoea in children (Henker, 2007).

So far it is not clear whether bacteria used as starter cultures for milk fermentation, such as *L. bulgaricus* and *S. thermophilus* may have beneficial effects (Szajewska *et al.*, 2006).

Although there seems to be a beneficial effect of probiotic intake on antibiotic associated diarrhoea due to *C. difficile* colonisation, no benefit on diarrhoea of other bacterial aetiology such as infection by *Salmonella*, *Shigella*, *Campylobacter*, *Yersinia* or *Entamoeba* has been proven (Guandalini *et al.*, 2000). This lack of activity in bacterial-induced diarrhoea may be an explanation why probiotics have shown clear effects in children in developed countries (predominance of viral infections) compared to reduced efficacy in developing countries (higher incidence of bacterial infections) (Szajewska *et al.*, 2006; Van Niel *et al.*, 2002).

Taken together it seems to be clear that probiotics do play a beneficial therapeutic role by reducing the duration of diarrhoea and frequency of stools particularly in young children. This strain-dependent, dose-dependent effect has been shown most consistently for *L. rhamnosus* GG (Szajewska *et al.*, 2006; Van Niel *et al.*, 2002). The exact mechanism of action is not clear and involves most likely several mechanisms including anti-microbial activity, microbial competition, epithelial adherence and anti-inflammatory activities.

Another interesting and highly relevant aspect for the use of probiotics is the prevention of nosocomial diarrhoea. The incidence of nosocomial diarrhoea in children admitted to a children's hospital ranges from 4.5 to 22.6 cases per 100 admissions (Ford-Jones *et al.*, 1990; Ponce *et al.*, 1995; Szajewska *et al.*, 2001). Nosocomial diarrhoea prolongs hospital stay and leads to increasing costs in medical health care. Transmission typically occurs by faecal-oral route, possibly also via droplet infection (Caul, 1994). Infants and younger children are at a higher risk to acquire nosocomial diarrhoea which is typically induced by virus infection. Bacterial infection occurs only in one percent of cases of nosocomial diarrhoea in children. Among these, *C. difficile* infection is the most frequent cause.

Few controlled trials in children intended to investigate the potential of probiotics for the prevention of nosocomial diarrhoea. These studies were performed using acidified formula milk supplemented with *B. animalis* subsp. *lactis* Bb12 and *S. thermophilus* (both Christian Hansen Laboratories Copenhagen) (Chouraqui *et al.*, 2004; Saavedra *et al.*, 1994) or powder containing *L. rhamnosus* GG (Mastretta *et al.*, 2002; Szajewska *et al.*, 2001). Results were partially contradictory. The initial RCT performed in 1994 evaluated infant formula supplemented with *B. animalis* subsp. *lactis* Bb12 and *S. thermophilus* on 55 infants in a chronic care hospital (Saavedra *et al.*, 1994). Compared with placebo it revealed a significant reduction of the prevalence of nosocomial diarrhoea within the probiotic group (7% versus 31%) as well as shortening of rotavirus shedding. The number needed to treat was 5 (95% CI 3-20). However, if the results of the study were presented as the number of episodes per patient-month instead of numbers of episodes per patient in each group, the results between both groups would not have

been significantly different (Szajewska *et al.*, 2006). A further RCT evaluated the effect of *L. rhamnosus* GG on the prevention of diarrhoea (Mastretta *et al.*, 2002). 220 children under the age of 18 months admitted to a children's hospital for other reason than infectious enteritis were included. Probiotic supplementation did not show any benefit on reduction of the incidence of diarrhoea (Chouraqui *et al.*, 2004; Mastretta *et al.*, 2002). In one trial *L. rhamnosus* GG reduced the incidence of nosocomial diarrhea and rotavirus gastroenteritis but there was no difference in the incidence of rotavirus infection (Szajewska *et al.*, 2001).

In summary there are some promising results showing the effectiveness of *L. rhamnosus* GG or *B. animalis* subsp. *lactis* Bb12 plus *S. thermophilus* in prevention of nosocomial diarrhoea. However, lacking consistent evidence for efficacy, general advice for the use of probiotics for prevention of nosocomial diarrhoea cannot be given so far.

Antibiotic-associated diarrhoea is regarded as otherwise unexplained diarrhoea in patients treated with antibiotics (for other reasons than diarrhoea) (Bartlett, 2002). Results that show beneficial effects of probiotics in antibiotic-induced diarrhoea in adults are encouraging (Sazawal *et al.*, 2006). There is also a moderate beneficial effect in children (Correa *et al.*, 2005; Kotowska *et al.*, 2005). Improving of antibiotic-associated diarrhoea has been shown for certain strains like *L. rhamnosus* GG, *S. boulardii*, *B. animalis* subsp. *lactis* Bb12 plus *S. thermophilus* (Szajewska *et al.*, 2006). There is one recent RCT evaluating the effect of a commercialised formula infant formula milk containing *B. animalis* subsp. *lactis* and *S. thermophilus* (Nan Probiotico by Nestle Brasil) that found a significant difference in the incidence of antibiotic-associated diarrhoea in children (31% versus 16%; NNT 7; 95% CI 4-62) (Correa *et al.*, 2005). These results suggest that probiotics could be a relevant supplement for antibiotic therapy in children but further RCTs are needed to propagate the regular use with the aim to prevent side effects of antibiotic therapy.

The potential mechanisms involved in the prevention and treatment of acute diarrhoea are discussed in more detail in Chapter 4.

3.2.4 Probiotics inhibit *Helicobacter* colonisation and support standard eradication therapy

Infection with the Gram-negative microaerophilic bacterium *Helicobacter pylori* is typically acquired in childhood. About 10% of patients develop symptoms of gastritis, peptic ulcer disease or MALT lymphoma, but anaemia and growth retardation is also associated with *H. pylori* colonisation in children (Czinn, 2005). The infection is a relevant health problem since approximately half of the world population is infected with *H. pylori*. In particular, high prevalence rates exist in developing countries and in populations with low socio-economic standard and poor hygienic conditions. *H. pylori* can be eradicated by anti-microbial therapy (typically amoxicillin plus clarithromycin

or amoxicillin plus metronidazole) plus a proton pump inhibitor (Bourke *et al.*, 2005; Czinn, 2005; Elitsur and Yahav, 2005). However, using the current antibiotic combinations complete eradication rate has not been achieved and current therapy is associated with gastrointestinal side effects.

There is increasing evidence that probiotics do not completely eradicate *H. pylori* but maintain lower levels of this pathogen in the stomach (Gotteland *et al.*, 2006). This reduction in bacterial load seems to increase the efficacy of standard eradication therapies. Furthermore, the use of probiotics may reduce the rate of adverse effects of current standard therapies (Gotteland *et al.*, 2006).

School children from a low socioeconomic area of Santiago de Chile that received one month live *Lactobacillus johnsonii* La1 (but not *L. paracasei* ST11 or heat-killed *L. johnsonii* La1) showed moderately reduced DOB values in the (13)C-urea breath test, indicating a direct modulation in *H. pylori* colonisation by living *L. johnsonii* La1 (Cruchet *et al.*, 2003).

H. pylori infection and probiotic interventions in adults are discussed in Chapter 4.

3.2.5 Probiotics and the prevention of allergy in children

According to the hygiene hypothesis the increasing number of allergic diseases in Western countries is caused by reduced exposure to pathogens in early infancy, improved hygiene standards, changes in diet, different delivery mode and smaller family sizes (Schaub *et al.*, 2006; Strachan, 1989).

The analysis of the composition of the faecal microbiota from healthy and allergic children found in affected children higher amounts of the adult type *B. adolescentis* compared with healthy infants harbouring more *B. bifidum*. It was suggested that *B. bifidum* has greater adhesive qualities which may help to stabilise the mucosal barrier and prevent adsorption of antigenic proteins (He *et al.*, 2001).

Similarly, the intestinal microbiota of atopic children has been found to be different from non-atopic individuals. The bacterial cellular fatty acid profile in stools from atopic children was significantly different from the one in healthy children. These findings correlated with more clostridia and a lower content of bifidobacteria in the faeces of allergic individuals (Bjorksten *et al.*, 2001; Kalliomäki *et al.*, 2001a). This might be a strong indication of an interaction of the gut microbiota with the immune system influencing the onset of allergy and atopy.

Kalliomäki *et al.* investigated the long term effect of early colonisation of infants with probiotic bacteria on allergy prevention. One hundred and fifty-nine pregnant women who had a positive family history for atopy were supplemented during their last month of pregnancy with *L. rhamnosus* GG. Probiotic administration was continued in mothers and children for six months after delivery. The primary end point was chronic atopic eczema. Probiotic treatment led to a significant reduction in the prevalence in at-risk infants at

the age of two years (46 % versus 23 %) (Kalliomäki *et al.*, 2001b). However, there was no decrease in antigen-specific IgE by *L. rhamnosus* GG administration. The authors performed a four-year follow up of the study group. Sixty-seven percent of the initially randomised children were re-examined. In the probiotic supplemented population there was a significantly decreased prevalence of atopic eczema compared with the non-treated group (14 of 53 versus 25 of 54 children) (Kalliomäki *et al.*, 2003). No difference was found upon skin prick test reactivity. Another long-term follow up study was able to show that intentional colonisation of the intestine with *E. coli* 083 (K24LH31) after birth decreased the incidence of allergies after 10 and 20 years after colonisation (Lodinova-Zadnikova *et al.*, 2003).

Results for the use of probiotic organisms in the field of allergy prevention are encouraging. Effects on early intervention seem to lead to longer lasting effects.

3.2.6 Developing probiotic and prebiotic products for infants – potential and open questions

The development of probiotics for use in children is a challenging task. Due to the flexibility of the developing microbiota in early childhood there is a clear potential to influence and modulate the intestinal microbiota to achieve health benefits. Furthermore there are several defined applications where probiotics have been shown therapeutic benefit. The therapeutic applications include relevant disorders with high incidence like infectious enteritis, *H. pylori* infection or allergy. Already these few applications mean that probiotics could be relevant for potentially every child.

However, there are several risks and questions that have not been answered yet: there is still some uncertainty about the safety of probiotics in very young children with an immature immune system as addressed earlier. In fact, there are few reports of sepsis in patients treated with *L. rhamnosus* GG. A 6-week-old full-term baby with double outlet right ventricle and a 6-year-old child with jejunostomy and several infections are among those reported (Land *et al.*, 2005). There are also reports of fungaemia by *S. boulardii* (*Saccharomyces cerevisiae* Hansen CBS 5926) after therapeutic use as probiotic (Hennequin *et al.*, 2000; Herbrecht and Nivoix, 2005; Rijnders *et al.*, 2000). This was probably due to contamination of central venous catheters while opening capsules for enteral feeding. Very restricted information is available about the effects and side effects of probiotic use in the long-term administration over several years. To our knowledge there is only one published report spanning a two-decade post-treatment follow up of therapeutic modulation of the intestinal microbiota by postnatal administration of *E. coli* 083 (K24LH31) (Lodinova-Zadnikova *et al.*, 2003).

Most studies performed on children used a specific therapeutic formulation of the probiotic microorganisms. Only in rare cases have dairy products been used to investigate health effects in RCTs. However, the route of delivery is

crucial for probiotic microorganisms. It is therefore not directly possible to extrapolate from effects seen in a specific pharmaceutical formulation with a defined dosage, to judge effects of probiotic bacterial strains used as dietary dairy product supplements. The viability of probiotics varies in different commercial products ready for consumption. There is a large variability of therapeutic effects between different strains and even quite related strains do not necessarily exhibit similar effects. Therapeutic effects cannot be generalised and are therefore restricted on a certain probiotic strain tested for a defined indication. However, data that enable the comparison of different probiotics side by side are limited. Since study design and populations vary between different RCTs, comparison between studies is error prone and further studies are needed to find the most efficient strains.

Prebiotics are easier to manage in industrialised processes compared to living probiotics. However, effects in terms of modulation of the microbiota are difficult to study and no relevant surrogate markers are useful for judging health effects. More randomised controlled clinical trials are needed to study the effects of prebiotics on disease prevention and treatment.

The mass market for supplementation of dietary products with probiotics carries some risk of transmission of pathogenicity or antibiotic resistance between different bacteria and the probiotics used. This would be in particularly relevant when genetically modified bacteria enter the use in humans.

Although there is an increasing number of well designed RCTs indicating the benefits of probiotics, we still do not understand the mechanisms of their biological activity in detail. Consistent with this, the influence of long-term administration of probiotics on allergy, autoimmune diseases, and infection control has not been understood.

Non-pathogenic probiotic bacteria may become a relevant vehicle for the delivery of drugs as has been shown for IL-10 producing *Lactococcus lactis*. IL-10 secreting genetically modified lactococci were able reduce colitis in a mouse model (Steidler *et al.*, 2000). Strategies have been applied to use genetically modified probiotics for vaccination.

Probiotic organisms start to enter the food mass market as well as the paediatric medical routine. To address the open questions seems to be a precondition for the use of probiotics as therapeutics, to meet safety requirements of probiotics and dairy product in children and adolescents as well as to maintain patients and consumer confidence.

3.3 Other dairy products to improve infant health

Human breast milk is recognised as being the best functional food for infants due to its undisputed optimal health-promoting effects by specific and non-specific factors, such as enhancement of the immature immunologic system of the newborn baby and strengthening of defence mechanisms against infective agents. Breastfeeding seems to protect from infections, the development of

IBD and atopic disease (Corrao *et al.*, 1998; Hanson and Korotkova, 2002; Klement *et al.*, 2004; van Odijk *et al.*, 2003).

Infant formulas or so called breast milk substitutes aim to provide an efficient and safe alternative diet for infants of those women who are not able to continue breastfeeding until six months of life. Infant formula can be fed directly after birth when breastfeeding is not possible; follow-on formulas are designed for children after the sixth month of life. Breast milk substitutes aim to mimic the composition of human breast milk concerning protein, fat and carbohydrate composition. The only carbohydrate of infant formulas is lactose, whereas follow-on formulas contain other carbohydrates, too. Protein sources are mainly bovine whey or casein (in the standard cow's milk based formulas) or soy protein (for infants with lactose intolerance or cow milk protein allergy). The quality parameter for the evaluation of infant diets is the ability to allow normal physical growth as well as optimal neurological and mental development.

Hypoallergenic formulas are commonly classified by the degree of protein hydrolysis as 'extensively' or 'partially' hydrolysed protein products or they are mixtures of amino acids. Proteins of different sources such as bovine casein, whey and soy are further processed by heat treatment or ultrafiltration (Host and Halken, 2004). Only pure amino acid mixtures are considered to be non-allergenic. Partially hydrolysed products contain different degree of residual immunogenicity. There is clear evidence that extensively hydrolysed protein products can prevent allergy and atopy (Host and Halken, 2004). So far there are no generally accepted criteria to design hypoallergenic foods for prevention (Host and Halken, 2004).

Recent efforts aim to put more emphasis on the imitation of the functional outcome of the breastfed infant. For this purpose compounds that are not necessarily found in breast milk but which are supposed to exert specific health-promoting effects have been added to formulas. Among those are long-chain polyunsaturated fatty acids for brain development, probiotics and prebiotics to modulate the faecal microbiota and nucleotides for immune development and to promote protective immune responses. Changes in protein quantity and quality may influence early brain development and may have long-term effects on later development of overweight. Proteins are hydrolysed for the prevention of atopic disease and for the treatment of food allergies such as cow's milk allergy (Agostoni and Haschke, 2003).

There are a few high-quality RCTs evaluating the beneficial effects of prebiotics or probiotics to infant and follow-on formula. Two RCTs performed by Saavedra *et al.* (1994) and Chouraqui *et al.* (2004) evaluated the effect on prevention of nosocomial diarrhoea by infant formulas supplemented with a mixture or *B. animalis* subsp. *lactis* Bb12 and *S. thermophilus* (Chris Hansen Laboratories Copenhagen). The first one showed a statistical significant reduction on a small population. The second was not able to show a significant reduction of nosocomial diarrhoea. One other study was able to show beneficial effects of probiotic supplemented formula (mixture of *B. infantis*,

S. thermophilus and *B. bifidus*, Solgar, division of Wyeth Consumer Healthcare, Bergen Country NJ) on the reduction of necrotising enterocolitis in preterm infants (Bin-Nun *et al.*, 2005).

3.4 Sources of further information and advice

U.S. Food and Drug Administration – U.S. regulatory authority responsible for food safety, Nutrition, Dietary Supplements, infant formula

<http://www.fda.gov/default.htm>

International Scientific Association for Probiotics and Prebiotics

<http://www.isapp.net/index.html>

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4

Functional dairy products for gastrointestinal infections and dysfunction

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4.1 Introduction

Fermented milk products have traditionally been used to reduce the risk of abdominal problems and diarrhoea during antibiotic treatments. Similarly, they have been used to alleviate the symptoms and reduce the duration of diarrhoea. It has been less clear whether or not probiotics reduce the risk of infectious diarrhoea, but recently more evidence has been gathered in this field, too. During the past two decades, studies on particular strains, namely probiotics, have transformed information based on folklore into science-based research; infections not only in the intestine, but also in the stomach, have been a focus of the research. There is a growing body of evidence which shows that probiotics can be used as an adjunct therapy to antibiotics used for the eradication of *Helicobacter pylori*, the main causative agent of gastric ulcers. Intestinal disturbances are not always due to infections, but a disturbed microbiota, together with other thus far undefined mechanisms, is supposed to be connected to many irregular intestinal symptoms, and probiotics and prebiotics are suggested for use for the treatment of irritable bowel syndrome and relief of constipation. Although bacteria genera other than *Lactobacillus* and *Bifidobacterium* can also be used as probiotics, this article deals with studies conducted on strains mainly from those genera, suitable for use in fermented dairy products.

4.2 *Helicobacter pylori* infection

H. pylori was isolated from gastric mucosa and bacteriologically identified in 1983 (Marshall and Warren, 1984). Thereafter, a reduction in ulcer recurrence

rates after eradication of *H. pylori* from the stomach of peptic ulcer patients has been reported (Marshall *et al.*, 1988; Hantschel *et al.*, 1993). It has been established that *H. pylori* infection is a major cause of chronic gastritis and peptic ulcer disease. *H. pylori* was designated a first class definite carcinogen for stomach cancer in 1994 following epidemiological investigation by the International Agency for Research on Cancer (IARC, 1994), a subordinate organization of the World Health Organization. Furthermore, association of primary malignant gastric lymphoma with *H. pylori* has been reported in a large-scale cohort study (Parsonnet *et al.*, 1994).

Approximately half of the world's populations are infected with *H. pylori* (Go, 2002). It is generally acquired during childhood and usually persists indefinitely if left untreated. The infection rate of *H. pylori* has been suggested to be associated with age, ethnicity, socio-economic status, sanitary environments and lifestyle. The main reservoir of *H. pylori* is the human stomach, and the most likely mode of transmission is person to person (Vaira *et al.*, 2001).

H. pylori is sheltered from gastric acidity in the mucus layer. Colonization of the gastric mucosa by *H. pylori* evokes local inflammatory responses, which result in further mucosal injury but are not able to clear the infection. Production of urease, a vacuolating cytotoxin, and the cagA-encoded protein, is associated with injury to the gastric epithelium (Atherton, 1998). The spontaneous reduction in the lifetime incidence of *H. pylori* infection in developed countries to 10%–15% allows the remaining non-malignant gastroduodenal diseases associated with infection to be addressed with antibiotic treatment. Triple therapy, which combines a proton pump inhibitor with two antibiotics (amoxicillin and clarithromycin), is the current standard of therapy for eradicating *H. pylori* (Malfertheiner *et al.*, 2002). However, the treatment of infection is challenged by the rapid rate with which the bacteria acquire resistance to the drugs, the cost of those drugs and some of their side-effects. These facts provide the rationale supporting the efforts to develop better therapies against *H. pylori*.

The role of probiotics in the prevention and treatment of gastrointestinal infections is increasingly documented, as a complement or alternative to antibiotics, with the potential to decrease the use of antibiotics. Prevention and treatment of *Helicobacter* infections is one important area of potential for probiotics.

4.2.1 Clinical studies

Probiotics as a complement to antibiotics may have potential in reducing the adverse events of anti-*Helicobacter* treatment and improving the eradication rate. The first study provided evidence that *L. acidophilus* LB improved the eradication rate significantly in the probiotic group (Canducci *et al.*, 2000). Inactivated bacteria in spent supernatant preparation were given in an open trial for 10 days to 60 dyspeptic *H. pylori* infected volunteers as a supplement

to triple anti-*Helicobacter* therapy. However, in this study the supplementation did not alleviate the side-effects of the anti-*Helicobacter* treatment. In contrast, Armuzzi *et al.* (2001a, 2001b) reported in two separate studies that *L. rhamnosus* GG was able to reduce the occurrence of side-effects, such as diarrhoea, taste disturbance, nausea and bloating. The latter of these two studies was conducted in a double-blind fashion. Moreover, Sheu *et al.* (2002, 2006) reported in open trials that yoghurt containing *B. animalis* Bb12 and *L. acidophilus* LA5 was able to increase the eradication rate and also significantly decrease several side-effects of the triple therapy, such as vomiting, diarrhoea and metallic taste. Administration of *L. rhamnosus* GG, *Saccharomyces boulardii* or combination of *L. acidophilus* and *B. lactis* for two weeks also decreased adverse events during the triple treatment (Cremonini *et al.*, 2002a). However, the effect of probiotic supplementation seemed independent of the probiotic species used. Furthermore, probiotic combination of four strains (*L. rhamnosus* GG, *L. rhamnosus* Lc705, *B. breve* 99 and *Propionibacterium freudenreichii* ssp. *shermanii* JS) has also improved tolerance to the standard triple therapy and increased the eradication rate non-significantly in a double-blind randomized clinical study (Myllyluoma *et al.*, 2005). Tursi *et al.* (2004) also recently found that a 10-day quadruple anti-*Helicobacter* therapy with *L. casei* ssp. *casei* DG supplementation significantly increased the eradication rate. Also a fermented milk product containing *L. casei* DN-114001 was concluded to increase effectively the eradication rate of standard triple treatment in children with gastritis (Sykora *et al.*, 2005). In this study, the eradication rate was remarkably low in the standard treatment group with placebo.

There are not many studies on the attenuation of microbiota disturbances following an anti-*Helicobacter* triple treatment by probiotics. In a pilot study, Madden *et al.* (2005) found that probiotic combination including two strains of *L. acidophilus* (CLT60 and CUL21) and two strains of *B. bifidum* (CUL17 and Rhodia) suppressed the rise in the numbers of facultative anaerobes seen in the placebo group. Later, the same probiotic product was also able to suppress a rise in the amount of antibiotic-resistance among enterococci, the effect of which was seen in the placebo group during and after the triple therapy (Plummer *et al.*, 2005). However, despite the probiotic supplementation the microbiota in both studies were susceptible to the effects of the antibiotics administered to eradicate *H. pylori*.

Probiotics as an alternative to antibiotics have also been the topic of several studies. Administration of the culture supernatant or fermented milk containing the *L. acidophilus* La1 decreased *H. pylori* density, measured by ¹³C-urea breath test (UBT) in adults (Michetti *et al.*, 1999) and in children (Cruchet *et al.*, 2003), and also in two other trials measured by histological analysis (Felley *et al.*, 2001, and Pantoflickova *et al.*, 2003). Furthermore, a decrease in *H. pylori* infection-associated inflammation was evident in those studies. However, the regular intake of La1 did not eradicate *H. pylori* in any of the studies. Sakamoto *et al.* (2001) found *L. gasseri* OLL2716 (LG21) to be effective in suppression of *H. pylori* and reduction in gastric mucosal

inflammation as measured by ^{13}C -UBT and assays of serum pepsinogen I. In their study, 31 subjects infected with *H. pylori* ingested yogurt containing LG21 daily for an eight-week period. Also *L. casei* was shown to inhibit *H. pylori* growth and to reduce ^{13}C -UBT values (Cats *et al.*, 2003). Similar effects of yoghurt (containing *L. acidophilus* La5 and *B. lactis* Bb12) consumption for six weeks on the growth of *H. pylori* in 59 human volunteers have been reported (Wang *et al.*, 2004). Not all clinical trials have, however, shown effectiveness. In one open study, 27 *H. pylori* infected volunteers received yoghurt containing three *Lactobacillus* strains (*L. casei* and *L. acidophilus*) and a commercial starter culture for one month (Wendakoon *et al.*, 2002). At the end of the trial, ^{13}C -UBT values remained positive in 26 of the 27 subjects. This study used strains that showed *in vitro* inhibition of *H. pylori*, but no other probiotic characteristics were documented.

Studies on the effects of synbiotics on *H. pylori* infection are very scarce, and, to our knowledge, no clinical studies on prebiotics exist. A randomized, open, eight-week study investigated the effects of *Lactobacillus acidophilus* LB in comparison with antibiotics and with the symbiotic combination of probiotic yeast *Saccharomyces boulardii* with inulin (Gotteland *et al.*, 2005). The *H. pylori* eradication rate was 66% (30/45) of the children from the antibiotic group, 6.5% (3/46) of those in the *L. acidophilus* LB group, and 12% (6/51) of those in the symbiotic group. The eradication rate was not significantly different between the two probiotic study groups.

4.2.2 Experimental studies

Preventive strategies using various probiotics have shown favourable effects in animal models of *H. pylori* infection. The first two studies presented a highly protective and therapeutic effect of oral administration of *L. salivarius* on an *H. pylori* infected gnotobiotic (raised in germ-free conditions or contain only specific microbes) BALB/c mice model (Kabir *et al.*, 1997) and a gnotobiotic murine model (Aiba *et al.*, 1998). Similarly, Coconnier *et al.* (1998) reported that *L. acidophilus* strain LB was able to protect against *H. pylori* infection in conventional mice. Inhibition of stomach colonization by *H. felis* (closely related to *H. pylori*) was observed and no evidence of gastric histopathological lesions was found. Recently, probiotic combination containing *L. acidophilus* R0052 and *L. rhamnosus* R0011 reduced the effects of *H. pylori* infection in a C57BL/6 mice model through reducing *H. pylori* colonization and ameliorating *H. pylori*-induced inflammation of the stomach (Johnson-Henry *et al.*, 2004). Also, the same probiotic preparation has proven effective in a Mongolian gerbil model of *H. pylori* infection via its attenuating effect on the *H. pylori* colonization, the mucosal inflammation, and the impairment of gastrin-somatostatin link (Brzozowski *et al.*, 2006). Studies by Sgouras *et al.* (2004, 2005) in a C57BL/6 mice model demonstrated that *L. casei* strain Shirota and *L. johnsonii* La1, both administered in drinking water, attenuated *H. pylori* infection-induced gastric mucosa inflammation.

However, only *L. casei* strain *Shirota* was able to down-regulate the colonization of *H. pylori* to gastric mucosa.

The development of effective vaccine is also an interesting area in the prevention of *H. pylori* infection. However, the ability of recombinant *Lactobacillus* or other probiotics to be used as an antigen-delivery vehicle to induce protective immune response have rarely been studied. In the first study by Lee *et al.* (2001), *Lactococcus lactis* producing cytoplasmic Urease B was shown to be unable to induce protection against *H. pylori* in a mouse model. In contrary, recombinant *L. plantarum* strain producing *H. pylori* Urease B subunit was found to induce successfully a partial mucosal protection against *Helicobacter* (Corth  sy *et al.*, 2005).

4.2.3 *In vitro* studies

Some probiotic species such as *L. salivarius*, *L. gasseri* and *L. acidophilus* have shown growth inhibition or anti-adhesion capacity against *H. pylori* in a gastric epithelial cell model (Midolo *et al.*, 1995; Coconnier *et al.*, 1998; Lorca *et al.*, 2001; Nam *et al.*, 2002; Mukai *et al.*, 2002; Tsai *et al.*, 2004; Sgouras *et al.*, 2004). Mukai *et al.* (2002) have also examined the competition of binding of nine *L. reuteri* strains and *H. pylori* to ganglioside GM1 and sulfatide, which are putative glycolipid receptor molecules of *H. pylori*, and identified a possible sulfatide-binding protein of the *L. reuteri* strains (JCM1081 and TM105). Furthermore, Coconnier *et al.* (1998) found that the anti-*Helicobacter* substance(s) in the *L. acidophilus* LB strain were different from lactic acid. Also, it was shown recently that *L. rhamnosus* GG is able to antagonize *H. pylori* induced TNF- α production by murine macrophages *in vitro* (Pe  a and Versalovic 2003). Furthermore, a recent study by Hutt *et al.* (2006) provided evidence of the antagonistic effect of *L. rhamnosus* GG, *L. paracasei* 8700:2 and *L. plantarum* 299v on *H. pylori*.

4.2.4 Possible mechanism of probiotic actions in *H. pylori* infection

The action mechanism of probiotics on *H. pylori* infection is unclear, but there are a number of proposed possibilities. Several *Lactobacillus* and *Bifidobacterium* strains have been shown to produce general antimicrobial substances such as organic acids and hydrogen peroxide. Other general mechanisms are competition with pathogens for nutrients and ecological sites, and stimulation of the non-specific and specific immune responses. Furthermore, probiotic bacteria strains have been found to produce bacteriocins with a more defined antimicrobial spectrum. Few *Bifidobacterium* strains release heat-stable proteinaceous antimicrobial compounds against *H. pylori* *in vitro* (Collado *et al.*, 2005). Another mechanism proposed recently is that *L. johnsonii* La1 expresses a cell surface-associated La1GroEL protein. Its recombinant variant expressed in *Escherichia coli* is able to induce aggregation of *H. pylori* but not that of other intestinal pathogens. It was also shown to

have pro-inflammatory activity thus favouring the activation of intestinal immunological defences (Bergonzelli *et al.*, 2006). However, it is not clear which of these mechanisms function in a very acidic environment in the stomach, where furthermore the pathogen is living intracellular on gastric mucosa.

To conclude, these observations suggest that consumption of certain strains of probiotics may be useful in combating *H. pylori* infection as a complement or alternative to antibiotics, and thus possibly act as an effective biotherapeutic agent against gastrointestinal diseases such as gastritis and duodenal ulcer. This far, eradication without anti-*helicobacter* therapy has succeeded in only a few patients in one clinical study. On the other hand, regular consumption of fermented dairy products with a specified probiotic strain as an alternative to antibiotics may have some potential in suppression of *H. pylori* infection and gastritis, but more clinical studies are needed to confirm the effect.

4.3 Acute diarrhoea

Acute diarrhoea is defined as a diarrhoeal disease (three or more times per day or at least 200 g of stool per day) lasting 14 days or fewer. It may be caused by drugs or inflammatory bowel diseases, but the main reason is an intestinal infection by bacteria, viruses or parasites. Although it is a primary cause of morbidity and mortality especially in children in developing countries, it is still a serious problem in developed countries, too (Gadewar and Fasano, 2005).

4.3.1 Reduction of the risk of diarrhoea

Breastfeeding is the best way to protect newborn and young infants against viral infections (Mastretta *et al.*, 2002). On the other hand, probiotics are supposed to reduce the risk of diarrhoea, and a few studies have been conducted to evaluate that potential. Table 4.1 summarises prophylactic studies in children, including those conducted by using infant formulae supplemented with probiotic bacteria, as well as those where probiotic strains were given in powder form or included in fermented milk products (see also Chapter 3 in this volume). Bifidobacteria are more commonly studied and used in infant formulae than are lactobacilli, maybe because bifidobacteria are the natural dominating bacteria in the intestinal microbiota of a breast-fed infant. Significant reduction in the incidence or number of episodes of diarrhoea is reported for both lactobacilli and bifidobacteria. Overall, the studies though few in number indicate that the incidence of diarrhoea may be reduced significantly. Furthermore, the strength of diarrhoeal episodes is milder in those children receiving probiotics, and they need less oral rehydration therapy.

The effect of probiotic intervention on the incidence of diarrhoea has also been studied in children who were admitted to hospital for reasons other than

Table 4.1 The effect of probiotic bacteria on the risk and morbidity of acute diarrhoea in infants and in children

n	Age, months	Mode of administration and duration of intervention, mo.	Probiotic strain(s)	Main effects on diarrhoea	Ref.
201	4–10	IF vs. control formulae, for 3 mo.	<i>B. lactis</i> BB12 or <i>L. reuteri</i> ATCC 55730	Number of episodes ↓ Duration per episode ↓	Weizman <i>et al.</i> , 2005
921	4–6	Fermented IF vs. control formulae, for 5 mo.	<i>B. breve</i> C50 <i>S. thermophilus</i> 065	Incidence of diarrhoea ± Duration per episode ± Cases of dehydration ↓ Medical consultations ↓	Thibault <i>et al.</i> , 2004
90	<8	Acidified IF with probiotics vs. non-acidified control formulae	<i>B. lactis</i> BB12 (<i>S. thermophilus</i> and <i>L. helveticus</i> for acidification)	Number of episodes ± Number of days with diarrhoea/child ↓ Daily probability of diarrhoea ↓ Relative risk of diarrhoea ↓	Chouraqui <i>et al.</i> , 2004
634	12–36	Milk formula vs. control formulae, for 12 mo.	<i>B. lactis</i> HNO19 + GOS	Incidence of diarrhoea ± Incidence of dysentery ↓	Sazawal <i>et al.</i> , 2004
118	3–24	IF vs. control formulae, for 7±4 mo.	<i>B. lactis</i> BB12 + <i>S. thermophilus</i>	Frequency of episodes ±	Saavedra <i>et al.</i> , 2004
81	1–36	Freeze-dried powder vs. placebo, for the duration of hospital stay	<i>L. rhamnosus</i> GG	Incidence of diarrhoea ↓ Duration per episode ±	Szajewska <i>et al.</i> , 2001a
928	6–24	Yoghurt ± probiotic, for 4 mo.	<i>L. casei</i> DN-114001	Incidence of diarrhoea ↓ Duration per episode ±	Pedone <i>et al.</i> , 2000
287	<24	Yoghurt ± probiotic vs. jellied milk, for 1 mo.	<i>L. casei</i> DN-114001	Incidence of diarrhoea ± Duration per episode ↓	Pedone <i>et al.</i> , 1999
204	6–24	Freeze-dried powder (capsulae) spread on gelatine for 15 mo.	<i>L. rhamnosus</i> GG	Number of episodes ↓ Duration per episode ±	Oberhelman <i>et al.</i> , 1999
55	5–24	IF vs. control formulae, for the duration of hospital stay	<i>B. bifidum</i> + <i>S. thermophilus</i>	Number of episodes ↓ Duration per episode ±	Saavedra <i>et al.</i> , 1994

IF = infant formulae; B. = *Bifidobacterium*; L. = *Lactobacillus*; S. = *Streptococcus*; GOS = galactooligosaccharide

diarrhoea. Interestingly, two studies indicated that a probiotic strain, *L. rhamnosus* GG, did not reduce the number of rotavirus infections, measured as the detection of rotavirus antigen in stool samples (25.4% vs. 30.2%, *L. rhamnosus* GG compared to the placebo, respectively, $p = 0.432$ in Mastretta *et al.*, 2002, and 20% vs. 27.8%, respectively, RR 0.72 by Szajewska *et al.*, 2001a). However, in one of these studies the incidence of gastroenteritis due to rotavirus was significantly lower in the probiotic group compared to the placebo (6.7% vs. 33.3%, RR 0.2, Szajewska *et al.*, 2001a). This opens the way for the hypothesis that probiotics may make it possible to have an asymptomatic viral infection and still receive immunoprotection against the virus (Kaila *et al.*, 1992; Majamaa *et al.*, 1995).

There are not many reports on the reduction by probiotics of the risk of adult infective diarrhoea. One study, conducted among young soldiers, showed that commercial yoghurt containing *L. casei* DN-114001 did not reduce either the incidence or the duration of diarrhoea (Pereg *et al.*, 2005). Two studies on the reduction of the risk of traveller's diarrhoea using *L. rhamnosus* GG indicated that it may give protection also to adults, but the effect was moderate. Infective agents were not analysed in these studies (Oksanen *et al.*, 1990; Hilton *et al.*, 1997). However, higher doses of probiotic bacteria and perhaps a combination of more strains with different modes and target sites of action might improve the effect in these multifactorial types of diarrhoea. There is also a dearth of data available on the prevention or treatment of diarrhoea caused by pathogenic bacteria. However, several *in vitro* and experimental studies indicate that probiotics have the potential to reduce enteric infections caused by pathogen bacteria (see review papers by Servin, 2004; Leahy *et al.*, 2005).

Antibiotic-associated diarrhoea and Clostridium difficile colitis

Diarrhoea occurs in about 5–25% of patients who receive antibiotics, especially in children (Turck *et al.*, 2003; Surawicz, 2005). Antibiotic treatment may disturb the balance of the intestinal microbiota and thus lead to disturbances of catabolism of dietary compounds and such processes as the formation of short chain fatty acids in the colon. The relative proportion of potentially pathogenic or opportunistic indigenous bacteria (e.g., enterobacteria, *C. difficile*) may increase, but often a particular pathogen colonisation is not found (Arvola *et al.*, 1999). In meta-analyses, the impact of probiotic treatments has been found to reduce the episodes of diarrhoea significantly (OR 0.37–0.39) (Cremonini *et al.*, 2002b; D'Souza *et al.*, 2002; McFarland, 2006). In particular, uses of *L. rhamnosus* GG for the prevention of gastrointestinal symptoms associated with antibiotic therapy have been effective in children and in adults (see meta-analyses of Hawrelak *et al.*, 2005 and McFarland, 2006). Also bifidobacteria and certain combinations of strains are known to reduce the incidence of antibiotic-associated diarrhoea (Correa *et al.*, 2005; McFarland, 2006).

The results of a few double-blind, placebo-controlled human intervention studies indicate that certain probiotic strains might reduce the recurrence of chronic diarrhoea caused by *C. difficile* (Wullt *et al.*, 2003; Plummer *et al.*, 2004). *C. difficile* is an opportunistic pathogen, causing persistent and serious chronic diarrhoea generally following antibiotic treatment. Adjunct therapy of antibiotic treatment on emergency clinic patients with a combination of *B. bifidum* and *L. acidophilus* reduced the number of patients carrying the pathogen in convalescence, compared to antibiotics and placebo-treated patients; especially toxin production was less frequently found (Plummer *et al.*, 2004). However, recent systematic reviews of studies indicate that the evidence is insufficient for the routine clinical use of *Lactobacillus* therapy for the prevention and treatment of *C. difficile*-associated diarrhoea (Dendukuri *et al.*, 2005; Katz, 2006; McFarland, 2006).

4.3.2 Treatment of acute infective diarrhoea

Diarrhoea is a common problem even in industrialised countries, but in developing countries it is the main cause of children's death (Cheng *et al.*, 2005). About 30–50% of cases are based on unknown aetiology, but viral infections, especially rotavirus, are common (Shornikova *et al.*, 1997; Guandalini *et al.*, 2000). The symptoms of infectious diarrhoea are generally self-limiting, but require assessment of the degree of dehydration in children. Oral rehydration solutions (ORS) are recommended for therapy by UNICEF/WHO (2001) (http://www.who.int/child-adolescent-health/New_Publications/NEWS/Expert_consultation.htm). There is a consensus of opinion that certain probiotic bacteria strains, especially *L. rhamnosus* GG, can help in the relief of acute diarrhoea in children. This is especially documented in diarrhoea caused by rotavirus (reviewed by Szajewska and Mrukowicz, 2001b and van Niel *et al.*, 2002). The duration of diarrhoea was reduced on average by one day in children admitted to hospital due to acute diarrhoea. The beneficial effects are more prominent with early probiotic interventions: the duration of diarrhoea was 2–3 days shorter compared to the placebo, in studies where the probiotic therapy was given to children being cared for at home (Guarino *et al.*, 1997; Rosenfeldt *et al.*, 2002). Probiotics also seem to help in relieving diarrhoea caused by non-identified agents (Fig. 4.1) (Shornikova *et al.*, 1997; Guarino *et al.*, 1997; Guandalini *et al.*, 2000), but there are insufficient data to draw conclusions as to the effectiveness on diarrhoea caused by various infective agents. As mentioned above, ORS is the standard therapy in the treatment of acute diarrhoea. The probiotic preparations used in most of the studies are in capsules or powder forms, which are easy to open and add also to ORS. However, if administered without ORS or after that, strains in fermented milk forms are shown to be as effective as is a pure bacteria preparation (Isolauri *et al.*, 1991; Kaila *et al.*, 1992; Pedone *et al.*, 2000).

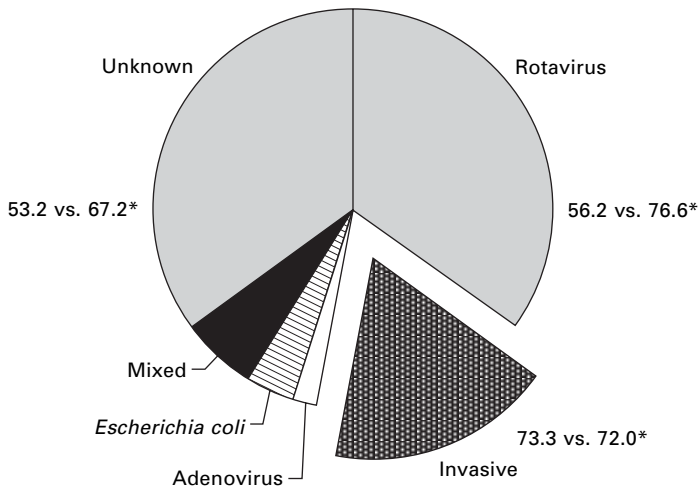


Fig. 4.1 The relative proportion of infective agents in a multicentre treatment study of acute diarrhoea. * Duration of diarrhoea (h) in children treated with *Lactobacillus* GG vs. placebo.

4.3.3 Potential mechanisms involved in prevention and treatment of acute diarrhoea

An intact intestinal epithelium with a normal indigenous microbiota creates a barrier, known as colonization resistance, against pathogens. The rationale behind colonization resistance is the ability of normal microbiota to occupy the same ecological niche as pathogenic organisms. Bacterial adhesion to host cells or mucosal surfaces is always the fundamental first step in the disease process, and therefore interruption of the pathogen adhesion could be of therapeutic benefit to the host. On the other hand, the adhesion of probiotic organisms to mucosal surfaces is one of the main properties of the beneficial health effects of probiotics, by which probiotics can prevent the attachment of pathogens, a phenomenon known as ‘competitive exclusion’ (Hentges, 1992). Adhesion of pathogens can be inhibited by steric hindrance, where a large number of beneficial bacteria may cover receptor sites in a non-specific manner, or by competing for specific carbohydrate receptors which would otherwise be available to pathogens. It has been suggested that lactobacilli are able to compete with *E. coli* and *Salmonella* through steric hindrance (Lee and Puong, 2002). Mucins, high-molecular-weight glycoproteins secreted by epithelial cells, may also bind to pathogens, thereby inhibiting their adherence to epithelial cells. *Lactobacillus plantarum* 299V and *L. rhamnosus* GG have inhibited the *in vitro* adherence of enteropathogenic *E. coli* to HT-29 intestinal epithelial cells by inducing intestinal mucin gene expression (Mack *et al.*, 1999).

In addition to the competitive inhibition of the mucosal adherence of pathogens, lactobacilli and bifidobacteria also demonstrate direct antimicrobial

activity against pathogens by producing antimicrobial substances such as organic acids, hydrogen peroxide, diacetyl, short chain fatty acids, biosurfactants and bacteriocins (for review see Servin, 2004). Bacteriocins are bactericidal proteinaceous molecules which have a relatively narrow killing spectrum, and are only toxic to bacteria closely related to the strain that produces them. However, antimicrobial substances produced by lactic acid bacteria and bifidobacteria are mainly active against pathogen species, such as *Clostridium*, *Bacteroides*, *Bacillus*, *Enterobacteriaceae*, *Pseudomonas*, *Staphylococcus*, *Streptococcus*, *Salmonella*, and *E. coli* (Servin, 2004). A recent study showed that the antagonistic activity of *L. rhamnosus* GG against *Salmonella typhimurium* is mainly due to strong formation of lactic acid (de Keersmaecker *et al.*, 2006), but it has also been shown to reduce the infection rate of *S. typhimurium* in an *in vivo* animal model (Hudault *et al.*, 1997). The anti-infective activity of probiotics may also partly be due to coaggregation with pathogens (Cesena *et al.*, 2001), whereby pathogens are exposed to high doses of potential growth-inhibiting factors produced by probiotics.

Whole live bacterial cells are needed to reduce the adhesion and/or to block the penetration of pathogen bacteria across the mucosal layer. *In vitro* studies showed probiotics to be effective only when added before the pathogen challenge. The penetration of an enterohaemorrhagic (Hirano *et al.*, 2003), an enteroinvasive (Resta-Lenert and Barrett, 2003) and an enteropathogenic (Michail and Abernathy, 2003) *E. coli* strain in cell culture monolayers was significantly reduced when the monolayers were challenged with certain strains of probiotic lactobacilli (*L. rhamnosus*, *L. acidophilus* or *L. plantarum*) before the pathogen infection. Intestinal bacteria contribute to the permeability of the gut mucosal barrier. Harmful bacteria may increase the permeability, thus enabling passage through the mucosal wall of bacteria and dietary macromolecules (Lee *et al.*, 2000). Several probiotic bacteria have been shown to prevent and repair such mucosal damages, whether caused by food antigens (Rosenfeldt *et al.*, 2004) or medicinal substances, by inhibiting the damages in tight junction proteins (Montalto *et al.*, 2004). The probiotic strains prevented the disruption of the cytoskeletal and tight junction proteins in the epithelial cells caused by the pathogen, thus improving the mucosal barrier function and preventing also the failures in the secretion of electrolytes (Michail and Abernathy, 2003; Resta-Lenert and Barrett, 2003). Lactobacilli also increased cell proliferation in villi of gnotobiotic and conventional rats (Banasaz *et al.*, 2002) and promoted recovery of intestinal mucosal damages by improving the cell survival (Yamaguchi *et al.*, 2003).

In rotavirus infection, probiotics may inhibit the adhesion of rotavirus by modifying the glycosylation of the receptor in epithelial cells by soluble factor(s) excreted by probiotics (Freitas *et al.*, 2003). Another potential mechanism of action in the treatment of diarrhoea might be the balancing of the microbiota. Bacterial imbalance may follow the osmotic phase of diarrhoea,

and probiotics provide a beneficial tool for preventing this (Isolauri *et al.*, 1994). Certain strains of probiotic bacteria, especially *L. rhamnosus* GG, are also known to enhance both unspecific antibody formation and specific immune response against rotavirus (Kaila *et al.*, 1992), but the response seems to be strain specific, as is the clinical effect (Majamaa *et al.*, 1995). Also, enhancement of secretory IgA production on intestinal epithelium may have a role in protection (Viljanen *et al.*, 2005) as well as enhancement of phagocytic activity, as documented in experimental studies (Gill and Rutherford, 2001).

4.4 Irritable bowel syndrome

Irritable bowel syndrome (IBS) is a widespread functional gastrointestinal disorder that affects 10–20% of the Western population (Drossman *et al.*, 2002). The main clinical features of IBS include abdominal pain, bloating, flatulence and variable bowel habit. Current treatments for IBS are regarded as relatively ineffective. The pathophysiology of IBS remains unknown, but there is evidence that at least in part of the patients an imbalanced intestinal microbiota is associated with the onset of disease.

4.4.1 The role of the intestinal microbiota in IBS

Studies comparing IBS patients with healthy control subjects show that the intestinal microbiota may be aberrant in IBS. The composition of the microbiota has been investigated both using conventional culturing methods and by DNA-based techniques. Indirect methods studying the metabolic activity of the microbiota have also been applied. Early studies by Balsari and colleagues (Balsari *et al.*, 1982) found that IBS patients had significantly lower numbers of coliforms, lactobacilli and bifidobacteria. Another study based on culturing methods produced a similar finding regarding lower numbers of bifidobacteria in IBS patients (Si *et al.*, 2004). In addition to lower numbers of bifidobacteria, an increase in *Enterobacteriaceae* was also observed. In contrast to Balsari *et al.* (1982), a recent study found a slightly higher number of coliforms in IBS (Mättö *et al.*, 2005). No differences were found in the mean culturable numbers of bacteroides, bifidobacteria, spore-forming bacteria, lactobacilli, enterococci or yeasts, but an increased aerobe:anaerobe ratio could be seen.

Extensive culturing-independent quantitative PCR analysis has revealed lower amounts of *Lactobacillus* spp. in diarrhoea predominant IBS and higher amounts of *Veillonella* spp. in constipation predominant IBS when these IBS subtypes were compared to healthy controls (Malinen *et al.*, 2005). Results also suggested a lower level of *Clostridium coccoides* and *Bifidobacterium catenulatum* in IBS. Another DNA-based method, denaturing gradient gel

electrophoresis (PCR-DGGE), has shown more temporal instability in the predominant bacterial populations of IBS patients compared to healthy controls (Mättö *et al.*, 2005). The same kind of general instability of the predominant microbiota combined with changes in the clostridial population was found to be typical for IBS in a study by Maukonen *et al.* (2006). An increased formation of colonic hydrogen (King *et al.*, 1998) and an abnormal pattern of short-chain fatty acids (Treem *et al.*, 1996) in irritable bowel syndrome also indicate an imbalanced microbiota. There is thus a growing body of evidence indicating that the intestinal microbiota in IBS may differ from a healthy microbiota. It is, however, unclear whether alterations in the microbiota are a cause of IBS or a result of, for instance, disturbed gut motility induced by the syndrome.

Dietary therapy may be useful in IBS as certain foods can aggravate symptoms in some patients (Drossman *et al.*, 2002). For instance, fatty foods, beans and other gas-producing foods, alcohol, caffeine and lactose are often mentioned as causing discomfort. Fibre is considered to have an established role in the treatment of constipation in IBS, but other forms of IBS do not benefit from added fibre.

4.4.2 Probiotics and prebiotics in irritable bowel syndrome

Indications of an aberrant microbiota in IBS have raised the hypothesis of probiotic therapy in IBS. Some intervention studies have reported improvements in IBS symptoms, while others have found probiotics to be ineffective. It should be emphasized that the quality of the trials, such as sample size and duration, vary considerably. Given the chronicity and fluctuating nature of functional gastrointestinal disorders, a minimum treatment duration of four weeks is generally recommended for short-term trials and six months for long-term efficacy evaluation (Irvine *et al.*, 2006). With few exceptions, the vast majority of studies are of a short-term nature.

The effect of *Lactobacillus plantarum* 299v on IBS symptoms has been investigated in three randomized placebo-controlled trials. In a four-week trial, Nobaek and colleagues (2000) demonstrated *L. plantarum* 299v to be significantly more efficient than placebo in reducing flatulence and abdominal pain. A second study with the same probiotic also found a reduction in abdominal pain (Niedzielin *et al.*, 2001), whereas a third study including only 12 patients was not able to confirm any improvement of symptoms (Sen *et al.*, 2002). Besides *L. plantarum* 299v, other strains of lactobacilli have also been clinically investigated. *L. rhamnosus* GG administration to adults resulted in a trend towards less diarrhoea, but no other improvements could be seen (O'Sullivan and O'Morain, 2000). Neither did *L. rhamnosus* GG alleviate abdominal pain in children suffering from IBS (Bausserman and Michail, 2005). Also a six-month *L. reuteri* ATCC 55730 therapy was concluded to be ineffective (Niv *et al.*, 2005). An early trial with *L. acidophilus* milk was unable to demonstrate any advantage (Newcomer *et al.*, 1983), whereas

heat-killed *L. acidophilus* capsules have demonstrated a therapeutic benefit (Halpern *et al.*, 1996).

In addition to lactobacilli, other probiotics and probiotic combinations have also been studied in IBS. In an eight-week trial, *B. infantis* 35624 has been able to alleviate IBS symptoms, and normalize an aberrant interleukin-10/interleukin-12 ratio closer towards healthy subjects (O'Mahony *et al.*, 2005). In the same setting, *L. salivarius* UCC4331 gave only mild relief to the participating IBS patients. *Streptococcus faecium* therapy has also demonstrated a positive effect (Gade and Thorn, 1989) in one intervention. A preparation containing *L. plantarum* LP01 and *B. breve* BR0 has also demonstrated slight improvements in a four-week controlled study (Saggioro, 2004). A probiotic combination consisting of eight different bacterial species, VSL#3, showed some favourable effects on bloating and flatulence scores in controlled settings (Kim *et al.*, 2003, 2005), as well as in a small uncontrolled intervention (Brigidi *et al.*, 2001). Another probiotic combination consisting of *L. rhamnosus* GG, *L. rhamnosus* Lc705, *B. breve* B699 and *Propionibacterium freudenreichii* ssp. *shermanii* JS was shown to be significantly superior to placebo in alleviating IBS symptoms in thus far the only six-month long-term intervention showing positive response (Kajander *et al.*, 2005).

In conclusion, the studies available suggest that certain probiotic strains and probiotic combinations are effective in the relief of IBS. Clinical trials reporting the effect of prebiotics or synbiotics in IBS are scarce, and no recommendation can be made from them. The effects of probiotics in IBS are obviously strain-specific, and hence all strains or combinations of strains have to be studied separately in carefully designed, double-blind human interventions.

4.4.3 Possible mechanisms behind probiotic therapy in IBS

There are several putative mechanisms that could explain the reduction of IBS symptoms by probiotics. Probiotics could influence the symptoms by balancing the microbiota, and thus restoring possibly aberrant gas-production or production of short chain fatty acids. An inflammatory component has also been suggested in IBS, especially in so-called post-infectious IBS, a form of IBS that affects 10–15% of patients after acute infectious enteritis (Spiller, 2003). Many probiotics can modulate the immune system for instance by balancing the ratio between pro-inflammatory and anti-inflammatory cytokines, and could hence alleviate a possible low-grade inflammation (Blum *et al.*, 2002; Ezendam and van Loveren, 2006). In addition to the balancing effect on the microbiota and the immunomodulatory effects, recent studies also suggest that probiotics may influence intestinal motility. *In vitro* studies on isolated intestines of guinea pigs have shown that probiotics, especially bifidobacteria, have a relaxing effect on the colon (Massi *et al.*, 2004). *Lactobacillus paracasei* seems also to attenuate post-infective dysmotility

and visceral hypersensitivity in murine models of IBS (Verdu *et al.*, 2004 and 2006).

4.5 Constipation

Constipation is a major health problem that occurs in up to 27% of people depending on demographic factors, sampling and definition (Pare *et al.*, 2001). The concept of constipation is complicated by disagreement about its nature, but according to the Rome criteria, functional constipation presents as persistently difficult, infrequent, or seemingly incomplete defecation, which do not meet the criteria for IBS (Longstreth *et al.*, 2006). Both IBS and constipation are known to reduce the quality of life in addition to causing an economic burden for the patient and the health care system (Chang, 2004).

4.5.1 The role of the intestinal microbiota in functional constipation

Relatively few studies have investigated the role of the intestinal microbiota in constipation. Using culturing methods, Khalif and colleagues (2005) found that subjects with functional constipation have an aberrant microbiota that presents as a suppression of bifidobacteria and lactobacilli as well as an increased pool of potentially pathogenic micro organisms, such as *E. coli* and enterobacteria. Patients suffering from severe constipation demonstrated the most pronounced changes. Another study focusing on constipated children revealed increased numbers of bifidobacteria and clostridia in constipation (Zoppi *et al.*, 1998).

The initial treatment of chronic constipation includes lifestyle management, such as adequate fluid intake together with increased dietary fibre and physical exercise (Bleser *et al.*, 2005). Treatment of constipation often also implies pharmacologic measures, such as laxatives, prokinetic drugs, and other therapies.

4.5.2 Probiotics and prebiotics in constipation

Since dietary management is the primary management approach in constipation, probiotic or prebiotic therapy in principle fits well into the regimen. The efficacy of lactobacilli, bifidobacteria, and a combination of lactobacilli and propionibacteria has been clinically investigated in the treatment of constipated subjects. *Lactobacillus* GG supplementation was found to be of no therapeutic value in an uncontrolled trial with elderly subjects suffering from difficulties in defecation (Ling *et al.*, 1992). Another double-blind placebo-controlled trial also found *L. rhamnosus* GG to be ineffective as an adjunct to standard lactulose treatment in constipated children (Banaszkiewicz

and Szajewska, 2005). Koebnick and colleagues (2003) investigated the effect of another strain of lactobacilli, *L. casei Shirota*, in 70 subjects diagnosed with chronic constipation. In comparison to placebo, *L. casei Shirota* supplementation resulted in improvement in the severity of constipation and in the stool consistency over a four-week period. A study in elderly subjects with difficulties in defecation demonstrated some relief of constipation with a combination of *L. rhamnosus* Lc705 and *Propionibacterium freudenreichii* ssp. *shermanii* JS, while the same study found no beneficial effects with *L. reuteri* (Ouwehand *et al.*, 2002). Finally, two studies have been conducted to investigate the effect of *B. animalis* DN-173010 in elderly subjects with prolonged colonic transit time (Meance *et al.*, 2001 and 2003). In both trials the probiotic therapy resulted in a significant decrease in intestinal transit time.

In addition to being a widely used laxative, lactulose may also be considered a prebiotic compound, as it has been shown to stimulate the amount of colonic bifidobacteria (Ballongue *et al.*, 1997; Bouhnik *et al.*, 2004). According to a recent systematic review, the clinical evidence for the use of lactulose in constipation is moderate (Ramkumar and Rao, 2005). Besides lactulose, different forms of oligosaccharides have been investigated for their treatment potential in constipated subjects. Galacto-oligosaccharides (GOS) have shown promise in two clinical trials. A cross-over trial with elderly constipated subjects demonstrated that supplementation with nine grams GOS daily resulted in a significant increase in stool frequency (Teuri and Korpela, 1998). A similar trial including subjects with constipation tendency showed that five or ten grams of GOS daily significantly increased defecation frequency and softened stools (Deguchi *et al.*, 1997). Inulin supplementation (20 or 40 grams daily) has also shown a significant laxative effect in elderly constipated subjects (Kleessen *et al.*, 1997).

4.5.3 Mechanisms behind probiotic and prebiotic therapy in constipation

Several mechanisms have been proposed to play a role in the laxative effects of probiotics and prebiotics. Probiotics and prebiotics are able to modulate the intestinal microbiota. The intestinal microbiota and its composition are known to affect gut functions, especially intestinal motility (Borriello, 1984). The mechanisms behind this are not well established. Modulation of the gut microbiota also changes the intestinal metabolic activity, such as production of gases and short chain fatty acids. There is evidence suggesting that short chain fatty acids are correlated with colonic transit time (Høverstad and Bjørneklett 1984; Cherbut *et al.*, 1997). Considering prebiotics, colonic fermentation of the carbohydrate residue leads to a larger faecal mass. This fibre-like bulking-effect of stools is known furthermore to activate the bowel function.

4.6 Future trends

Functional dairy products clearly possess potential to improve intestinal health. Milk as such is a healthy food. Certain lactobacilli strains already have established evidence for their shortening effect in the duration of acute diarrhoea, especially in rotavirus infections. However, vaccination against rotavirus is starting and the need for improved treatment may be reduced. There are still only a few studies on the successful treatment of invasive acute diarrhoea with native lactobacilli, but a promising experimental trial to bind cholera toxin with a genetically modified probiotic strain has recently been published (Focareta *et al.*, 2006).

The treatment of diarrhoea by dairy foods presents a communication challenge to get the message across to general consumers, since all medicinal claims are forbidden for food products. Also, the development and marketing of drugs based on effective probiotic products requires heavy documentation and registration and may not be in the interest of food companies. Risk-reduction claims are or will be accepted for foods in the EU in the near future, and will certainly be of interest to food companies, in this case perhaps in the form of the reduction of the risk of acute diarrhoea. How to communicate this in marketing and on yoghurt packages is again a challenge for producers.

Other intestinal disturbances, such as IBS, are also of interest to many dairy food producers, but this area still needs further studies to prove efficacy. Also a better understanding of the mechanisms and origin of the symptoms would certainly help in the development of effective preventive and treatment agents, including probiotics. In any case, more studies are needed, specific to single strains or combinations of strains. With regard to product development, also other factors such as lactose intolerance have to be taken into consideration. The concept of lactose-free dairy products will be more easily accepted than those with a normal lactose level.

4.7 Sources of further information and advice

Meta-analyses and review articles

Helicobacter pylori

Felley and Michetti, 2003

Gotteland *et al.*, 2006

Hamilton-Miller, 2003

Acute diarrhoea

McFarland, 2006

Sazawal *et al.*, 2006

Szajewska *et al.*, 2006

Szajewska and Mrukowicz, 2001b

van Niel *et al.*, 2002

Antibiotic-associated diarrhoea and *C. difficile*

Cremonini *et al.*, 2002b

D'Souza *et al.*, 2002

Dendukuri *et al.*, 2005

Hawrelak *et al.*, 2005

Irritable bowel syndrome

Hamilton-Miller, 2001

Madden, 2004

Verdu and Collins, 2005

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5

Probiotics, prebiotics, and inflammatory bowel disease

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5.1 Introduction

Crohn's disease and ulcerative colitis, collectively known as inflammatory bowel disease (IBD), are chronic idiopathic relapsing and remitting inflammatory disorders of the gastrointestinal tract. These disorders result in tissue-damaging inflammatory responses in the intestine that may result in much personal suffering and impaired quality of life. IBD is a substantial economic healthcare burden, and in developed countries, between 0.1 and 0.2% of the population are affected (Shanahan, 2000). Although the precise etiology of IBD remains unclear, its initiation and perpetuation arise from a complex interaction of environmental, genetic, and immunoregulatory factors (Elson, 2002).

Traditional therapeutic strategies for IBD target only the host inflammatory response. In many patients, such strategies remain sub-optimal in terms of disease management and are thwarted by various issues including expense, toxicity, and incomplete efficacy. More comprehensive approaches are needed to achieve sustained therapeutic responses in patients with IBD. Within the gastrointestinal tract, the resident microbiota is an essential health asset that crucially influences homeostasis and the normal structural and functional development of mucosal immunity. In healthy individuals, the mucosal immune system exhibits an exquisitely regulated restrained response to resident micro-organisms. However, in susceptible individuals, some components of the microbiota may become a liability. Genetically-influenced unrestrained mucosal immune activation in response to local bacterial signals is believed to contribute to the pathogenesis of IBD (Duchmann *et al.*, 1995; Rutgeerts *et al.*, 1995). It follows that manipulation of the intestinal microbiota to preferentially

enhance its beneficial components thus represents a potential therapeutic strategy for IBD.

Probiotics are commensal micro-organisms, usually bacteria, which can be harnessed for health benefits. The term 'probiotic' traditionally refers to live micro-organisms. However, this definition may be restrictive as live bacteria may not be an absolute requirement for therapeutic efficacy. Dead organisms, bacterial constituents such as DNA, or biologically active secreted metabolites such as bacteriocins also account for some of the anti-inflammatory effects of probiotics. Thus, the term 'pharmabiotic' has been coined to encompass any form of therapeutic exploitation of the gut microbiota (O'Hara and Shanahan, 2006). Prebiotics are non-digestible food ingredients, usually polysaccharides or oligosaccharides, which beneficially affect the host by selectively modulating the composition of the enteric microbiota. Therefore, pharmabiotics also embrace prebiotics, as well as synbiotics (combinations of probiotics and prebiotics) and genetically modified commensal bacteria. In recent years there has been a marked resurgence of interest in eukaryotic-prokaryotic interactions at the mucosal surface. In this chapter we present an overview of our increasing understanding of the host-microbe interface within the gut, as well as the therapeutic rationale, role, and range of pharmabiotic options in IBD.

5.2 The host-microbe interface within the gut

Therapeutic modification of the intestinal microbiota with any type of pharmabiotic poses a significant challenge when one considers the complexity and scale of this internal ecosystem. Intestinal bacteria comprise more than 1000 different species, outnumber human somatic and germ cells 10-fold, and have a collective metabolism that is equivalent to that of an organ within an organ (O'Hara and Shanahan, 2006). In the past, investigation of this living bacterial organ was impeded by the limitations of conventional microbiology techniques. However, the constituent species and temporal stability of the microbiota are now being examined using modern molecular techniques. Host genotype significantly impacts on bacterial diversity within the gut (Zoetendal *et al.*, 2001). Although the composition of the microbiota is relatively stable and individualistic after weaning (Vaughan *et al.*, 2000), it may alter with diet, lifestyle and age (Hopkins *et al.*, 2001; Moore and Moore, 1995). Within the alimentary tract, surface adherent and luminal microbial populations differ (Eckburg *et al.*, 2005), and the ratio of anaerobes to aerobes is lower at mucosal surfaces compared to within the lumen. Moreover, the composition of the microbiota increases in complexity and variety from foregut to hindgut, and in the large intestine rises to an estimated 10^{12} bacteria per gram of colonic content (Coyne *et al.*, 2005). It follows that strategies that modify the microbiota require design specifications that consider

hostile environments such as gastric acid and bile, as well as determinants of viability and replication during gut transit.

5.2.1 Sensing the mucosal microenvironment

Mutually beneficial interactions between the host and resident bacteria are mediated by bi-directional host-microbial exchange and, eukaryotic-prokaryotic signalling appears to be central to pharmabiotic action. The mucosal immune system recognises and responds rapidly to pathogenic bacteria, whilst exhibiting a restrained responsiveness to the resident microbiota. This ability to distinguish commensal from pathogenic organism demands exquisite regulation of discriminatory responses. In the gut, pattern recognition receptors (PRR) play a central role in the interpretation and discrimination of incoming bacterial signals (Cario, 2005).

The two major host PRR systems are the toll-like receptors (TLR) and nucleotide-binding oligomerisation domain/caspase recruitment domain (NOD/CARD) proteins. These PRRs contribute critically to the activation of pro-inflammatory cascades in immunosensory cells in response to specific microbial-associated molecular patterns. These include lipopolysaccharide, lipoteichoic acid, peptidoglycan, and bacterial DNA. Immunocytes probably use multiple PRRs simultaneously to recognise the features of a specific microbe. In the intestine, the main types of immunosensory cells include enterocytes, M cells, and dendritic cells. It is now well established that mucosal homeostasis requires continual signalling from bacteria within the lumen of the gut, and PRRs are crucial for bacterial-host dialogue. Decreased enterocyte proliferation and levels of cytoprotective factors have been observed in TLR-defective mice, and TLR signals mediated by commensal bacteria or their ligands are essential for intestinal barrier function and repair of the gut (Fukata *et al.*, 2005; Rakoff-Nahoum *et al.*, 2004).

5.2.2 The enteric microbiota as a health asset

The microbiota fundamentally impact gastrointestinal physiology, as well as pathophysiology, and intestinal bacteria are not uniform in their capacity to drive mucosal inflammatory responses. Some commensal species such as *Bacteroides vulgatus* are pro-inflammatory (Sartor, 1997). In contrast, others species lack inflammatory capacity, and certain bacteria including strains of bifidobacteria and lactobacilli can even attenuate inflammatory responses (Kelly *et al.*, 2004; Neish *et al.*, 2000; O'Hara *et al.*, 2006; Otte and Podolsky, 2004). Normally, commensal bacteria are an essential health asset that exert a conditioning and protective influence on intestinal structure and homeostasis (Fig. 5.1).

Intestinal bacteria protect against infection, and exchange developmental and regulatory signals with the host that prime and instruct mucosal immunity (O'Hara and Shanahan, 2006; Shanahan, 2002). Interactions between gut-

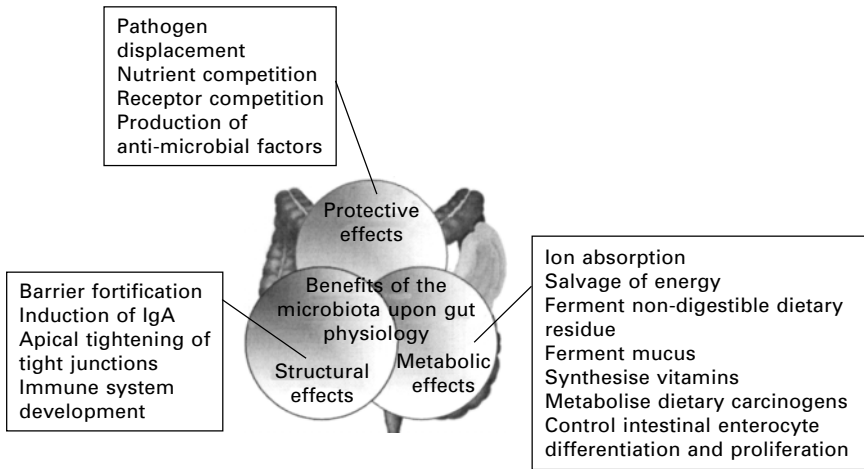


Fig. 5.1 Functions of the intestinal microbiota. Commensal bacteria exert a miscellany of protective, structural and metabolic effects on the intestinal epithelium.

associated lymphoid tissue and the microbiota early in life are crucial for appropriate development of mucosal and systemic immunoregulatory systems, and the composition of the colonising microbiota may influence individual variations in immunity (Cebra, 1999; Shanahan, 2002). Bacterial metabolism confers many benefits to intestinal physiology (Fig. 5.1), and commensal bacteria represent a rich repository of metabolites that can be mined for therapeutic benefit. Genome sequencing of commensal and probiotic bacteria will help divulge properties that are essential for beneficial signals within the gut (Claesson *et al.*, 2006; Pena *et al.*, 2004; Xu *et al.*, 2003).

5.3 The pathogenesis of IBD

Both Crohn's disease and ulcerative colitis represent the clinical outcome of a complex interaction of immune, genetic, and environmental factors. The normal physiological response to indigenous micro-organisms is one of immunological quiescence. Deviations from this, and in particular, genetically-influenced aberrant immune responses to luminal antigens are now recognised to underlie IBD. The intestinal barrier can be impaired in IBD. Defects in epithelial barrier function may precede the onset of inflammation and lead to persistent immune activation (Irvine and Marshall, 2000). Leukocyte recruitment from the gut vasculature contributes to the initiation and perpetuation of mucosal inflammatory responses. Upregulation of various transcription factors including nuclear factor (NF)- κ B, the master coordinator of immune responses to danger signals, drives the subsequent excessive local release of a diverse array of immune mediators. These include cytokines,

growth factors, reactive oxygen metabolites, nitric oxide, and leukotrienes. Characteristically, the activated immune response of IBD is dominated by mucosal CD4⁺ T lymphocytes. Crohn's disease bears the immunological signature of an exaggerated Th1 response, with excess interleukin (IL)-12, IL-18, interferon- γ , and tumour necrosis factor- α (Cobrin and Abreu, 2005). Conversely, but less clearly, ulcerative colitis is associated with a dominant atypical Th2 cytokine response that is probably driven by the production of IL-13 (Targan and Karp, 2005).

5.3.1 Genetic influences on microbial perception in IBD

Family studies and gene searches have established an important role of genetic predisposition in the pathogenesis of IBD (Peeters *et al.*, 2000). At least ten genomic regions that harbour genes that can contribute to IBD have been identified (Korzenik and Podolsky, 2006). Of these, mutations of *CARD15*, which encodes NOD2, and certain TLR polymorphisms have been strongly associated with Crohn's disease. This implicates PRR dysfunction and impaired innate immune responses in the pathogenesis of IBD. Moreover, expression of some TLRs, including TLR4 which recognises bacterial lipopolysaccharide, is differentially altered in Crohn's disease and ulcerative colitis (Cario and Podolsky, 2000). It may be that increased TLR4 expression in patients with IBD confers hyperresponsiveness to lipopolysaccharide. Alternatively, it may reflect a loss of response.

NOD2 is involved in the recognition of muramyl dipeptide, a bacterial cell wall component, and intracellular triggering of NOD2 activates NF- κ B. Three major polymorphisms in the *CARD15* gene have been specifically associated with ~15% of Crohn's disease patients (Gaya *et al.*, 2006). The risk of developing Crohn's disease is 20–40-fold higher in individuals who carry two copies of the risk alleles (Bairead *et al.*, 2003; Macdonald and Monteleone, 2005). Nevertheless, this risk factor is neither sufficient nor necessary for development of the disease (Inoue *et al.*, 2002), and the functional phenotypic consequences of the genetic defect are controversial and remain to be elucidated (Mueller and Macpherson, 2006). In selective populations, promoter polymorphisms in the *TLR9* gene that encodes the receptor for bacterial DNA, and a polymorphism in the *TLR4* gene that encodes a lipopolysaccharide receptor have been associated with IBD (Cario, 2005; Franchimont *et al.*, 2004; Torok *et al.*, 2004). Together with the reported hyperreactivity to flagellins in sera from Crohn's disease patients (Lodes *et al.*, 2004), these findings indicate that PRR polymorphisms could lead to impaired bacterial clearance and, thus, an increased load of bacterial antigens in the lumen. This is supported by evidence for defective handling of bacteria and increased bacterial numbers in the mucosa of patients with Crohn's disease (Sitaraman *et al.*, 2005).

5.3.2 The enteric microbiota as a disease liability – the environmental component?

An environmental contribution to the pathogenesis of IBD is underpinned by the lack of complete concordance of IBD among monozygotic twins and the absence of a family history in the majority of cases (Tysk *et al.*, 1988). Numerous environmental factors have been reported to be associated with either form of IBD. These include smoking, diet, appendectomy, non-steroidal anti-inflammatory drugs, socio-economic conditions and more sanitary conditions during childhood in developed countries (Bernstein *et al.*, 2006; Shanahan, 2004). These factors are undoubtedly complex. For example, smoking which enhances the risk of Crohn's disease, unexpectedly appears to protect against ulcerative colitis (Rubin and Hanauer, 2000).

After the lesson of *Helicobacter pylori* and peptic ulcer disease, an infectious contribution to the pathogenesis of IBD is plausible. Despite intensive pursuit of a specific infectious cause for IBD, this subject remains controversial. However, an aetiological role for a single pathogenic micro-organism in the pathogenesis of IBD has not been established (Shanahan, 2004). On the other hand, compelling data from murine models of colitis as well as circumstantial evidence in patient-related studies, implicate the enteric microbiota in the pathogenesis of both Crohn's disease and ulcerative colitis (Table 5.1). The level at which the dysregulated immunity to commensal organisms occurs has not been identified. It remains unclear whether the associated inflammatory responses, both within the gut and at extra-intestinal sites, are elicited in response to a specific subset of intestinal microbes. Alternatively, sensing of commensal bacteria in general may be affected. Nevertheless, what is clear is that exposure to the microbiota is a pre-requisite for expression of the disease (Shanahan, 2002). Together the evidence implicating the microbiota in IBD underpins the rationale for using pharmabiotics as a therapeutic strategy. Pharmabiotics could beneficially modulate mucosal and systemic immunity, as well as improve nutritional and microbial balance. Of the various pharmabiotic options available, probiotics and prebiotics have been most extensively studied.

5.4 Modifying the intestinal ecosystem

Criteria for designating a bacterial strain as a probiotic include human origin, acid and bile resistance, survival of gastrointestinal transit, non-pathogenic, production of anti-microbial substances, and immune modulatory activity (Dunne *et al.*, 2001). The most commonly used probiotics include lactobacilli and bifidobacteria species that lack inflammatory activity. However, other bacteria including non-pathogenic *Escherichia coli*, and multi-strain cocktails such as VSL#3 have been used for probiotic effect also. VSL#3 comprises *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subspecies *bulgaricus*, *Bifidobacterium infantis*,

Table 5.1 Commensal bacteria play a role in IBD – the evidence

Experimental evidence from animal models of IBD

Commensal bacteria influence mucosal immune development and function

Bacterial colonisation is a pre-requisite for an inflammatory phenotype

Germ-free animals do not develop IBD-like lesions

Alteration of the microbiota with antibiotics can be beneficial

Disease can be propagated by adoptive transfer of T cells reactive with enteric bacteria but not dietary or epithelial antigens

Administration of probiotics can delay onset of disease and attenuate the inflammatory process

Experimental evidence from human studies

Cellular and humoral immune reactivity against components of the microbiota is evident in patients with IBD

Linkage of the *CARD15* polymorphisms and other PRR polymorphisms with a subset of human Crohn's disease incriminates defective interpretation of the local microenvironment

Observational evidence from patient-related studies

Bacterial numbers within the mucosa of IBD patients are increased compared with that of non-inflamed and inflammatory disease controls

Lesions occur in areas of the bowel with highest bacterial counts

Faecal stream diversion has a beneficial effect on the clinical course of disease

Relapse occurs consistently with restoration of faecal stream

Administration of antibiotics has therapeutic efficacy in IBD patients

Administration of probiotics has therapeutic efficacy in IBD patients

Enteroadherent and intramucosal bacteria are increased in Crohn's disease

Metabolic diseases involving defective phagocytic microbicidal function tend to develop Crohn's-like lesions that respond to antibiotics or correction of immune defect

Bifidobacterium breve, *Bifidobacterium longum*, and *Streptococcus salivarius* subspecies *thermophilus*. It is noteworthy that the potential for targeting host-microbial interactions for therapeutic effects in IBD is not limited to prokaryotes. Non-bacterial organisms such as *Saccharomyces boulardii* have demonstrated probiotic properties, and helminths and helminthic antigens are under investigation with encouraging results in animal models of inflammation and in humans (Elliott *et al.*, 2005; Guslandi *et al.*, 2003; Summers *et al.*, 2005).

5.4.1 Mechanisms of probiotic action

Increasing evidence supports a therapeutic role for probiotic strategies for treating enteric infections, post-antibiotic syndromes, necrotising enterocolitis, and irritable bowel syndrome (Allen *et al.*, 2004; D'Souza *et al.*, 2002; Lin *et al.*, 2005; O'Mahony *et al.*, 2005). Although an early report described the use of a retention enema containing exogenous bacterial microbiota to induce

remission in a patient with ulcerative colitis (Bennet and Brinkman, 1989), the use of microbial therapy for IBD has only recently attracted serious interest from clinicians.

Multiple microbe-microbe and host-microbe interactions probably account for the versatility of probiotic action. The beneficial effects of probiotics may be either direct, or indirect through modification of the intestinal ecosystem, epithelial barrier function, or the immune response. Figure 5.2 illustrates several mechanisms of probiotic action that are relevant to IBD therapy: (1) competition with pathogenic bacteria for receptors and essential nutrients; (2) secretion of antimicrobial factors that suppress pathogen overgrowth or pro-inflammatory activity; (3) inhibition of apoptosis in epithelial cells; (4) enhancement of mucosal barrier function; (5) priming and stimulation of humoral and cell-mediated immunity; (6) modulation of immune responses and cytokine secretion; and (7) induction of T cell apoptosis in the lamina propria. Oral consumption of probiotics is associated with immune engagement and demonstrable systemic immunologic changes (McCarthy *et al.*, 2003), and it appears that probiotics serve to mimic the commensal microbiota and exploit host-microbial signalling pathways. It remains to be established which potential probiotic mechanism(s) relate to various clinical diseases. Experimental models have revealed that probiotic strains differ greatly in their mechanisms of action; any singular mechanism is unlikely to account for all of their clinical effects.

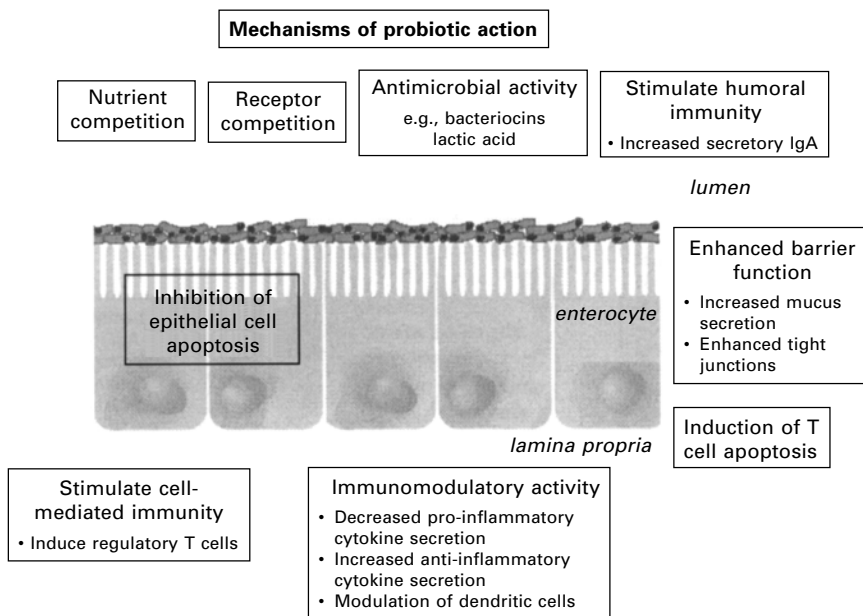


Fig. 5.2 Mechanisms of probiotic action in inflammatory bowel disease (IBD).

5.4.2 Probiotic and prebiotic studies in animal models

Encouraging results of some probiotic therapies have been observed in various animal models of experimental colitis. The reported beneficial effects range from protection against the severity of intestinal inflammation or the recurrence of colitis, or the enhancement of disease resolution. These effects are associated with a reduction in pro-inflammatory cytokines and an induction of regulatory cytokines (McCarthy *et al.*, 2003; Schultz *et al.*, 2002).

Administration of *Lactobacillus reuteri* R2LC, but not *Lactobacillus rhamnosus* GG, significantly attenuated inflammation in acetic acid- or methotrexate-induced colitis in rats (Fabia *et al.*, 1993; Holma *et al.*, 2001; Mao *et al.*, 1996). Conversely, *L. rhamnosus* GG and VSL#3 demonstrated a protective effect in the iodoacetamide model of colitis (Shibolet *et al.*, 2002). *Bifidobacterium infantis* 35624 also limited inflammation, and its protective effects were evident outside the context of established inflammation and at the pre-inflammatory stage (McCarthy *et al.*, 2003; Sheil *et al.*, 2006). In the IL-10 deficient model of spontaneous colitis, VSL#3, *Lactobacillus reuteri* and *Lactobacillus plantarum* 299v have demonstrated therapeutic effects (Madsen *et al.*, 2001, 1999; Schultz *et al.*, 2002). VSL#3 also enhanced epithelial barrier function in this model. *L. rhamnosus* GG, but not *L. plantarum* 299v, prevented the recurrence of colitis in gnotobiotic transgenic rats (Dieleman *et al.*, 2003). It is noteworthy that although not all probiotic preparations have a beneficial effect in all models, they do not appear to be harmful to any model.

Interestingly, non-viable irradiated probiotic bacteria and the subcutaneous administration of DNA derived from the VSL#3 cocktail have demonstrated protective effects in a number of animal models of colitis (Rachmilewitz *et al.*, 2004). The effects were shown to be mediated not by bacterial metabolites or ability to colonise the colon, but by the DNA through TLR9 (Rachmilewitz *et al.*, 2002; Rachmilewitz *et al.*, 2004). These findings in particular, as well as the supportive finding that DNA from VSL#3 can attenuate NF- κ B-mediated signalling in intestinal enterocytes (Jijon *et al.*, 2004), challenge the traditional assumption that probiotic bacteria must be live and intact.

A number of prebiotic combinations have been tested in animal models of intestinal inflammation. Lactulose, germinated barley foodstuff, inulin, and fructo-oligosaccharide are some of the prebiotic preparations that have been shown to reduce inflammation in dextran sodium sulphate, IL-10 knockout mice, and trinitrobenzene sulphonic acid models of colitis (Cherbut *et al.*, 2003; Fukuda *et al.*, 2002; Madsen *et al.*, 1999; Videla *et al.*, 2001). Further investigation of the potential synergistic effects of co-administration of prebiotics with probiotics would also be of interest.

5.4.3 Probiotic and prebiotic studies in pouchitis

Despite the encouraging data from probiotic studies in animal models of colitis, the role of probiotics in human IBD has proven more complex. The

most impressive studies have been in patients with pouchitis (Table 5.2). Pouchitis is a common complication, an estimated 24–46% of patients experience at least one episode following pouch surgery for ulcerative colitis. Pouchitis is characterised by non-specific inflammation of the ileal reservoir. Although many patients respond to antibiotics, approximately 10% of patients develop chronic pouchitis (Fazio *et al.*, 1995; Sandborn *et al.*, 1994). Altered luminal microbiota may be a risk factor for pouchitis. Reduced numbers of bifidobacteria and lactobacilli, as well as increased counts of *Clostridium perfringens* and other species have been recorded in stool samples of patients with pouchitis (Ruseler-van Embden *et al.*, 1994).

A number of studies have indicated that altering the microbiota in the pouch by administering probiotic bacteria can be effective in maintaining remission in chronic pouchitis, or preventing the development of pouchitis in the first place (Gionchetti *et al.*, 2000, 2003; Mimura *et al.*, 2004) (Table 5.2). Two randomised controlled trials involving a total of 76 patients evaluated VSL#3 as a maintenance therapy. In both studies the relapse rate was 15% in the VSL#3 group compared to 100% (Gionchetti *et al.*, 2000) or 94% (Mimura *et al.*, 2004) in the placebo group. In one of these studies all those in remission following probiotic treatment relapsed on completion of the trial (Gionchetti *et al.*, 2000). An uncontrolled open-label study of the effectiveness of VSL#3 in clinical practice was less encouraging. Of 31 patients with antibiotic-dependent pouchitis, <20% maintained remission on VSL#3 during 8 months of follow up (Shen *et al.*, 2005). There were some inherent problems with this study. Patients had to purchase and bear the cost of medication, and although the medication was self-administered, adherence was not monitored.

In a randomised controlled trial, VSL#3 was shown to reduce the risk of post-surgical prevention of pouchitis, and also reduce the stool frequency of patients without clinical pouchitis (Gionchetti *et al.*, 2003). A recent study reported that the diversity of the bacterial microbiota was increased, whereas fungal diversity was repressed, in patients in VSL#3-maintained remission compared to placebo treatment (Kuhbacher *et al.*, 2006). These findings support a therapeutic role of probiotic bacteria in the restoration or maintenance of a protective intestinal microbiota.

Lactobacillus rhamnosus GG did not demonstrate efficacy for the treatment of active acute pouchitis, although in a case controlled study, a decreased cumulative risk of pouchitis was observed in patients taking *L. rhamnosus* GG (Gosselink *et al.*, 2004; Kuisma *et al.*, 2003). Trials using a fermented milk product, Cultura®, which contains *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* subspecies *lactis* Bb-12, have also shown some benefit in the prevention and treatment of active pouchitis (Laake *et al.*, 2003, 2004, 2005). Several of these studies are encouraging. However, the wider, open clinical experience with probiotics in pouchitis patients is inconsistent. Whether this relates to variability in patients populations, or the quality and choice of probiotic preparation, is unclear. Further studies should

Table 5.2 Clinical trials of probiotics in pouchitis

Study	<i>n</i>	Duration	Probiotic	Response to probiotic	Reference
RCT; maintenance of antibiotic-induced remission	40	9 months	VSL#3	85% response vs 0% in placebo group	Gionchetti <i>et al.</i> , 2000
Open-label; treatment of acute active pouchitis	10	1 month	Cultura®	50% endoscopic improvement; 0% histologic improvement	Laake <i>et al.</i> , 2003
RCT; treatment of active acute pouchitis	20	3 months	<i>Lactobacillus rhamnosus</i> GG	No benefit on clinical or endoscopic response	Kuisma <i>et al.</i> , 2003
RCT; post-operative prevention	40	12 months	VSL#3	90% response vs 60% in placebo group	Gionchetti <i>et al.</i> , 2003
RCT; maintenance of antibiotic-induced remission	36	12 months	VSL#3	85% response vs 6% in placebo group	Mimura <i>et al.</i> , 2004
Case control study; post-operative prevention	117	3 years	<i>Lactobacillus rhamnosus</i> GG	Decreased risk of pouchitis; cumulative risk at 3 years 7% vs 29%	Gosselink <i>et al.</i> , 2004
Controlled clinical trial; prevention of acute pouchitis	51	1 month	Cultura®	Improvement in PDAI, but not histology	Laake <i>et al.</i> , 2004
Clinical trial; prevention of acute pouchitis	61	1 month	Cultura®	Improved symptoms and endoscopic response	Laake <i>et al.</i> , 2005
Open-label; management of antibiotic-dependent pouchitis	31	8 months	VSL#3	Only 6 completed trial; no differences in PDAI vs placebo	Shen <i>et al.</i> , 2005
RCT; role of the microbiota in maintenance of remission	15	12 months	VSL#3	Increased bacterial diversity, reduced fungal diversity in probiotic group	Kuhbacher <i>et al.</i> , 2006

VSL#3 comprises *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subspecies *bulgaricus*, *Bifidobacterium infantis*, *Bifidobacterium breve*, *Bifidobacterium longum*, and *Streptococcus salivarius* subspecies *thermophilus*. Cultura® contains *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* subspecies *lactis* Bb-12. *n*, total number of patients; PDAI, pouchitis disease activity index; RCT, randomised controlled trial.

also address whether there is a need for antibiotic pre-treatment to induce remission prior to administration of probiotic therapy.

5.4.4 Probiotic and prebiotic studies in ulcerative colitis

The results of the published clinical trials with pharmabiotics in the treatment of ulcerative colitis are shown in Table 5.3. In earlier trials, VSL#3 and *Escherichia coli* Nissle 1917 were shown to reduce remission of acute ulcerative colitis (Kruis *et al.*, 1997; Rembacken *et al.*, 1999). However, these studies were not adequately powered for equivalence, but the observed efficacy was similar to that of mesalazine in the maintenance of remission. Mesalazine is the standard treatment used to prevent ulcerative colitis relapses. In a later and larger study involving 327 patients with inactive ulcerative colitis, *E. coli* Nissle 1917 was deemed statistically equivalent to mesalazine in the maintenance of remission (Kruis *et al.*, 2004). *Lactobacillus rhamnosus* GG has also shown the same efficacy as mesalazine in maintaining remission of ulcerative colitis (Zocco *et al.*, 2006). VSL#3 has demonstrated efficacy in the induction and maintenance of remission, and significant concentrations of the probiotics present in VSL#3 were identified in faecal cultures (Bibiloni *et al.*, 2005; Venturi *et al.*, 1999).

One randomised control trial of a bifidobacteria-fermented milk administered as maintenance therapy over one year recorded fewer relapses in the treatment arm (27% vs 90% in control group), but no difference in the endoscopic score (Ishikawa *et al.*, 2003). This milk product contained *Bifidobacterium breve* (Yakult Co. Ltd., Tokyo, Japan), *Bifidobacterium bifidum* (Yakult), and *Lactobacillus acidophilus* YIT 0168. However, the study was limited by the absence of blinding among the investigators and patients and a lack of correlation in the outcome measures. Similarly, following the administration of BIFICO® (Shanghai Sine Pharmaceutical Corp. Ltd., Shanghai, China), a capsule containing *Bifidobacterium*, *Enterococcus*, and *Lactobacillus acidophilus*, the relapse rate in the probiotic group was 20% compared with 93% in the placebo group. Furthermore, NF- κ B activation was significantly attenuated and IL-10 expression was elevated in the probiotic group (Cui *et al.*, 2004). More recently, in an *in vitro* model, treatment with *Bifidobacterium longum* was shown to inhibit NF- κ B activation and downregulate inflammatory cytokine secretion from inflamed tissues of active ulcerative colitis (Bai *et al.*, 2006). In combination with inulin-oligofructose, this species was used as a synbiotic in a randomised controlled trial of 18 patients with active ulcerative colitis (*Bifidobacterium longum*/Synergy I, Orafit, Tienen, Belgium). Although endoscopic differences were not significant, there was a significant reduction in inflammatory cytokines in the synbiotic group that was accompanied by reduced inflammation and regeneration of epithelial tissue (Furrie *et al.*, 2005).

Germinated barley foodstuff containing glutamine-rich protein and hemicellulose-rich fibres have alleviated symptoms in patients with ulcerative

Table 5.3 Clinical trials of probiotics in ulcerative colitis

Study	<i>n</i>	Duration	Probiotic	Response to probiotic	Reference
RCT; maintenance of remission	120	4 months	<i>Escherichia coli</i> Nissle 1917	84% response to probiotic vs 89% response to mesalazine	Kruis <i>et al.</i> , 1997
RCT; maintenance of remission	116	12 months	<i>Escherichia coli</i> Nissle 1917	26% response to probiotic vs 25% to mesalazine	Rembacken <i>et al.</i> , 1999
RCT; acute active disease	116	3 months	<i>Escherichia coli</i> Nissle 1917	68% response to probiotic vs 75% to mesalazine	Rembacken <i>et al.</i> , 1999
Open-label; maintenance of remission	20	12 months	VSL#3	75% in remission	Venturi <i>et al.</i> , 1999
RCT; maintenance of remission	21	12 months	Bifidobacteria-fermented milk	73% response to probiotic vs 10% response in control group	Ishikawa <i>et al.</i> , 2003
Open-label; acute active disease	25	1 month	<i>Saccharomyces boulardii</i>	68% in clinical remission	Guslandi <i>et al.</i> , 2003
Open-label; treatment of chronic active disease	6	1 week	Faecal enemas derived from healthy donors	100% remission; off standard medications by 4 months; disease-free during follow-up	Borody <i>et al.</i> , 2003
RCT; maintenance of remission	327	12 months	<i>Escherichia coli</i> Nissle 1917	Statistically equivalent to mesalazine	Kruis <i>et al.</i> , 2004
RCT; maintenance of remission	30	8 weeks	BIFICO	80% remission rate vs 7% in placebo group	Cui <i>et al.</i> , 2004
Open-label; induction of remission	34	6 weeks	VSL#3	53% remission rate	Bibiloni <i>et al.</i> , 2005
RCT; treatment of active disease	18	1 month	<i>Bifidobacterium longum</i> with inulin-oligofructose	Significant reduction in inflammatory cytokines	Furrie <i>et al.</i> , 2005

Table 5.3 Continued

Study	<i>n</i>	Duration	Probiotic	Response to probiotic	Reference
Open-label; treatment of ulcerative colitis	12	4 weeks	Bifidogenic growth stimulator (prebiotic)	Statistically significant improvement in clinical activity index score	Suzuki <i>et al.</i> , 2006
Open-label; maintenance of remission	187	12 months	<i>Lactobacillus rhamnosus</i> GG	No difference compared to mesalazine	Zocco <i>et al.</i> , 2006

VSL#3 comprises *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subspecies *bulgaricus*, *Bifidobacterium infantis*, *Bifidobacterium breve*, *Bifidobacterium longum*, and *Streptococcus salivarius* subspecies *thermophilus*. The bifidobacteria-fermented milk product contains *Bifidobacterium breve* (Yakult Co. Ltd., Tokyo, Japan), *Bifidobacterium bifidum* (Yakult), and *Lactobacillus acidophilus* YIT 0168. BIFICO® (Shanghai Sine Pharmaceutical Corp. Ltd., Shanghai, China) capsules contain *Bifidobacterium*, *Enterococcus*, and *Lactobacillus acidophilus*. *n*, total number of patients; RCT, randomised controlled trial.

colitis (Bamba *et al.*, 2002). These prebiotics are thought to work by decreasing stool frequency, increasing local butyrate levels, and increasing the numbers of bifidobacteria and eubacteria. An example of a bifidogenic growth stimulator is a prebiotic preparation produced by *Propionibacterium freudenreichii* isolated from Swiss cheese. Oral administration of this prebiotic to 12 patients over one month resulted in a significant improvement in the clinical activity index scores of the patients (Suzuki *et al.*, 2006). Interestingly, the yeast, *Saccharomyces boulardii* (Biocodex Laboratories, Montrouge, France) has been shown to induce remission rates similar to those reported for VSL#3, *E. coli* Nissle 1917, and mesalazine (Guslandi *et al.*, 2003). To further define the role of probiotics, prebiotics, or synbiotics in ulcerative colitis there is a need for larger placebo controlled trials to be performed.

An alternative pharmabiotic strategy, faecal bacteriotherapy, was employed with interesting outcomes in a study by Borody *et al.* (2003). Faecal enemas were prepared from healthy adult donors, and administered once daily for five consecutive days as enemas to six ulcerative colitis patients on high-fibre diets. The bacteriotherapy achieved 100% remission, all patients were off standard medications within 4 months, and were disease-free for 1–13 years follow-up despite the fact that no maintenance therapy was used. These reports are striking, and clearly worthy of further investigation both from a pathogenesis as well as a therapeutic perspective.

5.4.5 Probiotic and prebiotic studies in Crohn's disease

The evidence for therapeutic efficacy of probiotics in Crohn's disease is varied and inconclusive (Table 5.4). There have been very few randomised controlled clinical trials. Small patient numbers, differences in disease activity and variations in disease distribution have confounded most trials. One of the earliest studies examined the use of *Saccharomyces boulardii* (Biocodex Laboratories) in patients with moderately active Crohn's disease. There was a significant decrease in the Crohn's disease activity index (CDAI) compared with the control group (Plein and Hotz, 1993). *S. boulardii* has been used also in combination with mesalazine in the maintenance of remission. There was a significant difference in the relapse rate after one year between the probiotic and placebo groups (Guslandi *et al.*, 2000). Despite this encouraging result, no further Crohn's disease studies with *S. boulardii* have been published.

In a randomised, double-blind, placebo-controlled pilot study involving 28 patients with active colonic Crohn's disease, 70% of patients who received *Escherichia coli* Nissle 1917 daily for one year remained in remission compared with 30% in the placebo-treated group (Malchow, 1997). *Lactobacillus salivarius* UCC118 has yielded encouraging results in treating acute active Crohn's disease also (McCarthy *et al.*, 2001). Furthermore, Campieri *et al.* reported that a combination of antibiotic and VSL#3 treatment was efficacious in preventing post-operative recurrence of Crohn's disease when compared with mesalazine (Campieri *et al.*, 2000).

Table 5.4 Clinical trials of probiotics in Crohn's disease

Study	<i>n</i>	Duration	Probiotic	Response to probiotic	Reference
RCT; treatment of acute disease	20	2 weeks	<i>Saccharomyces boulardii</i>	Decreased diarrhoea and CDAI index in probiotic group	Plein <i>et al.</i> , 1993
Open-label; treatment of paediatric Crohn's disease	14	10 days	<i>Lactobacillus rhamnosus</i> GG	Increased secretion of immunoglobulin A, enhanced barrier function	Malin <i>et al.</i> , 1996
RCT; maintenance of remission	28	12 months	<i>Escherichia coli</i> Nissle 1917	Relapse rate of 30% in probiotic group vs 70% in placebo (not significant)	Malchow <i>et al.</i> , 1997
Open-label; treatment of active disease	4	6 months	<i>Lactobacillus rhamnosus</i> GG	Improved intestinal permeability and CDAI	Gupta <i>et al.</i> , 2000
RCT; maintenance of remission	40	12 months	VSL#3	Endoscopic remission of 80% vs 60% in mesalazine group	Campieri <i>et al.</i> , 2000
RCT; maintenance of remission	32	6 months	<i>Saccharomyces boulardii</i>	94% remission in probiotic group compared to 38% mesalazine group	Guslandi <i>et al.</i> , 2000
Open-label; treatment of active disease	25	3 months	<i>Lactobacillus salivarius</i> UCC118	Reduction in CDAI and steroid use	McCarthy <i>et al.</i> , 2001
RCT; maintenance of remission	45	12 months	<i>Lactobacillus rhamnosus</i> GG	No difference between two groups at 1 year	Prantera <i>et al.</i> , 2002
RCT; induction and maintenance of remission	11	6 months	<i>Lactobacillus rhamnosus</i> GG	No benefit in induction or maintenance of remission	Schultz <i>et al.</i> , 2004
RCT; maintenance of remission in children	39	2 years	<i>Lactobacillus rhamnosus</i> GG	No difference in the median time to relapse compared to placebo	Bousvaros <i>et al.</i> , 2005
RCT; maintenance of remission	48	6 months	<i>Lactobacillus johnsonii</i> LA1	No benefit in preventing recurrence of disease	Marteau <i>et al.</i> , 2006

VSL#3 comprises *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subspecies *bulgaricus*, *Bifidobacterium infantis*, *Bifidobacterium breve*, *Bifidobacterium longum*, and *Streptococcus salivarius* subspecies *thermophilus*. CDAI, Crohn's disease activity index; *n*, total number of patients; RCT, randomised controlled trial.

Lactobacillus rhamnosus GG has been used in two open-label studies in paediatric Crohn's disease. One study reported elevated levels of gut immunoglobulin A following probiotic treatment, but without clinical improvement (Malin *et al.*, 1996). The other study reported improved intestinal permeability and clinical scores in four children with mildly active Crohn's disease (Gupta *et al.*, 2000). Of note, three patients had relapse of their Crohn's disease within 4–12 weeks of discontinuation of the *Lactobacillus* treatment. In more recent randomised controlled trials, *L. rhamnosus* GG did not demonstrate significant efficacy as a maintenance therapy in 75 children with Crohn's disease, in 11 patients with moderate to active Crohn's disease, or in 45 patients after curative surgery (Bousvaros *et al.*, 2005; Pranter *et al.*, 2002; Schultz *et al.*, 2004). Similarly, *Lactobacillus johnsonii* LA1 did not achieve statistical significance in reducing endoscopic recurrence of Crohn's disease post-operatively (Marteau *et al.*, 2006). Collectively, the result of these studies is not encouraging, and at the moment, probiotics as a therapeutic option for Crohn's disease is not scientifically validated.

Crohn's disease is a complex condition, variable in its location as well as its manifestation. Variability in the composition and diversity of the microbiota along and over the cross-sectional axis of the gastrointestinal tract suggests that depending on the topographic distribution of lesions in Crohn's disease, a single probiotic may not be equally suited to different subsets of patients. Colonic location of disease seems to respond better to antibiotics, and may, as a result, be more susceptible to pharmabiotic therapy. Investigators need to determine whether we are dealing with the wrong probiotic, the wrong dose, the wrong indication. Are probiotic combinations or other pharmabiotic approaches needed? There may be a role for probiotics in the enhancement of epithelial barrier function in the very early stages of Crohn's disease. Larger well-powered randomised control trials are clearly needed to conclusively determine whether there is a role for probiotics in Crohn's disease.

5.4.6 Safety of probiotics

Probiotics are not selected among pathogens, and by definition, have a high safety profile and the tolerance is usually excellent. Many of the commercial probiotic products have been officially designated as 'generally regarded as safe', and the deliberate ingestion of lactobacilli or bifidobacteria does not pose a greater risk of infection than that associated with the commensal strains (Borriello *et al.*, 2003). Nonetheless, some reports of infections probably caused by probiotics have been published (De Groote *et al.*, 2005; Riquelme *et al.*, 2003). Probiotic strains adhering to the intestinal mucosa could translocate, inducing bacteraemia and sepsis. However, this is very rare and it is estimated that infection due to probiotics represent between 0.05% and 0.4% of cases of infective endocarditis or bacteraemia (Gasser, 1994). Most of these rare cases have occurred in immunocompromised patients or those

with severe underlying disease. Obviously, the administration of probiotics to these patients groups should be approached with caution. Several studies have administered probiotic preparations to children; they are well tolerated and safe (Gupta *et al.*, 2000; Malin *et al.*, 1996; Vanderhoof and Young, 1998).

5.5 Future trends

Although naturally occurring probiotics may have insufficient efficacy in Crohn's disease, the genetic modification of commensal bacteria for the site-specific delivery of therapeutic molecules represents a realistic pharmabiotic strategy. Proof of principle has already been demonstrated in animal models of enterocolitis. Genetically engineered *Lactococcus lactis* has been used to deliver anti-inflammatory IL-10 or the cytoprotective trefoil factor locally to the gut (Steidler *et al.*, 2000; Vandenbroucke *et al.*, 2004). The safety issues related to genetic modification have been addressed by replacing the thymidylate synthase (*thy A*) gene in *L. lactis* with a synthetic therapeutic transgene. When the modified bacteria are deprived of thymine or thymidine they are not viable. Neither thymine nor thymidine are readily available in the external environment, thereby limiting the viability of the excreted organism. Moreover, the transgene would be eliminated from the bacterial genome if the engineered organisms re-acquire *thy A* from a wild-type strain (Steidler *et al.*, 2003). Recently, in the first human trial with genetically-engineered therapeutic bacteria, ten Crohn's disease patients were treated with modified *L. lactis* in which *thy A* was replaced with a synthetic sequence encoding human IL-10. The treatment was safe, disease activity was reduced, and the modified bacteria were biologically contained (Braat *et al.*, 2006). This encouraging study indicates that bacterial-based topical delivery of biologically active proteins represents a highly promising and safe therapeutic strategy for combating mucosal diseases. Designer probiotics are also being engineered to express molecular mimics of host receptors on their surface, these receptors bind bacterial toxins, thereby preventing enteric infections (Paton *et al.*, 2006). In a different setting, *Lactobacillus jensenii*, a commensal of the female genital tract, has been engineered to confer protection against HIV infectivity *in vitro* (Chang *et al.*, 2003). The potential for these designer probiotics is limited only by one's imagination, but public health and other safety concerns must be resolved before routine clinical use in humans.

5.5.1 Looking ahead

It is clear that significant differences exist not only between probiotic bacterial species, but also between certain strains. In addition to specific interactions between probiotic bacteria and host immune cells, microbe-microbe interactions also exist. This could explain some of the varying results observed within

the different clinical trials. Several unresolved issues impede the clinical evaluation of probiotics (Shanahan, 2003, 2004). These include determination of optimal dose and vehicle of delivery, development of reliable predictors of *in vivo* survival and performance, regulation and verification of product stability, determination of which combinations of probiotics or other pharmabiotics are synergistic or antagonistic, and strain-strain comparisons of probiotic performance in different indications. Furthermore, the microbial, immunological, and functional characteristics of individual probiotic strains and their mechanisms of action in different clinical settings require clarification. Individual variability in composition of the enteric microbiota may also be a determining factor for optimal strain selection. Another unresolved issue is whether probiotic administration should be preceded by antibiotic treatment to open the microbial niche as may have been achieved in some of the pouchitis trials. Clearly, rigorously designed, controlled, statistically-powered clinical trials are needed. There is evidence to suggest that many of the commercially available probiotics do not contain the advertised bacterial strain or the claimed concentration (Coeuret *et al.*, 2004). To protect consumers, there is a pressing need for more stringent regulation of unsubstantiated health claims.

Engagement with host immune cells is central to pharmabiotic action. However, therapeutic manipulation of the indigenous microbiota with any form of pharmabiotic remains sub-optimal due to incomplete understanding of the commensal microbiota, their immunoregulatory properties, and host-microbial interactions. Further studies of physiological interactions within the complex network of host cell-commensal-PRR-ligand signalling in gut health and disease should lead to the optimal exploitation of pharmabiotic approaches to alleviate mucosal inflammation in IBD and possibly other intestinal diseases. Mining the microbiota for metabolites that impact on host physiology is a promising source of new therapeutics. The increasing availability of commensal genomes should facilitate the identification of commensal effector molecules or other components with pharmabiotic potential. The possibility of using these molecules to specifically target distinct points of intracellular signalling cascades might alleviate inflammation in a target area and overcome the global immunosuppressive effects associated with current therapies. In patients with severe IBD, the use of designer probiotics may offer a new strategy for more targeted delivery of anti-inflammatory molecules to the inflamed mucosa.

5.6 Sources of further information and advice

5.6.1 Patient support groups

Canada: Crohn's and Colitis Foundation of Canada

60 St Clair Avenue East, Suite 600, Toronto, ON M4T 1N5, Canada.

Tel: + 1 416 920 5035 (1800 387 1479)

www.ccfc.ca

Europe: European Federation of Crohn's and Ulcerative Colitis Associations
C/o Tor Erik Jorgensen
Parallellen 13A, N-1430 As, Norway.
Tel: + 47 (0) 64 94 1671
www.efcca.org

Ireland: Irish Society for Colitis and Crohn's Disease
Carmichael Centre, North Burnswick St., Dublin 7.
Tel: + 353 (0)1 872 1416
www.iscc.ie

UK: National Association for Colitis and Crohn's Disease
4 Beaumont House, Sutton Road, St Albans, Hertfordshire AL1 5HH, UK.
Tel: + 44 (0) 172 784 4296/(0) 845 130 2233
www.nacc.org.uk

USA: Crohn's and Colitis Foundation of America
386 Park Avenue South, 17th Floor, New York, NY 10016-8804
Tel: + 1 212 685 3440/1800 932 2423
www.ccfa.org

5.6.2 Further websites

www.idbforum.com
<http://ibd.patientcommunity.com>
<http://www.digestivedisorders.org.uk/>
<http://virtual.vtt.fi/virtual/proeuhealth/>
www.ibdclub.org.uk
www.gastrohep.com
<http://apc.ucc.ie>
<http://www.uegf.org/>
<http://www.eufic.org>

5.6.3 Books

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Greig ER, Rampton DS. *Management of Crohn's disease*, London: Martin Dunitz, 2003.

Kirsner JB, Shorter RG, eds. *Inflammatory Bowel Disease*, 4th edn, Philadelphia: Lea and Febiger, 1995.

Targan SR, Shanahan F, Karp LC, eds. *Inflammatory Bowel Disease. From bench to bedside*, 2nd edn, Dordrecht: Kluwer Academic Publishers, 2003.

Rampton, DS, Shanahan, F. *Fast facts: Inflammatory Bowel Disease*, 2nd edn, Health Press Ltd., Oxford, UK, 2006.

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6

Dairy products and HIV/AIDS

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6.1 Introduction

One of the recommendations of the Expert Panel assembled in 2001 by the Food and Agriculture Organization of the United Nations and the World Health Organization on evaluation of health and nutritional properties of powder milk and live lactic acid bacteria, was that 'Efforts should be made to make probiotic products more widely available, especially for relief work and populations at high risk of morbidity and mortality' [1]. This was in recognition of the immense suffering and death due to diarrheal illness and spread of HIV, and the research to that point in time, which showed efficacy of probiotics for diarrhea and the ability of some strains to prevent bacterial vaginosis, a condition associated with increased risk of HIV.

It is difficult to know the extent to which this recommendation has been embraced by member nations, but based upon literature searches, grant announcements and knowledge of research being undertaken in countries particularly affected by HIV/AIDS, the signs are not promising. One difficulty has been the lack of availability of proven commercial products in these regions, due apparently to poor distribution channels, lack of availability of refrigerators required for shelf-life, and low income of the populations.

This chapter will explore the scientific and clinical rationale for use of wild type and recombinant probiotics, and prebiotics in reducing mortality and morbidity caused by HIV/AIDS. The ability to deliver these products in dairy formulations will be discussed. A specific example of an initiative in Tanzania will be given, in which a community-based project is allowing local families to produce and disseminate a probiotic yogurt.

6.2 HIV/AIDS – threatening health and the economy and viability of nations

The United Nations AIDS program (UNAIDS) has estimated that around 22 million people around the world have died from AIDS, and over 40 million more are currently infected with the virus. Most affected is sub-Saharan Africa, where over 18 million deaths have occurred and close to 30 million people are infected. In some African countries, over 30% of the adult population is HIV-positive [2]. In Nigeria, with about 25% of the African population, there are close to 4 million people living with HIV/AIDS [3]. Disturbingly, the age group 15–24 is particularly highly infected, the consequence of which are high rates of infected pregnant women, over 1.5 million infected children, and parents dying and leaving behind over 12 million orphans [4]. If the disease remains unchecked, the number of African children orphaned by AIDS will rise 20 million by 2010 [5].

In Africa, AIDS has surpassed armed conflict as the leading cause of death [6]. Less than 1% have access to life-prolonging antiretroviral drug therapy. With such therapy and good medical care, one might expect 1 in 20 infected subjects to succumb to HIV/AIDS in any given year, while in Africa, where only 30,000 of the 28.4 million people infected receive antiretroviral treatment, the death rate is 1 in 12. The loss of able-bodied members of the community has a devastating economic impact, as has the loss of teachers and the need for children to care for dying parents. Educated and able-bodied workers drive a country's economy, whether it is food production, manufacturing exports, tourism, or creation of new businesses. For example, maize production in Zimbabwe has fallen 61% due to the death of farmers with AIDS [7]. The World Bank has concluded that HIV/AIDS is responsible for a reduction of the annual growth rate of GDP in African countries of 0.8 to 1.4 percentage points per year and a 0.3 percentage point reduction in the annual growth rate of GDP per capita [8]. A dramatic example comes from Kenya, where the impact of AIDS on the GDP was projected at 14% lower in 2005 than it would have been without AIDS [9].

While men are often the breadwinners in Africa, women play a critical role in maintaining the households and family fabric. Thus, it is most disconcerting to note that more women than men now are becoming infected with HIV, with the number exceeding 7,000 each day. In sub-Saharan Africa, over one quarter of females under the age of 30 have HIV and an estimated half billion are at risk of acquiring the virus through sexual contact. A healthy vagina colonized predominantly with lactobacilli, has an acidic pH that can kill the virus [10] and displace pathogens [11] that induce inflammation and produce toxins which may make it easier for HIV to infect. Seminal fluid raises the pH within eight seconds of entry into the vagina, therefore increasing the opportunity of the virus to infect the host. Although condoms can effectively prevent infection if used properly, they are often not easily obtained, or men are unwilling to use them. The use of spermicides, long recommended to kill

the virus, has actually been shown to have serious adverse effects which can increase the risk of the virus infecting the host [12]. Thus, women have few options to protect themselves from becoming infected. In addition, younger men often leave communities in search of employment, and return infected with the virus, and pass it on to females in that community.

Such is the breadth of the problem, many interventional programs are needed to address sexual, mother-to-child and transfusion-related HIV transmissions, in addition to caring for infected subjects and their families. The decline in health of an HIV-positive subject coincides with a reduction in CD4+T helper cell counts, from normal levels between 500 and 1600, to 350 when anti-retrovirals are recommended (www.thebody.com/nmai/tcell.htm). Diarrhea (>200 g/day of adult stool and frequent liquid stools) is the primary cause of morbidity amongst AIDS patients, and is often the ultimate cause of death. Normally, the small intestine secretes and reabsorbs 10 liters fluid/day and effectively extrudes chloride from villus crypt cells. However, in secretory or osmotic diarrhea, there is a rapid loss of sodium, fluid and damage to the microvilli. Unless the patient is rehydrated, he/she will die. In AIDS patients with a depleted immune response, the range of microbes that cause diarrhea is larger and the inoculum needed to induce the event is lower than in healthy subjects. Microbial overgrowth can occur in the intestine, leading to shock and other adverse outcomes. Chronic diarrhea causes severe wasting as appetite is lost and essential nutrients are not adsorbed. Death from AIDS often occurs with gastrointestinal infections and diarrhea, and indeed, many non-AIDS deaths are due to these infections. It has been estimated that a child dies every 15 seconds from diarrheal diseases (Fig. 6.1). Without proper water and food sanitation, it is impossible to prevent gastroenteritis, but early hydration should reduce the death rate, unless the immune system is severely impaired.

The increasing number of AIDS patients with tuberculosis (TB) is also problematic. There are more than 400 million people infected with TB and over 14 million cases suffering from the disease [13]. Drug resistance and an immuno-compromised status in AIDS patients make the disease particularly difficult to manage, especially in African countries lacking in suitable medical facilities and supplies.

In summary, the HIV/AIDS epidemic is having an enormous, multi-faceted impact on global health, with particularly severe adversity amongst African nations. In countries like Botswana, where half of pregnant women are infected, the long term viability of the nation comes into question. In developed countries such as Canada, healthcare expenditures, without any major HIV/AIDS problem, already consumes almost half of the annual government revenues. With an estimated 4000 cases of sexually transmitted diseases detected each day in Canada (one tenth of US figures) [14], the spread of HIV is certainly feasible, and if it occurred amongst the general population, the consequences for the economy of the country would be dire. Just so, in

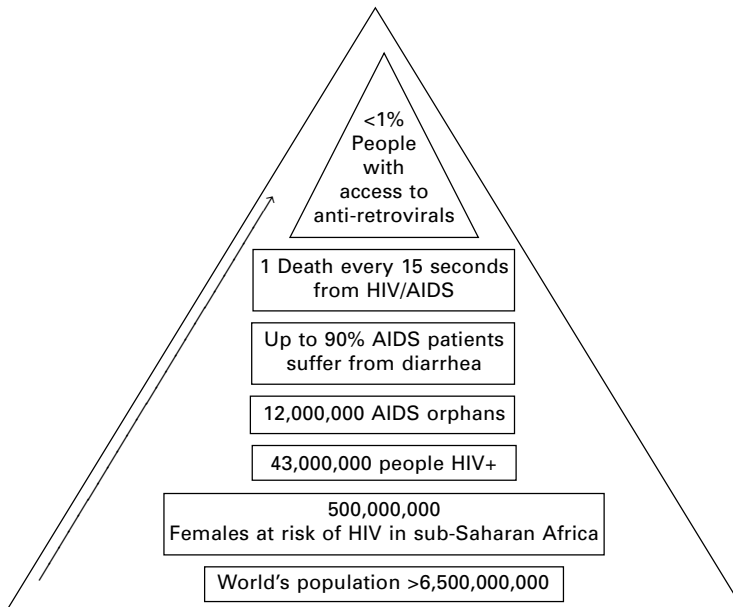


Fig. 6.1 The burden of HIV/Aids.

African nations without the income of Canada, the HIV/AIDS problem is crippling short and long term development and productivity.

6.3 The scientific and clinical rationale for probiotic and prebiotic interventions

In 2001, at the request of the government of Argentina, an Expert Panel was assembled by the Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) to examine probiotics in food and prepare an appropriate definition and set of guidelines for what constitutes a probiotic. Major reasons for the request were the lack of consensus on what a probiotic was at the time, how a product should be prepared and administered, and whether or not there was sufficient evidence to demonstrate efficacy in preventing or treating disease.

Subsequently, a definition was derived: 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' [1]. Unlike other definitions, this one more appropriately recognized that probiotic uses are not restricted to the intestinal tract, or to a mode of action such as adherence to cells. While some people appear not to have yet embraced this definition and either continue to use, or make up new ones that refer to the intestine, perhaps because they were not members of the Expert Panel, major organizations such as the International Scientific Association for Probiotics

and Prebiotics (ISAPP) have endorsed it [15]. The key to the definition is twofold: there needs to be sufficient numbers of viable organisms, and the product needs to have been shown to cause a physiological effect on the host, over and above any nutritional effect. It has been argued that dead bacteria can also induce a physiological response, leading to the suggestion that probiotics do not need to be alive [16], but such products need to find another name (bacterial products or deadbiotics or the like), rather than alter a probiotic term conceived in 1954, to mean 'for life' [17].

The Guidelines for Probiotics [18] provide a more detailed description of the essential components that make a probiotic. These reflect the historical observations of Nobel Laureate Elie Metchnikoff, who recognized at the turn of the last century, an association between lactic acid bacteria and long life; in other words specific bacterial types and a specific end point. In order to call a product probiotic, each strain must be taxonomically classified based upon DNA-DNA hybridization or equivalent molecular means, and given a strain designation to differentiate it from other strains in the same species. The latter is important as not every strain expresses the same properties.

The necessity to prove health benefits in appropriately designed and peer-reviewed human studies might seem an obvious milestone, but it represents a step not traditionally taken by companies selling food and dietary supplements (which is mostly the form used for probiotics). Low profit margins and an inability to make health claims have been cited as reasons for not doing clinical trials, but these can no longer be acceptable. This is not only necessary because of the growing interest in probiotic applications to medicine, but also because the expectation of consumers is that probiotics are not just foods, but they confer other health benefits. Companies are often quick to make wide sweeping health claims about their 'probiotic' products, yet too many are unwilling to invest in the research required to ensure that their products do in fact function as promised. The need for human studies is therefore ethical and responsible.

In terms of what constitutes an appropriate level of evidence for human studies, the Guidelines and a subsequent weight of evidence paper [19] provide good instructions. Sample size calculations should enable conclusions to be drawn from statistically significant differences when the product is compared with placebo or standard therapy. For example, is there a significant increase in CD4+ count in AIDS patients with a baseline mean count of 350, when ingesting a probiotic each day for three months? Or, how many days fewer do patients suffer from diarrhea? Then, assuming the product's viable count at end of shelf-life is consistent with that used in the studies, the company should claim what has been proven. Unfortunately, regulatory agencies often only allow vague claims on foods and dietary supplements, but this is changing in Europe with the implementation of disease risk reduction claims in 2006.

The market for probiotics is growing rapidly (expected to triple by 2010) [20], fueled to a large extent by failures of pharmaceutical agents, demands

for natural products, and studies showing encouraging results for probiotics. This has led to more drug-like properties being investigated, such as for the use of VSL#3 to ameliorate symptoms of inflammatory bowel disease [21]. With this comes a need to develop product standards higher than have been expected in the past. The FAO/WHO Guidelines represent a major roadmap for reaching this standard. Just as a consumer wants to, and needs to, know how much antibiotic to take, how often and what to expect from its usage, so too, the same consumer needs to be reassured that the consumption of a daily milk-based probiotic will provide tangible benefits. This can only be achieved by defining the product contents clearly, ensuring that manufacturing, packaging and storage conditions are optimal for retention of the required viable counts, and then proving that the health outcomes are verifiable. A number of websites mention that strains adhere to cells or mucus *in vitro*, or inhibit pathogens on agar, or survive acid pH, but while this is useful information, it does not make the organisms probiotic. Human studies are the ultimate proof, and peer-reviewed publication allows the general community to assess the level of evidence. Some so-called probiotics have undergone human trials with sample sizes so small, or study design so poor that it is difficult to understand how the manuscript was ever accepted. On the other hand, pilot studies or those with small numbers can provide useful information, albeit not sufficient for efficacy without placebo or standard therapy as control. The publication of a study per se does not necessarily provide sufficient verification of a probiotic. Some studies are published in non-peer reviewed textbooks, or in journals with an impact factor less than 0.5 and where acceptance of manuscripts may not always undergo the rigor of proper peer review. While few studies will be accepted in the *New England Journal*, *JAMA* or *Lancet*, the higher the impact of the journal, the more credible the reception given to the outcomes.

The selection of probiotic strains for use in preventing or treating HIV/AIDS, takes into consideration the end goal of the therapy. For AIDS patients, the ability of probiotic organisms to increase the CD4+ count, prevent and/or reduce the duration of gastrointestinal, respiratory or other infections, improve the efficacy and compliance of anti-retrovirals, or reduce the viral shedding and transmission to sexual partners, are all laudable goals. The mechanisms through which these benefits could be conferred are worthy of discussion. The following are some possible examples.

6.3.1 Maintenance or increase in the CD4+ count

The CD4+ T lymphocytes are important for immune responses to antigens, as well as being primary target cells for HIV binding. Once infected by the virus, the CD4 cell dies. The progressive loss of these cells results in an inability to cope with infection, leading to death of the patient. CD4, expressed on T helper cells, is one of the several glycoproteins termed ‘cluster of differentiation (CD) antigens’, expressed on the surface of lymphocytes. The Center for Disease Control in Atlanta, USA divides HIV-positive persons

into three CD4 count categories: (1) $>500/\mu\text{L}$; (2) $200\text{--}499/\mu\text{L}$; (3) $<200/\mu\text{L}$ (together with three parallel clinical stages A, B, and C). A low CD4 count ($<100/\mu\text{L}$), and a low CD4/CD8 ratio (<0.2) are highly predictive for death from AIDS-related complications, such as *Pneumocystis carinii* pneumonia [22]. A CD4 cell count of $200/\mu\text{L}$ is roughly equivalent to 20% of the lymphocyte count. The normal range for CD4 cell values varies among laboratories, but they are approximately $500\text{--}1300/\mu\text{L}$ for the absolute count, and 38–65% for the percentage. The rate of progression to AIDS in two years is around 50% when the CD4 cell count falls below $250/\mu\text{L}$. Thus, suppression of HIV replication is one way to replenish the CD4 pool, while another way is to stimulate more production of the cells.

It has been suggested that a rapid redistribution of CD4+ T lymphocytes trapped in lymphoid tissue, rather than the proliferation of new cells, is responsible for the initial rapid rise of CD4+ T cells. This could be induced by cytokine and chemokine level decline. In the next phase, an increase in CD4+ T cells may be due to newly regenerated cells [23]. The mechanism of probiotic therapy increasing the CD4 count is unknown, but it could be due to the induction of newly regenerated CD4 positive cells, or even killing of the virus [10]. In BALB/c mouse studies, seven days ingestion of *L. casei* did not increase the T cell population [24], suggesting that skewing rather than more production may be occurring, as supported by studies in which human myeloid dendritic cells activated with lactobacilli skewed CD4+ T cells to T helper 1 [25]. In human studies, *L. reuteri* administration induced a significantly higher amount of CD4+ T cells in the ileal epithelium [26].

In a study of 24 AIDS patients with CD4+ cell counts exceeding 325, daily ingestion of yogurt supplemented with *L. rhamnosus* GR-1 and *L. reuteri* RC-14, resulted in rapid amelioration of diarrhea and increase in the mean CD4+ count by 7 within 15 days, compared to a CD4+ decline following ingestion of unsupplemented yogurt [27]. This pilot study suggests that probiotic bacteria may provide some adjunctive therapy for AIDS patients, through maintenance of CD4+ counts. The same effects do not appear to occur with regular yogurt, as supported by an animal study which showed no increase in CD4+ T cells following yogurt ingestion [28]. More studies are needed to investigate the clinical and mechanistic effects in AIDS patients.

6.3.2 Prevent and/or reduce the duration of gastrointestinal, respiratory or other infections

Respiratory infections caused by tuberculosis have become the leading cause of death amongst AIDS patients [29], followed by other respiratory diseases and gastroenteritis [30]. Given the track record of probiotics in reducing the duration of diarrhea [15], it is surprising that no randomized, placebo-controlled studies have been performed on AIDS patients with chronic diarrhea. Two of the most documented probiotics, *L. rhamnosus* GG and *L. reuteri* SD2112 have been shown to be safely administered to HIV-infected patients. In the

former case, a placebo-controlled study of 17 subjects with diarrhea for more than 1 month, had no alterations in frequency or consistency of diarrhea after receiving 1.5×10^{10} cfu/dose) twice daily for two weeks [31]. In a study showing safety of *L. reuteri* SD2112, 39 subjects consumed 1×10^{10} cfu/dose or a placebo for 21 days in a double-masked, parallel design experiment [32]. These safety studies are interesting because some concerns have been raised about the use of viable bacteria (as in probiotic products) in immunocompromised subjects [33]. A study showed increased intestinal permeability among white but not black HIV-infected patients without diarrhea; however, when diarrhea was present in white or black African patients with HIV/AIDS, all subjects had marked malabsorption and increased intestinal permeability [34]. This implies that intestinal permeability need not necessarily lead to diarrhea. Still, when diarrhea is present, intestinal permeability is compromised, as supported by a study of 35 AIDS subjects which showed that there was a low, chronic rate of bacteria and/or bacterial products which crossed a compromised colonic wall and caused a chronic low stress response [35]. These data would suggest that AIDS patients with diarrhea do have compromised intestines, yet the use of *L. rhamnosus* GG or *L. rhamnosus* GR-1 with *L. reuteri* RC-14, did not induce infection.

If the combination approach, using *L. rhamnosus* GR-1 and *L. reuteri* RC-14, is effective in treating diarrhea in a larger number of AIDS subjects [27], it raises the question what is the mechanism(s) of action? The ability of lactobacilli to repair defects in gut permeability is now being studied. It is clearly not a universal phenomenon and negative studies in animals have been reported. One positive study using *L. farciminis* has shown that ingestion of this strain can prevent stress-induced hypersensitivity to distension, and decrease colonic paracellular permeability, via an inhibition of the contraction of colonic epithelial cells cytoskeleton and, the subsequent tight junction opening, and perhaps through an indirect effect of nitric oxide [36]. Cell culture studies have shown that organisms often used in dairy production, namely *S. thermophilus* and *L. acidophilus*, induce transepithelial resistance by maintenance (actin, ZO-1) or enhancement (actinin, occludin) of cytoskeletal and tight junctional protein phosphorylation [37].

The mechanisms involved in probiotics ameliorating or preventing respiratory infections is unknown, but presumably involves a systemic effect following ingestion of the organisms. No data exists on interference with tuberculosis, apart from perhaps reducing side effects of its treatment [38]. The evidence for effects against viral and bacterial infections is somewhat circumstantial, with reduced duration [39] or occurrences reported in a portion of subjects who have ingested various probiotic products during winter [40, 41]. A Russian study proposed that orally ingested *B. forte* functioned in the respiratory tract by enhancing the induction of alpha- and gamma-interferon and decreasing the production of serum interferon [42].

The benefits found for the urogenital tract can be explained, in part at least, by emergence of probiotic strains from the rectal area and their passive

transfer to the perineum and vagina, several centimetres away [43, 44]. The presence of these lactobacilli leads to acid and often hydrogen peroxide, bacteriocin and biosurfactant production, which helps displace pathogens and create a normal, healthy environment [11]. Use of milk to deliver *Lactobacillus* strains GR-1 and RC-14, has been shown to be effective [45]. Another mechanism could be the ability to reduce the pathogen load from the rectum to the vagina, or through signaling factors that reduce the virulence of some organisms [46].

Immune modulatory effects in the vagina and bladder are also likely induced by oral intake of probiotics, as noted from studies of patients with bladder cancer, in which recurrences were reduced by regular intake of *L. casei* Shirota in a sweetened skim milk based drink [47]. The mechanism was not investigated in this cancer study, but it seems reasonable to assume that it is due to either an immune adjuvant type effect that is elicited via the mucosal system from gut to bladder, or that the recurrences are caused by events occurring in the gut (for example nitrosamine production by bacteria) which are reduced by interference of the *L. casei*. Clearly, more studies are needed.

6.3.3 Improve the efficacy and compliance of anti-retrovirals

Highly active antiretroviral therapy (HAART) is the current mainstay of management of HIV/AIDS patients. These comprise a variety of drugs, mostly protease inhibitors and nucleoside reverse transcriptase inhibitors (NRTI) and non-NRTI (NNRTI). The increase in HIV reverse transcriptase or protease activity, through genetic mutations in the virus, continues to be a concern [48]. Additional problems arise when combination therapy is needed, such as for the treatment of tuberculosis, which often requires a four-drug initial phase (two months of rifampicin, isoniazid, pyrazinamide, and ethambutol) and a two-drug continuation phase (either four months of rifampicin and isoniazid or six months of isoniazid and ethambutol). HIV increases case fatality rates, and rates of recurrent tuberculosis after completion of therapy [49]. The NNRTIs and protease inhibitors are metabolised mainly through cytochrome P450 enzymes. Rifampicin induces CYP450, leading to a reduction in the plasma concentration of nevirapine by 30% and efavirenz by 20–25%. Increasing the dose of nevirapine to compensate for this interaction thereby increases the risk of toxic side-effects. One solution is to stagger the antibiotic and antiviral therapies. However, the concept of using one treatment to augment the activity of the other is worthy of consideration.

The apparent ability of probiotic *L. rhamnosus* GR-1 and *L. reuteri* RC-14 s to augment the activity of metronidazole [50] provides one example. The former might function by disrupting the pathogens, making it easier for the antibiotic to function effectively. In the case of HIV and tuberculosis or other pathogenic organism, the targets and location of infections differ, making it a challenge to combine the activities of the drugs. Recently, a viral fusion

inhibitor, enfuvirtide, was shown to increase antiviral activity of a combination of lopinavir/ritonavir, efavirenz, lamivudine and tenofovir in HIV-infected patients [51]. In the *Lactobacillus* GG study of HIV patients receiving antiretroviral therapy, the combination was safe [31], but the study did not examine any improved antiviral effects. The rationale for adding probiotics to the treatment of tuberculosis patients on HAART therapy is that long-term chemotherapy of tuberculosis leads to dysbacteriosis of the large intestine, that significantly decreases tolerance of tuberculosis drugs, provokes persistence of tuberculosis intoxication and retards involution of tuberculosis process in the lungs. Use of probiotics has been shown to alleviate this problem [38].

Another approach would be to use a probiotic organism as the delivery system for antiretroviral therapy. This is being investigated by at least three laboratories. The first published report came from Chang *et al.* [52] in which a human vaginal isolate of *L. jensenii* was engineered to secrete two-domain CD4 proteins which recognized a conformation-dependent anti-CD4 antibody and bound HIV type 1 gp120. The idea was to have the virus bind to the lactobacilli rather than the host. One criticism is that if the virus is not killed and the lactobacilli colonize the host, then the therapy may actually deliver the virus to the host. In the second reported study, a *Lactococcus* organism, not known for its ability to colonize the vagina, was bioengineered to secrete a prototypic virucidal compound cyanovirin, with a view to killing any approaching viruses [53]. Assuming this approach receives approval for human studies, it could be effective until the virus mutates to become resistant to this drug. In a multi-treatment approach, four HIV entry or fusion inhibitors, CD4D1D2-IgG2HC and CD4D1D2-IgKLC, MIP-1beta and T-1249 were fused to native *L. reuteri* RC-14 secretion signals of BspA, Mlp or Sep, or to full-length BspA, and the integration vectors were stably inserted into the *L. reuteri* chromosome [54]. As this strain is known to survive in the vagina, it is feasible that it could be used in place of toxic antiretrovirals. Whether or not any of these approaches can reduce viral shedding or transmission to sexual partners, remains to be seen. In a study of cervicovaginal lavage from 406 women, the HIV load was found to be lower in women with a normal lactobacilli dominated vagina [55].

Prebiotics are defined as 'a non-digestible substance that provides a beneficial physiological effect on the host by selectively stimulating the favorable growth or activity of a limited number of indigenous bacteria' [56]. At present, there are no studies showing effects of prebiotics on HIV/AIDS. The ability to modulate the intestinal microbiota could help to prevent or alleviate diarrhea, and as these compounds, including inulin and fructooligosaccharides can be included in dairy products, studies could be carried out on HIV/AIDS subjects without too much difficulty.

6.4 The challenge of delivering probiotics in dairy formulations

Probiotics are most often incorporated in yogurt and fermented milk, but other food lines are being developed and numerous products are sold in tablet, capsule, and powder forms. In the parts of the world where HIV/AIDS is most prevalent, namely Africa and Asia, probiotic products are not widely available. However, many communities use fermentation as a means of food preparation, for example in Africa, where raw milk, koko, bushera, cassava, and togwa are fermented; some of these have been reported to improve the nutritional quality, protein digestibility, and availability of amino acids [57]. Starter cultures differ amongst the regions. In India, lactococci and yeast are used to make yogurt [58], while in other countries, it is *L. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. None of these strains are true probiotics in the sense that they are designed to ferment the milk, not confer specific health benefits on the host. One study using Indian Dahi has shown a reduction in duration of diarrhea in children [57]. The problem with Dahi and yogurt is that there are variations in how the products are made, and in the starter cultures used, even if the species themselves are the same. It would be more reliable if a standard procedure was outlined and preferably each product formulation tested and shown to be probiotic. The argument has been made that *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* should be regarded as probiotic, as yogurt can stimulate immunity, as shown by increased expression of CD69 on T lymphocytes increased after yogurt consumption [59], and improve lactose digestion and symptoms of intolerance in lactose maldigesters [60]. But the evidence for the latter is scant and often undefined yogurts, or ones supplemented with *L. acidophilus* have been tested [61] without a positive outcome [62]. Therefore, a carte blanche definition of yogurt and its effects as a probiotic cannot be made, albeit this view is not shared by everyone [63].

Dairy formulations, such as Actimel drink (*L. casei* DN-114 001, Danone, France) Activia (*B. lactis*, Danone), Yakult (*L. casei* Shirota, Japan), LC1 (*L. johnsonii* LJ1, Nestle, Switzerland), Proviva (*L. plantarum* 299v, Probi, Sweden), Reuterin (*L. reuteri* ATCC 55734, BioGaia, Sweden) and Gelfilus (*L. rhamnosus* GG, Valio, Finland) are mostly not yet available in the developing world. The necessity for refrigeration for transportation, point of sales and in homes, makes it difficult for companies to enter these regions, especially when most of the population cannot afford 'Western' prices. Outsourcing the production of dairy probiotic products brings with it stringent requirements for standard operating procedures, good manufacturing practices, and other procedural stipulations that may or may not be commonplace in, for example, sub-Saharan Africa. In addition, legislation has to be in place to protect product formulations or enforce patents, to prevent copycat products being made. Thus, most of the companies named above, would insist upon setting up and controlling their own production facilities, and without an appropriate

return on investment, they may not do so. Still, companies can provide assistance in other ways, such as importing proven probiotic organisms/products that can be used in locally made foods, or sold as dried products that retain shelf-life under the climatic conditions. Governments too need to become involved, by fast tracking clinically proven probiotics, allowing importation tax breaks, funding local research on probiotics, and setting up mechanisms to monitor safety and impact on consumers, plus invest in local companies wishing to enter this market.

6.5 Western Heads East – a tangible example of what is possible

A receptivity survey for probiotic products among pre-menopausal female students in an African university, showed that 82% of participants stated they would welcome probiotic products in capsular form for vaginal instillation or as daily oral form to improve vaginal health [64]. Over one third of the women indicated they would use probiotic products as part of their daily self-care and 39% said they were willing to buy probiotic products for vaginal instillation, while 82% preferred yogurt or milk-based food forms of the products. This showed that lack of progress was not due to a different viewpoint on the merits of probiotics, but rather more likely a result of lack of knowledge and availability of products.

In an effort to try and acquaint local people whose lives are made more difficult by malnutrition and HIV/AIDS, scientists, social scientists, staff and students at the University of Western Ontario (UWO) established a 'Western Heads East' program (www.westernheadseast.ca), designed to set up a local probiotic yogurt facility in Mwanza, Tanzania. Through various grass roots fund raising and volunteering, the program has now sent nine student interns for 3–6 month spells to a rural community, where they have established a family kitchen, community centre and sustainable farming plot, at which local women produce probiotic yogurt. In collaboration with Kivilini Women's Group, the Tanzanian Medical Research Centre, and Kenya Medical Research Institute, the project is now in its third year. Probiotic yogurt was produced and local women have been taught how to perform the manufacturing and quality control steps. Local researchers, including health officials, are setting up projects designed to examine if and how the probiotics alters disease parameters ranging from diarrheal episodes to HIV spread in women.

To date, this project has impacted the lives of over 150 people through intake of the yogurt. It has been awarded the 2006 AUCC Scotiabank Prize for Excellence in Internationalization, and has been funded by the Canada Corps. Cows have been purchased and processes are underway to ensure sustainability and longevity of the project. The long-term goal is to

re-introduce an important natural health concept, train young people new skills (food science, microbiology, business, social science, etc.) and empower local women.

6.6 Future trends

The probiotic market is growing each year at double figures. In addition, scientific studies are being published on the breadth and scope of probiotic usage. Studies with recombinant organisms are showing great promise in delivering therapies to prevent and treat HIV/AIDS, but more urgently proven products are needed for use, and for study in countries ravaged by this disease. The opportunity exists for probiotics strains, including those delivered in foods, to help reduce the risk of HIV and to augment the management of people with AIDS (Table 6.1).

Less data have been obtained on prebiotics, but the ability to influence the human microbiota and immune system through natural ingredients, remains a worthy goal. It is impossible for pharmaceutical remedies to reach all the people around the world who suffer from HIV/AIDS. This is particularly the case with communities that are poor or lie outside the main medical care areas. The hope is that grass roots initiatives, like Western Heads East, can stimulate dissemination of the importance of the microbiotia for health, leading to new foods and supplements that enhance human and animal health.

Table 6.1 Potential for probiotics to prevent HIV and augment the care of AIDS subjects

Use of probiotics to potentially reduce the risk of HIV infection acquired through heterosexual contact	Treating HIV positive subjects with probiotics to alleviate symptoms or improve quality of life
Lactobacilli to restore and maintain a healthy vagina microbiota, and eradicate bacterial vaginosis [65]	Prevention or adjunct treatment of diarrhea and provide nutrition [27]
Lactobacilli to induce a cytotoxic T cell response to kill the virus [66]	Increase or maintain a stable CD4+ count to help host defenses against infection [27]
Recombinant lactobacilli and lactococci delivering microbicides and vaccines to prevent HIV infection [52, 53, 54]	Reduce the side effects of antiretroviral and antituberculosis treatment
	Augment the use of antibiotics to eradicate infection

6.7 Sources of further information and advice

The information contained in this article was obtained from PubMed searches (<http://www.ncbi.nlm.nih.gov>), manuscripts known to the author, and websites.

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7

Dairy products and oral health

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7.1 Introduction

Good oral health is essential to an individual's overall well being. The ability to communicate, smile, chew, and eat are essential in our daily lives. Inability to do any of these leads to lowered self-esteem and could ultimately lead to death by malnutrition. Oral diseases affect a significant proportion of the world's population and are costly to the human race in terms of morbidity and mortality (Petersen, 2003). Conditions classified as oral diseases include dental caries, periodontal diseases, dental erosion, tooth loss, dental fluorosis, oral mucosal lesions and cancers, and noma. The World Health Organization reports that despite great improvements in the oral health status of populations around the world, problems still persist (Petersen, 2003). Oral diseases are the fourth most expensive ailment to treat in the industrialized nations, and cost unspecified amounts for lost time at school and work. The prevalence and costs of oral disease are likely to grow in developing countries as they transition to unhealthy diets high in sugars and begin using more tobacco. Furthermore as the global population ages, older people have very different oral health needs compared with children and young adults. The implications to dental costs of this particular population shift are not estimated.

Dental caries and periodontal disease are the most common and important global oral health problems. Despite major investments in preventative procedures and educational activities by oral health professionals the problems still persist. Nearly 100% of the global adult population and 60–90% of school-aged children are affected by dental caries (Petersen *et al.*, 2005). Overall, improvements in developed countries have reduced incidence of dental caries and periodontal disease, but lower income and socially

disadvantaged groups experience a disproportionately higher level of these diseases than others. For example, although overall severity of dental caries in Europe is fairly low, Eastern Europe has a much higher severity rate than the average.

Milk is symbolized as being essential for energy and nutrition of newborn animals and humans that ultimately leads to survival. This biological fluid is now recognized as having additional importance than just as a nutritive source as more biological activities are identified. Proteins, peptides, and other milk components have bioactivities that influence multiple physiological processes throughout the body, and in particular the oral cavity, including mineral metabolism, bacterial inhibition, cell proliferation, blood clotting, and antioxidation. In fact, milk and dairy products reduce the incidence and lessen the severity of some oral health problems. The objectives of this chapter are to provide a brief overview of common oral diseases with an intensive focus on cariogenicity, demonstrate the role of milk and dairy products in preventing dental caries, focus on particular bioactive fractions in reducing incidence of oral diseases, and present examples of good research reduced to practice by product commercialization.

7.2 Oral diseases and cariogenicity

Oral refers to the mouth, and includes the teeth and gums (gingival) and their supporting tissues, the hard and soft palate, the mucosal lining of the mouth and throat, the lips, salivary glands, chewing muscles, and upper and lower jaw bones. Digestion begins in the oral cavity, and there are numerous supporting structures for the mouth including the nervous, vascular, and immune systems. Humans contract oral diseases for a number of reasons including genetics, poor hygiene, poor nutrition, alcohol and tobacco use, drug abuse (Shaner *et al.*, 2006), and complications from other diseases such as diabetes (Sandberg *et al.*, 2000, Twetman *et al.*, 2002), cancer (Woo *et al.*, 1993), obesity (Ritchie and Kinane, 2003), and osteoporosis (Norlen *et al.*, 1993). Oral infections themselves may play a role in progressing pathogenesis of many systemic diseases in healthy individuals, ill patients, and those immunocompromised (Ridker *et al.*, 1998). The theory is that oral infections, specifically periodontitis, elicit a hyper-inflammatory response. Indirect damage to the heart is caused by a release of inflammatory mediators eliciting different host-related reactions including release of C-reactive protein. This section reviews the two most common oral diseases – dental caries and periodontitis.

7.2.1 Dental caries

Dental caries has caused a great deal of discomfort and pain for the majority of mankind. This ubiquitous disease is usually not a fatal condition but has extolled tremendous costs to its victims including monetary and personal

appearance. Untreated dental caries leads to periodontal disease, tooth loss, and potentially jawbone deterioration. Patients that ignore their dental cavities present symptoms of having difficulty to eat, swallow, speak, and possess a different social personality.

Human teeth are highly vascularized, calcified structures coated with a biofilm of indigenous and endogenous microorganisms (Fig. 7.1). This very nutritional substrate becomes the primary focus for dental caries. Caries lesions result from interactions of odontopathogenic bacteria that colonize the tooth surface (Fig. 7.2). In Fig. 7.2 oral microbial flora embedded in the plaque biofilm utilize dietary sugars to produce mutans and organic acids. The organic acids demineralize calcium and other cations from the tooth's hydroxyapatite crystals. The body counteracts demineralization by the salivary protein statherin binding calcium to remineralize the tooth's surface. Dental caries worsen if odontopathogenic bacteria overcome the body's ability to remineralize the tooth. Dairy proteins, especially caseinophosphopeptides, have a role similar to statherin. Severe periodontitis, an anaerobic infection in the gingival crevice and crevicular fluid, elicits an inflammatory reaction in gum tissue. Interaction with dietary constituents, especially sugar, enables the bacteria to form a plaque layer on the tooth's enamel surface. This microecological niche is colonized by millions of bacteria that secrete glycosyltransferases to metabolize carbohydrates consumed by the host and trapped in plaque matrix (Fig. 7.3). Odontopathogenic bacteria secrete glucans as constituents of their outer layer and this contributes to plaque build-up. The plaque layer reportedly contains over 20% carbohydrate by dry weight (Bowen *et al.*, 1977).

The function of bacteria in causing dental caries is a source of continual controversy. The debate has been whether a specific bacterial species or a

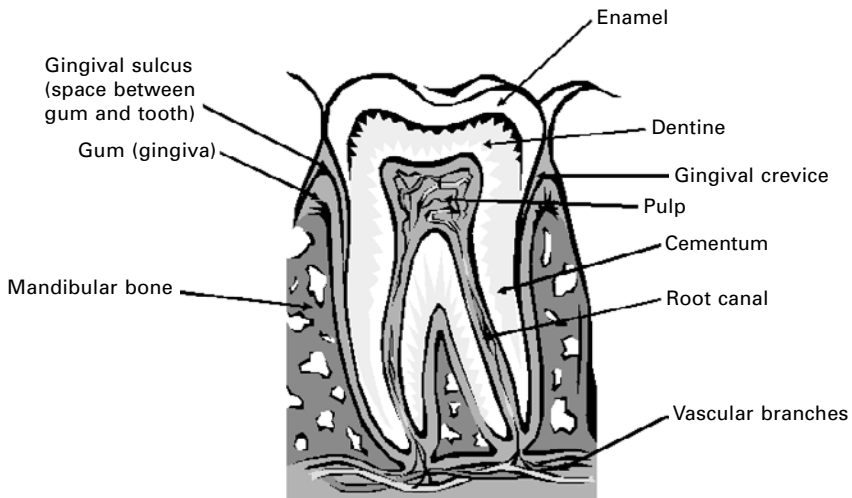


Fig. 7.1 Tooth anatomy.

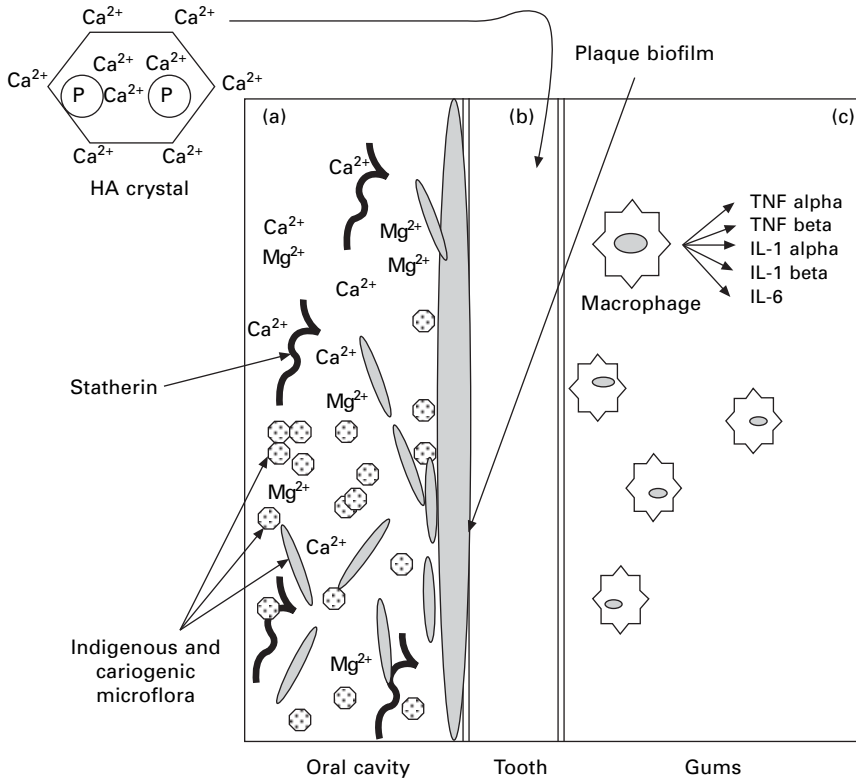


Fig. 7.2 Exaggerated representation of the oral environment illustrating dental caries mechanisms.

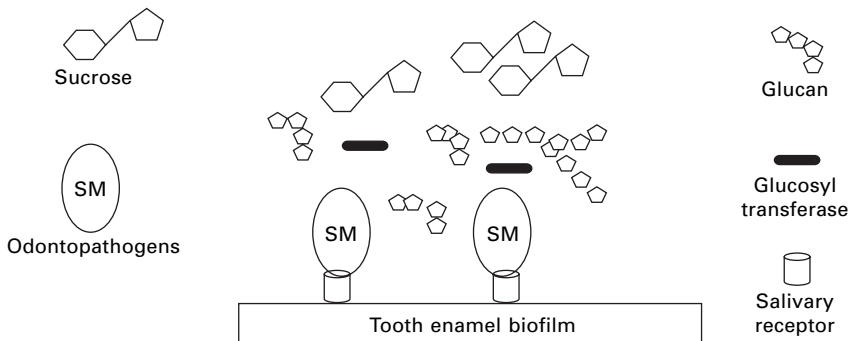


Fig. 7.3 Dental plaque formation by mutans streptococci (SM). Dietary sugars are transported into SM by glucosyl transferases to be metabolized and excreted as polysaccharides (mutans). The plaque layer thickens as mutans accumulate on the tooth surface.

non-specific mixed bacterial flora is the agent responsible. Also debated is whether dental caries is an infectious bacterial disease in the classical sense or an ecological overgrowth (Kleinberg, 2002). Frequent isolation and identification of *Lactobacillus acidophilus* and *Streptococcus mutans* with caries activity gave credibility to them being specific cariogens. There does not seem to be as much question whether these bacteria are responsible for dental plaque formation. When genes responsible for glucan production by mutans streptococci are deleted, the organisms lose their virulence to induce dental caries in experimental animals (Yamashita *et al.*, 1993). However, many other indigenous oral bacteria are capable of producing substantial amounts of organic acid from fermentable carbohydrates providing arguments for non-specificity. Numerous studies have shown some indigenous bacteria are capable of remineralizing tooth enamel to prevent dental caries.

Dental cavity formation results from a complex series of interactions occurring on the tooth enamel surface inside of the plaque biofilm. Generally, cariogenic bacteria produce organic acids that demineralize the calcified surface (Fig. 7.4). Once cavitation has begun, the tooth is continually under siege due to the different metabolic rates of cariogenic bacteria. Organic acid penetrates through the plaque biofilm to the tooth's enamel surface and begins diffusing into hydroxyapatite through water-filled interprismatic spaces. Loss of apatite crystals in the enamel is demineralization. The first visible change in tooth enamel is a translucent zone, and represents approximately 1 to 2% mineral loss from the enamel (Fig. 7.5). Tooth cavity formation is still reversible at this stage by calcium (or other minerals) and phosphate diffusing into the subsurface lesion and remineralizing the tooth. If further demineralization occurs to approximately 25% of a lesion in the enamel a visible cavitation occurs. Robinson *et al.* (2000) present a thorough review on enamel cavitation and dental caries.

Although dental sealants have reduced dental caries incidence in children, tooth decay affects more than 20% of American children aged 2 to 4 years, 50% of those aged 6 to 8, and nearly 60% of those aged 15 years old

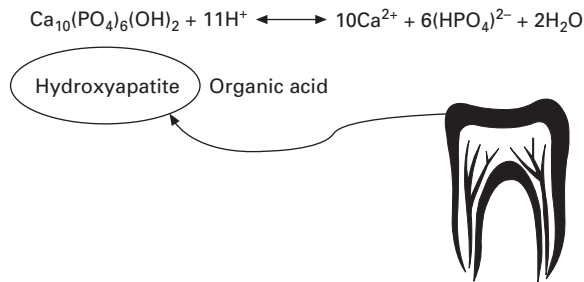


Fig. 7.4 The chemical reaction of dental caries formation. Hydroxyapatite is hydrolyzed from the tooth's enamel by organic acids secreted from mutans streptococci and other cariogenic bacteria to solubilize calcium and phosphate.

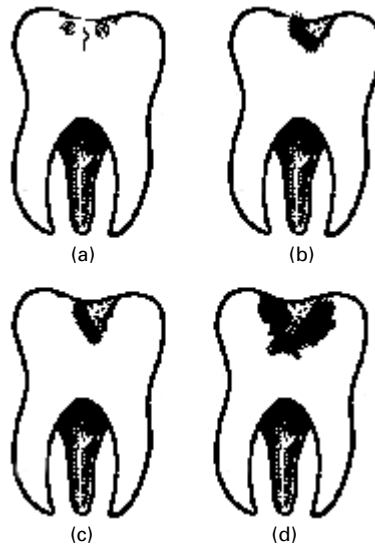


Fig. 7.5 Stages of dental caries formation. Caries usually begin in pits and crevices in the tooth enamel surface (a). This stage is known as the initial surface zone. Odontopathogenic bacteria colonize in crevices and pits to begin demineralizing the tooth. A loss of approximately 1% mineral (usually as magnesium and carbonate) occurs during stage the translucent zone (b). Enamel erosion progresses at an irregular pace as the body attempts to counteract demineralization using its own defenses (immune response and statherin), dietary factors (e.g., milk and other dairy products), and oral hygiene (various dentifrices and other products) to remineralize the carious lesion. In the positively birefringent zone (c) calcium phosphates are precipitated deeper in the tooth often reaching into the dentin layer. Surface remineralization can still occur if the salivary pellicle is saturated with minerals (especially calcium and fluoride), but the tooth has lost approximately 5–10% of mineral at this stage, and cavity formation may not be reversible. Visible cavitation (d) occurs when demineralization exceeds 25%, and is referred to as the body of the lesion zone.

(Carmona, 2005). Children's teeth become infected with potential odontopathogenic bacteria between the middle of the second year and the end of the third year of life – the 'window of infectivity' (Caufield *et al.*, 1993). The primary infection source for infants is maternal, but certain environmental conditions, such as infants born into a high caries-prone population, can also favor non-familial infection (Mattos-Graner *et al.*, 2001). Children not infected by a high maternal dose by three years of age remain minimally colonized by odontopathogenic bacteria until secondary teeth eruption.

7.2.2 Periodontal disease

The gingival sulcus environment provides a selective habitat for establishment of a mixed, predominantly anaerobic, microflora. Periodontal disease is a

chronic infection of tissues (gums) supporting the teeth. The infectious vector for chronic periodontitis is predominantly opportunistic Gram-negative anaerobic bacteria that colonize in crevices below the gum line. Specifically, the etiology in adults is caused by *Bacteroides forsythus* and *Porphyromonas gingivalis*. Juvenile patients are infected by *Actinobacillus actinomycetemcomitans* that results in a localized, aggressive periodontitis (Moore and Moore, 1994). The organisms utilize a variety of virulence factors to colonize and cause periodontium infection. Although periodontitis etiology is bacterial, the pathogenesis of periodontal disease is progressed by host response associated with increased production of reactive oxygen species and other inflammatory factors to anaerobic bacteria in gingival spaces below the teeth (Moore and Moore, 1994). If these reactive oxygen species are not buffered, damage to the host cells and tissues occurs (Moynihan, 2005). For more information on periodontal inflammatory response consult Van Dyke and Serhan (2003).

Periodontitis is diagnosed by observing loss of gum attachment to teeth and probing periodontal pocket depth along the tooth and gum line. The prevalence and severity of periodontal disease in different age groups have been measured in several global populations. Although periodontal disease is observed in all populations in varying degrees, severity is 10 to 20% higher in industrialized nations especially America (Petersen and Ogawa, 2005). Periodontal disease prevalence and severity is higher in older age groups as compared to younger age groups (Petersen, 2003). The disease is also more prevalent in lower socio-economic classes. Poor oral hygiene is a high risk factor for disease progression, but other risk factors contribute to severe periodontal disease including tobacco use, excessive alcohol consumption, drug abuse, stress, malnutrition, and diabetes mellitus (Genco *et al.*, 1999, Nishida *et al.*, 2000, Shaner *et al.*, 2006, Tezal *et al.*, 2001). Al-Zahrani (2006) utilized data collected from the Third National Health and Nutrition Examination Survey (NHANES III) to correlate dairy product intake with reduced periodontitis prevalence. Individuals in the highest quintile of dairy product consumption were 20% less likely to have periodontitis.

Diabetes patients exhibit a very high prevalence of periodontal symptoms, and the disease is much more aggressive (Grossi *et al.*, 1996). Periodontitis is recognized as the sixth complication of diabetes. If a diabetic does not maintain very high oral hygiene and consistently regular visits to their dentist, the disease will rapidly deteriorate the gum structure resulting in tooth loss and an impaired quality of life.

7.3 The role of dairy products in preventing dental caries

Milk is a biological fluid providing significant nutrients, immunological protection, and biologically active peptides to both infants and adults (Clare and Swaisgood, 2000). Nutritionists have consistently recommended

consumption of several servings of milk and other dairy products on a daily basis for all age groups. Despite this recommendation, fluid milk consumption globally has declined over the past decade. However, consumers have continued to ingest large amounts of dairy products including cheese and yogurt as an alternative supplement to the nutritional benefits of milk. Milk and dairy products are an important source of calcium to attain proper bone development and maintenance of bone mineral density. Likewise, milk and dairy products serve as a source of calcium for tooth development and mineralization (Wise *et al.*, 2002).

An individual's dietary and social patterns are major contributors to one's oral health. The quality of life can be greatly impacted as a result of poor oral health leaving a negative impact on self-esteem, eating ability, and social functioning (Moynihan, 2005). Several oral diseases can be linked back to poor nutrition, and as teeth deteriorate the conditions are exacerbated. Studies (Johansson *et al.*, 1994, Norlen *et al.*, 1993) have shown edentulous individuals are more apt to have inadequate dietary intake (high carbohydrate, high fat, low nutrient density foods) than dentate individuals. Sugars, specifically sucrose, are recognized as being a major contributor to dental caries' etiology. Other social factors such as alcohol and tobacco use, drug abuse, poor hygiene, and poor nutrition are also cited as being major contributory factors to oral diseases.

Saliva secretions supersaturated with calcium phosphate continually moisturize the tooth surface. Saliva serves as a reservoir of minerals (Fig. 7.2) to replenish tooth enamel hydroxyapatite after demineralization by plaque bacteria fermenting dietary sugars to organic acids. If there is a disruption in the calcium and phosphate balance, or other modulators of mineralization are deficient or missing, carious lesions in tooth enamel begin forming eventually leading to dental caries if untreated.

Most foods are noncariogenic, and do not contribute to oral diseases. However, some foods have anticariogenic properties that prevent tooth decay and other oral diseases. Although milk contains the sugar lactose, there is significant research to indicate milk and associated dairy products are anticariogenic. The high buffering capacity of milk is a contributing factor to pH control in the mouth after milk consumption (Mor and McDougall, 1977). Lactose is less fermented by indigenous oral microflora than sucrose only lowering the biofilm microenvironmental pH to around 6.0 compared to pH 5.0 with sucrose (Rugg-Gunn *et al.*, 1985). Most anticaries properties are attributed to presence of calcium, phosphate, and casein, and their modulation of tooth enamel mineralization.

7.3.1 Fluid milk as an anticariogenic food

Shaw *et al.* (1959) first identified milk as an anticariogenic food in 1959 when they reported a reduction in dental caries incidence in rats fed milk and flavored milks. Rat diets were supplemented with milk, chocolate drink,

chocolate milk, and a shake-like mixture that contained milk or chocolate milk plus vanilla ice cream. In addition, they also included a group that consumed cheese. All of the groups with milk (except the chocolate drink) demonstrated caries reduction with the cheese variable having best results.

Weiss and Bibby (1966) utilized an *in vitro* test with extracted teeth and with similar test variables (milk and flavored-milk) to show tooth enamel demineralization was reduced when teeth were exposed to cariogenic substances in an acetate buffer (pH 4.0) system. They concluded casein proteins were rapidly absorbed onto the tooth enamel surface and provide resistance to acid demineralization. Concurrently, Jenkins and Ferguson (1966) studied the effects of milk on acid production in human plaque. Subjects refrained from tooth brushing for three days to allow plaque accumulation on their teeth. Subsequently, milk was orally rinsed, and a microelectrode was used to measure the plaque pH. Milk showed a minimal plaque pH drop. The authors concluded the extent and duration of the pH neutralization would be non-cariogenic compared to a cariogenic positive control (sucrose solution). Bibby *et al.* (1982) studied how several dairy products and foods impacted acid production and enamel demineralization in an *in vitro* oral model (Orofax). Human milk was later shown to reduce demineralization and raise plaque pH in a manner similar to bovine milk (Rugg-Gunn *et al.*, 1985). The authors also identified casein as an important component to buffer plaque environmental pH, by showing cheese consumption after drinking sweetened (sucrose) milk minimized pH decline observed with control variables.

The role of milk minerals was also investigated about this same time (Harper *et al.*, 1986). Casein-free milk mineral concentrates were prepared from whey, and tested in rat models to study enamel mineralization and demineralization culminating in dental caries formation. The mineral concentrates contained varying levels of calcium, phosphate, and some residual whey proteins, and were used to study anticariogenicity in rats fed a high (20%) sucrose diet. Although all mineral concentrate diets studied reduced buccal caries, the concentrate with highest calcium (22.4 g/100 g) and phosphate (38.3 g/100 g) was most effective in anticariogenicity of smooth surface caries formation. An explanation for these results was not given.

A disturbing trend is occurring in global beverage consumption. Many consumers have stopped drinking milk as a predominant beverage to consuming soft drinks and other beverages highly sweetened with sucrose and other sweeteners. Sadly, the trend is occurring in young children as well as adults. Data analyzed from the National Health and Nutrition Examination Survey I (NHANES) found caries incidence was positively associated with elevated consumption of soft drinks in 9 to 29 year olds (Ismail *et al.*, 1984). Marshall *et al.* (2003) reported an increased incidence rate for dental caries in young children (4 to 7 years old) when they consumed higher than median intakes of soft drinks, powdered beverages (sweetened with sugar), and to some extent fruit juice during their 'window of infectivity' (2 to 5 years old). They also showed this age group to have inadequate intakes of other nutrients

during the same time period (2 to 5 years old). Consumption of fluid milk higher than the observed median had a neutral association with dental caries in these children. A similar study done by Levy *et al.* (2003) showed in 5 year old children there was a negative association of dental caries with bovine milk consumption from age 24 to 36 months. However, bovine milk and sugared beverage consumption from 6 weeks to 12 months were positively associated with dental caries in 5 year olds. Levine (2001) reported dairy beverages containing less than 5% added sugars have a negligible or low cariogenic potential in older children (> 16 years old).

Marketers in recent years have encouraged increased milk consumption by offering more flavored milks. Many flavored milks are sweetened with fermentable sugars including sucrose and high fructose corn syrup. Non-nutritive (minimal caloric contribution to the diet) sweeteners are an alternative means to sweeten dairy beverages. Polyols are very effective non-nutritive sweetening agents, and some have shown effectiveness in preventing dental caries. Castillo *et al.* (2005) studied acceptance of milks sweetened with xylitol or sorbitol by Peruvian children aged 4 to 7 years old. Xylitol (0.042 g/ml) was preferred most, followed by sorbitol (0.042 g/ml), xylitol (0.021 g/ml), and plain milk.

Jensen *et al.* (2000) studied the role of between meal snacks by adults on tooth enamel mineralization and demineralization. Enamel demineralization and caries progression was observed when apple juice, a cola soft drink, and sweetened (sucrose) yogurt were consumed as snacks. Dairy products were also studied, and found to remineralize tooth enamel after consumption. Cheddar cheese was the most effective dairy product to remineralize tooth enamel, but whole milk was most effective at changing the mineral content of dentin lesions.

7.3.2 Cheese as anticariogenic food

As mentioned several times in the previous section, cheese is identified as a potent anticariogenic food. Cheese is a concentrated source of casein proteins, milk fat, calcium, phosphorus, and other minerals. In addition, cheese will contain bioactive peptides released from the casein primary amino acid sequences by enzymatic and microbial digestion as cheese ages.

König (1966) was the first to report cheese anticariogenic properties from a study in rats being fed a highly cariogenic diet. Animals fed Emmental cheese developed fewer and less severe caries than the control group. Rugg-Gunn *et al.* (1975) fed humans cheese after a very sugary snack and observed a blunted plaque pH decline. Edgar *et al.* (1982) conducted similar experiments and reported cheddar cheese stimulated saliva flow, and reduced the number of smooth surface (sides of teeth) caries. Morrissey *et al.* (1984) also conducted similar experiments with aged cheddar cheese and reported few smooth surface caries, but an intermediate number of sulcal caries. Rosen *et al.* (1984) observed cheddar cheese was cariostatic in rats when fed in conjunction

with sucrose. The mechanism of action was not a direct antimicrobial effect on potentially odontopathogenic bacteria. Jensen *et al.* (1982) studied cheese maturity as a factor in pH buffering. They found aged cheeses (Cheddar, Gouda, blue, Monterey Jack, mozzarella, and Swiss) allowed no to slight pH declines after a meal, whereas young cheeses (Cheddar, cream, feta, and provolone) gave pH minima lower than 5.0.

Most authors from these studies proposed the following mechanism of action. After ingestion, cheese forms a film over the outer tooth surface within the biofilm, and there is resultant saliva flow stimulation. As pH declines after eating, cheese buffers the acidic environment and there is less demineralization. However, Krobicka *et al.* (1987) fed cheddar cheese to desalivated rats and observed fewer and less severe caries lesions in animals ingesting a cariogenic diet. Therefore, the presence of calcium, phosphate, and possibly bioactive peptides must have a direct role in demineralization and remineralization (Moynihan, 2000).

7.3.3 Dairy proteins as anticariogens

Early researchers observed that dairy products (milk, casein, caseinates, and cheeses) have anti-caries activity (Schweigert *et al.*, 1946b, Shaw, 1950). Several studies proved casein was an effective anticariogenic substance, but casein's adverse organoleptic properties and the large amount required for efficacy disqualified its use as a food or toothpaste. Acid casein as an active ingredient in toothpaste was effective at reducing dental caries, but was required at very high levels for efficacy (Bavetta and McClure, 1957, Schweigert *et al.*, 1946a). Sodium caseinate solublized in water and fed to rats in a caries model was shown to be anticariogenic (Reynolds and Del Rio, 1984). Sodium caseinate as an ingredient in a chocolate confectionary reduced cariogenicity, but high levels of caseinate (17%) were required to demonstrate an effect and the product was unpalatable (Reynolds and Black, 1987, 1989).

Tryptic digestion of caseinate enhances the proteins' ability to modulate enamel mineralization in a human oral caries model (Reynolds, 1987). Analysis of human dental plaque samples found elevated concentrations of casein peptides, calcium, and phosphorus. It was concluded these peptides were caseinophosphopeptides (CPP) derived from specific tryptic activity on α_{s1} -, α_{s2} -, and β -caseins. This prompted investigators to focus on casein peptides in subsequent research.

Dental caries prevention by milk-derived bioactive peptides is a complex physical and chemical sequence of cascading events. In general, bioactive peptides with anticariogenic activity have multiple functions to prevent dental lesions including bacterial inhibition; competitive exclusion to enamel binding sites, improved buffering capacity in the pellicle surrounding teeth, reduced enamel demineralization, and enamel remineralization. The interaction with salivary secretions is not well understood, but appears to be important for some of these prophylactic events.

Caseinophosphopeptides

Peptides identified to sequester calcium and other minerals, hence acting as biocarriers, are called phosphopeptides. The term was first given to casein-derived phosphorylated peptides that enhance vitamin D-independent bone calcification in rachitic infants (Mellander, 1950). Embedded within casein primary sequences are motifs of phosphopeptides that sequester calcium and other minerals (Fig. 7.6). Milk micelles contain physiologically significant amounts of calcium and phosphorus. The vast majority of bovine caseins exist in a phosphorylated form, with individual variants possessing as little as one phosphorylated residue (κ -casein) and as much as 13 for others (α_{s2} -casein). Phosphorus in milk is bound via monoester linkages to casein serine residues. The calcium and inorganic phosphate residues associated with bovine milk caseins are greater than expected from the physico-chemical solubility of calcium phosphate in milk (Kitts, 2005). The presence of phosphorus and calcium bound to casein helps to maintain thermodynamically stable casein micelles in fluid milk.

Tryptic digestion of caseins either *in vitro* or *in vivo* yields phosphoseryl peptides that sequester divalent metal ions (Kitts and Yuan, 1992). Caseinophosphopeptides (CPPs) can be found in the stomach and duodenum after milk ingestion (Chabance *et al.*, 1998). Casein phosphopeptides released from casein molecules are resistant to further proteolytic breakdown in the intestinal tract. Further evidence for intact passage through the upper gastrointestinal tract to the distal ileum was confirmed by identifying CPPs in ileostomy fluid (Meisel *et al.*, 2003). In addition to *in vivo* proteolysis of

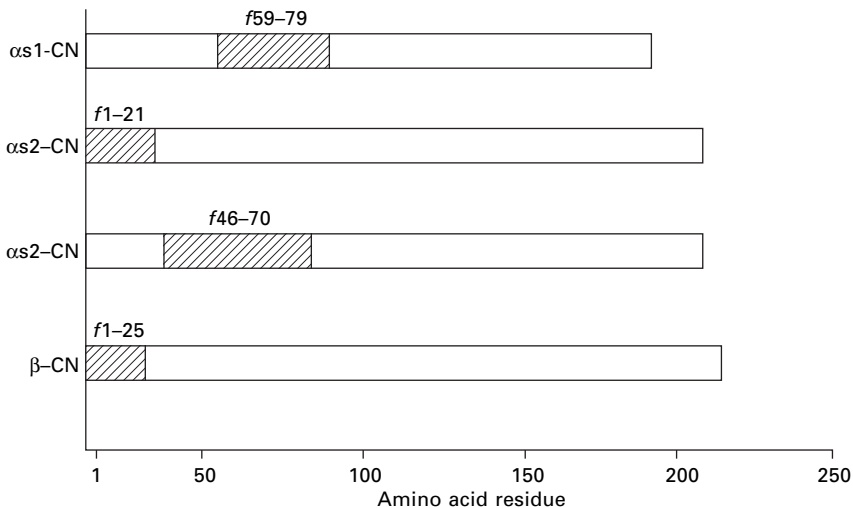


Fig. 7.6 Position of caseinophosphopeptide motifs embedded in major casein primary sequences. The motifs contain a common sequence within them of SerP-Serp-SerP-Glu-Glu.

milk casein to release CPPs, lactic acid bacteria possess proteolytic enzymes that release CPPs during cheese ripening. CPPs are found as natural constituents in Comté, Cheddar, and Grana Padano cheeses (Pellegrino *et al.*, 1997, Roudot-Algaron *et al.*, 1994, Singh *et al.*, 1997).

A major physiological role for CPPs is their ability to sequester minerals. The phosphate residues, corresponding to about 30% of the phosphorus content in milk, are present as monoesters of serine and occur mainly as clusters in the primary sequence of caseins, especially α_{s1} -, α_{s2} -, and β -caseins (Silva and Malcata, 2005). Of note, it is interesting most CPPs have a common sequence of three phosphoserine residues followed by two glutamic acid residues (SerP-SerP-SerP-Glu-Glu) (Meisel, 1997). This high concentration of negative charges is responsible for CPPs' resistance to further digestion in the digestive tract (Clare and Swaisgood, 2000, Reynolds *et al.*, 1994). Furthermore, phosphate groups and negatively charged side chains are binding sites for divalent minerals such as calcium, magnesium, and iron (Meisel, 1998), and renders them more bioavailable (Hansen *et al.*, 1996, Peres *et al.*, 1999). This mineral binding ability is important for CPPs' role in tooth mineralization.

The antioxidant potential of proteins derived from milk and dairy products is known (Allen and Wrieden, 1982). Casein has antioxidant activity at concentrations comparable to bovine fluid milk sources, but whey is not as effective at similar concentrations (Allen and Wrieden, 1982). Antioxidant activity of milk proteins is due to sequestering of iron and copper metals by phosphoserine residues located on the casein micelle surface. Another possible mechanism is that whey proteins donate hydrogen to reduce free radicals (Colbert and Decker, 1991), and free sulfhydryl groups from cysteine are effective at inhibiting lipid auto-oxidation (Taylor and Richardson, 1980a, 1980b). Buttermilk powder was shown to have antioxidant properties in a model lipid emulsion system (Wong and Kitts, 2003). A stronger affinity was noted for ferrous than ferric ions.

Likewise, the ability to sequester divalent cations also presents an opportunity for CPPs to act as antioxidants (Diaz *et al.*, 2003). Activities of CPP and less defined casein hydrolysates have antioxidant properties in a muscle food system (Diaz and Decker, 2004). Another research group reported CPP antioxidant activity in aqueous and emulsion model systems (Taylor and Richardson, 1980a). Antioxidation activity may also have a physiological role in humans in reducing inflammatory agents produced in response to reactive oxygen species. Calcium enriched CPP stimulates release of the anti-inflammatory, interleukin-6 (IL-6) in a human gastrointestinal cell culture line (Kitts and Nakamura, 2006). This CPP bioactivity may be important in reducing inflammatory response elicited during periodontal disease.

Demineralization and remineralization modulation

Phosphoserine peptides execute important functions at the organic-inorganic interface in the bioprocesses of biomineralization and calcium phosphate

stabilization for bones and teeth. Disruption of biomineralization processes leads to consequences in bone and tooth hypomineralization or hypermineralization. Insights into molecular dynamics of these processes are beginning to assist scientists and clinicians in understanding how to design products that lead to applications in the biomineralization arena to repair damaged teeth and bones.

Caseinophosphopeptides released from casein tryptic digests account for approximately 10% by weight of the total casein protein. The major tryptic CPP are released from β -casein (*f* 1–25), α_{s1} -casein (*f* 59–79), and α_{s2} -casein (*f* 1–21 and *f* 46–70) (Fig. 7.6). All of these peptides contain three continuous phosphoserine residues capable of sequestering their own equal weight in amorphous calcium phosphate and forming colloidal nanocomplexes (Cross *et al.*, 2005). The amount of calcium bound and calcium phosphate stabilized is influenced by peptide net charge, length, primary sequence, and peptide conformational folds and shapes. In general, longer peptides bind more calcium. In the presence of phosphate ions, CPP binds additional calcium above and beyond that bound in the absence of phosphate. This reaction appears to be pH dependent, and more calcium and phosphate are bound as pH increases (Cross *et al.*, 2005). More detailed information is written on the molecular conformation changes and stabilization of calcium and phosphate by CPP (Cross *et al.*, 2006, Cross *et al.*, 2004, Laila *et al.*, 2005).

Tryptic digests of casein significantly reduced enamel subsurface demineralization in a human *in situ* caries model (Reynolds, 1987). In that study, the authors speculated CPP was incorporated into dental plaque, and serves several purposes in anticariogenicity including a role in mineralization, buffering acids produced by plaque bacteria, and amino acid transport. In a subsequent study, CPP purified from a tryptic digest of sodium caseinate was used to confirm utility of CPP in mineralization of the tooth's surface of rats (Reynolds *et al.*, 1995). A dephosphorylated peptide with the same amino acid sequence as CPP showed no anticariogenicity. Results from this study also indicated specific amino acid residues and/or conformational specificity, such as in the longer α_{s1} - and β -peptides, are required to increase the level of mineralization (Wikiel *et al.*, 1994). Further studies in humans using *in situ* caries models (Reynolds, 1995, Reynolds, 1997) showed CPP was effective in reducing frequency and severity of dental caries lesions. The mechanism proposed is CPP stabilizes calcium phosphate as amorphous calcium phosphate (ACP). The multiple phosphorylserine residues in CPP bind ACP to form size-limiting nanocomplexes in a metastable solution. In turn, the nanoclusters' growth is controlled to a critical size required for nucleation and phase transformation at the enamel surface. Enamel subsurface caries lesions are also remineralized by CPP-ACP nanocomplexes (Reynolds *et al.*, 1999).

The bioactivity of CPP can be compared to the saliva protein statherin. Milk casein and statherin have been mapped to the q arm of human chromosome 4 (Laila *et al.*, 2005), and it is speculated the salivary protein ancestral gene evolved from the caseins (Kawasaki and Weiss, 2003). Both proteins control

crystal nucleation and growth of hydroxyapatite in the tooth microenvironment (Stayton *et al.*, 2003). Secondary sequence predictions of the N-terminus of statherin suggest α -helix formation (Gururaja and Levine, 1996). However, NMR spectral data for α_{s1} -casein (*f* 59–79) CPP does not indicate either α -helical or β -strand conformation (Cross *et al.*, 2004). Despite this difference, both peptides adopt conformations that allow specific amino acid sequences to interact with calcium ions to control nucleation and/or biomineral growth. In the case of CPP, glutamyl and phosphoserine side chains form nanocomplexes with calcium (Cross *et al.*, 2004), whereas statherin structures calcium on the phosphorylated serines and carboxylate-containing aspartic and glutamic acid residues (Stayton *et al.*, 2003).

Whey proteins from cottage cheese manufacture or acid casein precipitation were less effective than CPP in precluding Ca and phosphate solubilization from hydroxyapatite (Warner *et al.*, 2001). Proteose peptone fractions 3 and 5 were shown to inhibit hydroxyapatite demineralization *in vitro* (Grenby *et al.*, 2001). Both proteose peptone fractions are embedded within β -casein and are liberated by plasmin hydrolysis (Eigel and Keenan, 1979, Eigel, 1981). Plasmin is an endogenous milk protease. Proteose peptone fractions would elute with whey proteins after casein insolubilization. Although whey proteins may not prevent enamel demineralization, it has been suggested whey may exert a topical anticariogenic effect by acting as a buffer (Reynolds and Del Rio, 1984).

Several patents have been issued to produce and utilize CPP as anticariogenic compounds (Han and Shin, 1998, Reynolds, 1991, 2002, 2004). Others have utilized this technology to develop commercial products for prophylactic treatment of dental caries. For example, Reynolds *et al.* (1992) patented a dentifrice composition (toothpaste) containing CPP to prevent demineralization that leads to gingivitis and dental caries. Later, Reynolds (1993) was issued a United States patent for specific CPP cation complexes that when formulated into mouthwash, toothpaste, lozenge, tablet, foodstuff, beverage, or other pharmaceutical compositions incorporates into plaque. Once CPP-cations are incorporated, they are resistant to endogenous phosphatase or peptidase activity and inhibit calculus activity. Reynolds (1999) extended this technology further by incorporating a phosphatase or peptidase inhibitor into dentifrice formulations containing CPP. Caseinate calcium fluorophosphates combined with CPP were patented (Bannister, 2003) for use in confectionary-like food forms to prevent gingivitis and halitosis. Sodium bicarbonate can be incorporated into chewing gums with CPP-AMP to provide dental health benefits (Luo and Wong, 2005). Dixon and Kaminski (2005) were awarded a patent for creating stable oral compositions containing CPP-AMP nanocomplexes in combination with a fluoride ion source, and a calcium chelator. Han (2006) invented an interproximal toothbrush coated with CPP that is recoated after each use by storing the brush in an accompanying storage and sealing unit.

Experimental evidence is available for efficacy of these patented

formulations. For example, Shen *et al.* (2001) reported a sugar-free gum containing CPP-AMP nanocomplexes remineralized enamel subsurface lesions in a human *in situ* model system. Reynolds *et al.* (2003) reported chewing gum containing CPP-ACP produced higher levels of enamel remineralization than other calcium sources. In a follow-up study (Iijima *et al.*, 2004), they demonstrated tooth enamel remineralized by CPP-AMP was more resistant to an acid challenge than control (chewing gum without CPP-AMP) remineralized enamel. A mouthwash that contained CPP-ACP significantly increased plaque calcium and inorganic phosphate levels. Sugar-free lozenges are a suitable composition to deliver CPP-ACP and promote enamel remineralization (Cai *et al.*, 2003). They observed a dose-related response in a human *in situ* model system. Glass-ionomer cement (GIC) is used to chemically adhere restorations to tooth tissues. The incorporation of CPP-ACP into GIC allows slow release of ions into dentin to provide further protection during acid challenges without any negative effects to compressive strength, net setting time, or microtensile bond strength to the dentin (Mazzaoui *et al.*, 2003). Lennon *et al.* (2006) reported amine fluoride gel (12,500 ppm fluoride) was more effective at protecting enamel demineralization after acid challenge than CPP-AMP, NaF (250 ppm), or a combination of CPP-AMP and NaF.

Control of odontopathogenic bacteria

Tooth surfaces are colonized by a variety of bacterial species that hydrolyze dietary carbohydrates to organic acids (Figs 7.2 and 7.3). This commensal relationship establishes a complex microbial ecosystem composed of aerobic, microaerophilic, and anaerobic microorganisms. These bacteria are integral components of plaque. Some of them are opportunistic cariogenic pathogens that demineralize tooth surfaces and form lesions that develop into dental caries. Plaque composition is a complex mixture of viable and dead bacterial cells, carbohydrate glucans, proteins, and a variety of minerals (Margolis and Moreno, 1994).

In the oral microbial ecosystem, bacteria must adhere to a surface for colonization. There are over 400 different indigenous microbial species in the human oral cavity (Moore and Moore, 1994). Different bacterial species colonize the tooth's surface than found in the gingival crevice. Tooth surface bacteria interact with saliva, host diet constituents, and growth factors to colonize. Bacteria usually identified on the tooth surface are *Streptococcus mutans*, *S. sanguis*, *S. sobrinus*, *S. gordonii*, and *S. oralis*. This bacterial group is usually referred to as mutans streptococci because of their ability to secrete polysaccharides that add to the tooth's biofilm layer. Researchers frequently isolate *Lactobacillus acidophilus*, *L. gasseri*, and *L. fermentum* from teeth of healthy individuals, and predominantly *L. plantarum* from periodontal patients (Koll-Klais *et al.*, 2005).

Dental caries etiology is quite complex and still being extensively studied. Although plaque is an important component in lesion development, other

factors such as the host's oral microflora and the microbial ecosystem in the lesion interact to progress decay (Figs 7.2 and 7.3). The organisms discussed above are frequently isolated from carious lesions. Furthermore, if genes responsible for mutans production are deleted from streptococci then lesions do not form in model systems. Therefore presence of these organisms in dental plaque indirectly indicates they could be an important factor in caries formation. Total elimination of mutans streptococci in the complex oral environment would not totally eliminate caries formation, but it would be at a significantly reduced level (Bowden, 2000).

Bacteria found in gingival crevicular fluid depend on environmental and host conditions to colonize. Most of these organisms are strict anaerobes, and capable of eliciting a host inflammatory response if their population becomes too large. Anaerobic bacteria such as *Porphyromonas gingivalis*, *spirochetes* (such as *Treponema species*), *Bacteroides*, *Campylobacter*, and *Fusobacterium* are frequently isolated from gingival crevicular space. These bacteria are opportunistic pathogens and will cause gingivitis that could lead to the more serious periodontitis.

Saliva is a multi-function biological fluid that controls and stabilizes the oral ecosystem. The primary composition of saliva is mucinous glycoproteins, which coats the teeth. This results in an acellular insoluble membranous layer referred to as the salivary pellicle. The pellicle is strongly adhered to the enamel surface and has numerous functions (Bowen and Li, 1997, Tabak and Bowen, 1989). Saliva also contains secondary components important for mineral modulation (statherin), immune response (immunoglobulins), food digestion (amylase), and plaque formation (glucosyltransferases) (Lijemark and Bloomquist, 1996). Saliva is not a separate oral microbial ecosystem. Instead, it acts as a diluent to deliver bacteria to most surfaces in the oral cavity from the tongue, dental plaque, mucosal surfaces, and ingested food.

Nesser *et al.* (1994) studied milk casein derivative's ability to inhibit odontopathogenic bacteria by preventing bacterial adhesion to a simulated tooth surface. Sodium caseinate, CPP, and glycomacropeptide (GMP) inhibited adherence of oral bacteria to saliva-coated hydroxyapatite beads (S-HA). The potential dental pathogens, *S. sobrinus* OMZ 176 and *S. sanguis* OMZ 9 were competitively excluded from S-HA beads by casein derivatives. However the more favorable oral organism, *Actinomyces viscosus* Ny 1, adhered to S-HA beads. In addition, caseinate, CPP, and GMP were able to bind directly to cell walls of examined cariogenic bacterial strains. Anticariogenicity by these proteins and peptides was accomplished by selectively inhibiting streptococcal adhesion to teeth. Furthermore, microbial composition of dental plaque was modulated to favor establishment of less cariogenic species such as oral actinomyces.

Milk and individual caseins (α -, β -, and κ -caseins) were studied to determine adherence of *S. mutans* to saliva-coated hydroxyapatite discs (sHA) (Vacca-Smith *et al.*, 1994). Milk inhibited *in vitro* adherence of *S. mutans* GS-5. Individual caseins were also examined. No effect on streptococcal adherence

was observed when α - or β -casein was incubated with sHA. However, κ -casein inhibited adherence of *S. mutans* GS-5. Inhibitory properties were credited to a 40,000 dalton glycoprotein.

Micellar casein was shown to prevent oral colonization in rats by *S. sobrinus* OMZ 176, and to promote colonization by *A. viscosus* Ny1 (Guggenheim *et al.*, 1999). Sodium caseinate was not as effective as micellar casein at inhibiting streptococcal colonization. Whey proteins and soy protein isolate had no effect on microbial colonization.

Inhibition of cariogenic bacterial adherence to the salivary pellicle would protect teeth from developing lesions. Casein peptides, GMP and CPP, were incubated with saliva coated bovine enamel discs to study incorporation into the pellicle (Schupbach *et al.*, 1996). Electron microscopy confirmed both proteins were incorporated into the pellicle in exchange for albumin. The effect of incorporated GMP or CPP on adherence of mutans streptococci was studied. Adherence of *S. sobrinus* was reduced 49%, 75%, and 81% by GMP, CPP, and GMP+CPP, respectively. Adherence of *S. mutans* was more efficient with reductions of 64%, 83%, and 84% by GMP, CPP, and GMP+CPP, respectively. This relatively selective inhibition of mutans streptococci could eventually produce a non-cariogenic plaque. Rose (2000) reported CPP-ACP has a greater affinity for calcium than *S. mutans* R9 cells. As CPP-ACP binds to plaque, it provides a large reserve of calcium for remineralization.

A similar study evaluated GMP and CPP as adhesion inhibitors of oral bacteria using saliva-coated hydroxyapatite beads (sHA) (Nesser *et al.*, 1994). Both casein peptides blocked adhesion of *S. sanguis* OMZ 9 and *S. sobrinus* OMZ 176 to sHA. Neither peptide inhibited adhesion of *A. viscosus* Ny 1 to sHA. *S. sanguis* OMZ 9 also attaches to human buccal epithelial cells (HBEC). However, adherence is inhibited in the presence of GMP (Nesser *et al.*, 1995). Desialylation of either GMP or HBEC was less effective at inhibiting adhesion of *S. sanguis* OMZ 9. These results indicate bacterial adhesion to oral epithelial cells is dependent upon sialic acid presence. This is in contrast to tooth enamel adhesion where ionic interactions are important for adherence (Nesser *et al.*, 1995). This exemplifies the multiple mechanisms odontopathogenic bacteria utilize to establish in the oral microecology.

Malkoski *et al.* (2001) have further fractionated GMP using reversed phase high performance liquid chromatography to identify the peptide fragment responsible for *S. mutans* growth inhibition. They found the active form to be a non-glycosylated, phosphorylated peptide in residues 138–158. They designated the antimicrobial as kappacin. The non-phosphorylated peptide did not possess inhibitory activity.

Lactoperoxidase in combination with hydrogen peroxide and thiocyanate ion is a powerful bacteriostatic system found in milk, tears, and saliva. Inhibition of mutans streptococci (serotypes *a* through *g*) metabolism was studied in a washed cell model system (Thomas *et al.*, 1983). Serotypes (BHT, FA-1, OMZ 176) that produced large amounts of hydrogen peroxide were greater than 90% inhibited. Intermediate hydrogen peroxide producers

(B-13 and Ingbritt) were only inhibited 20 to 50%. No inhibition was shown with low peroxide producing strains (AHT, HS-6, GS-5, LM-7, OMZ 175, and 6715-15). Similar effects were observed on the metabolism of *S. mitis*, *S. sanguis*, *S. salivarius*, and *S. mutans* grown under aerobic and anaerobic conditions. Glyceraldehyde 3-phosphate dehydrogenase, an important enzyme in glycolytic pathways, was inhibited by hypothiocyanite (Carlsson *et al.*, 1983). A commercially available toothpaste formulated with lactoperoxidase showed elevated levels of hypothiocyanous acid and hypothiocyanite ions in the saliva of patients (Lenander-Lumikari *et al.*, 1993). However, no effect was noted on the oral microflora after 30 days treatment.

Saliva, milk, and tears also contain the bactericidal enzyme lysozyme. Lysozyme hydrolyzes $\beta(1 \rightarrow 4)$ -glucosidic linkages in bacterial cell wall peptidoglycan to kill susceptible organisms. This enzyme also has several other defenses to inhibit oral pathogens; for example, bacterial autolysins are activated (Labile and Germaine, 1985), bacterial cells are aggregated (Pollock *et al.*, 1987), and adherence (Iacono *et al.*, 1980) and metabolism (Lumikari and Tenovuo, 1991) are inhibited. Synergistic effects of lysozyme and subsequent lactoperoxidase exposure results in total inhibition of glucose metabolism by mutans streptococci (Lenander-Lumikari *et al.*, 1992).

Bovine milk lactoferrin inhibits saliva-induced *S. mutans* aggregation (Mitoma *et al.*, 2001). Amino acid residues 473 to 538 of lactoferrin are important for inhibition. Oho *et al.* (2002) subsequently reported lactoferrin peptide (f 473–538) inhibited adherence of *S. mutans* to sHA beads. Other milk proteins were also tested in this study. Lactoperoxidase and immunoglobulin G showed moderate inhibitory activity. Individual intact casein and whey proteins showed minimal inhibitory activity.

Several patents have been issued utilizing much of the technology discussed above. Micellar casein incorporated into an edible composition inhibits oral colonization of *S. sobrinus* (Berrocal *et al.*, 1995). Another patent (Berrocal *et al.*, 1998) protects a process to prepare micellar casein with 100 ppm fluoride for use in dentifrices. Two patents (Nesser, 1991a, 1991b) were issued to utilize intact and desialylated GMP in compositions for inhibiting buccal cavity bacteria. Gelatin combined with GMP and a surfactant (sodium lauryl sulfate) was formulated into a toothpaste or chewing gum (Zhang and Gaffar, 1998b). A patent (Zhang and Gaffar, 1998a) discloses a multi-component oral hygiene product for enhanced remineralization that contains GMP in one component and a fluoride ion in a second component. Xylitol and GMP combined in a composition remineralize teeth (Zhang and Gaffar, 2001). A couple of inventions claim periodontitis prevention by reduction of dental plaque and calculus deposition by using GMP and lactoferrin (Braun and Nimmagudda, 2002a and 2002b). Both patents show data for growth inhibition of *Actinobacillus haemophilus* and *S. pyogenes*. Valenti and Antonini (1998) formulated a topical protein preparation containing lysozyme. The composition is recommended for therapy of periodontitis and diseases of the teeth and oral cavity.

7.3.4 Effect of dairy components on other oral diseases

Oral mucositis is inflammation of oral mucosa from chemotherapeutic agents or ionizing radiation (Sonis, 1998). Oral mucositis typically manifests as erythema or ulcerations. This condition is sometimes called stomatitis, and is a major dose-limiting side effect of chemotherapy. Severity of oral mucositis is partially determined by epithelial cell turnover (Sonis and Sonis, 1979). Older patients (>60 years old) have a lower incidence of oral mucositis than pediatric patients (Sonis, 1998).

Clarke *et al.* (2002) reported a biologically active extract containing bovine whey proteins prevented oral mucositis in hamsters undergoing chemotherapy. They termed this extract whey growth factor extract – A (WGFE-A), and demonstrated the preparation contained mitogens that stimulate cells of mesenchymal origin and inhibit epithelial cell growth in culture (Belford *et al.*, 1997). The bioactive properties of WGFE-A include anti-proliferative, anti-apoptotic, and antimicrobial.

Naturally occurring growth factors contained in whey were formulated into a mouthwash (PV701) and tested in human patients undergoing chemotherapy (Prince *et al.*, 2005). Growth factors previously identified from whey preparations include insulin-like growth factor, platelet-derived growth factor, transforming growth factor β , fibroblast growth factor, and epidermal growth factor (Belford *et al.*, 1997, Rogers *et al.*, 1996). In addition, other bioactive compounds were reportedly present with bacteriostatic properties that would inhibit opportunistic infectious agents. PV701 mouthwash was used six times per day by patients with lymphoma starting six days before chemotherapy began, and continuing for five days after completion. Incidence and severity of oral mucositis was reduced in patients receiving PV701 compared to a placebo (Prince *et al.*, 2005). No adverse effects were reported with PV701 in this clinical trial.

7.3.5 Fermented foods and probiotics anticariogenicity

The word ‘probiotic’ is translated from the Greek language and means ‘for life’. A more modern definition for probiotics is living organisms that upon ingestion in certain numbers exert health benefits beyond basic nutrition (Aimutis, 2002). Many others have defined probiotics as live microbial food supplements that beneficially affect the host by improving its intestinal microbial balance. Thus they create a healthy gut environment. Most human probiotic products contain species of lactic acid bacteria, especially species of *Lactobacillus* or *Bifidobacterium*. Probiotics are beneficial to human health by demonstrating such diverse activities as regulation of the intestinal microbial balance, improvement of stool consistency, pathogen antagonism to prevent diarrhea, stimulation of immune response, and anti-cancer activity (Meurman, 2005, Sanders, 1999).

A randomized, double-blind, placebo-controlled intervention study was used to determine if milk containing *Lactobacillus rhamnosus* GG administered

to kindergarten children reduced their caries risk and initial caries development (Näse, 2001). Children consumed probiotic or normal milk 5 days a week for 7 months in their day-care facilities. Children consuming probiotic milk had less dental caries than the control group. In addition, the probiotic strains reduced numbers of mutans streptococci.

Cornelli *et al.* (2002) screened 23 dairy microorganisms for antagonistic behavior against cariogenic bacteria. They identified two *S. lactis* and two *Lactococcus lactis* strains that adhered to saliva-coated hydroxyapatite beads. *S. thermophilus* NCC 1561 and *L. lactis* ssp. *lactis* NCC2211 were incorporated into a biofilm mimicking dental plaque. *S. oralis* OMZ607, a cariogenic strain, was inhibited from growing in the plaque by *L. lactis* NCC2211.

Cheese containing *L. rhamnosus* GG and *L. rhamnosus* LC 705 was consumed by 74 subjects in a randomized, double-blind, controlled study with two parallel groups (Ahola *et al.*, 2002). Subjects ate 15 g of cheese five times a day preferably after meals or snacks. No statistically significant difference was observed between control and test groups in *S. mutans* counts after the intervention period, but during the post-treatment phase there was a significantly greater reduction in the intervention group compared to control. Mutans counts decreased 20% and yeast counts 27% in all subjects, regardless of treatment. This further confirms the beneficial effects of cheese to prevent dental caries.

Strains must be thoroughly screened for selection as a potential oral probiotic. Some species of lactobacilli are thought to be cariogenic. In a study by Matsumoto *et al.* (2005), *L. salivarius* LS1952R established itself in the oral cavity of rats and induced a significant level of dental caries. They confirmed *L. salivarius* could adhere to saliva-coated hydroxyapatite beads. Montalto *et al.* (2004) also showed patients receiving an oral administration of lactobacilli probiotic for 45 days had significantly higher lactobacilli counts in their saliva than placebo. *S. mutans* population was not significantly affected. Therefore, patients ingesting probiotic products should have their dental health closely monitored. *L. rhamnosus* GG reportedly does not colonize in the oral cavity (Yli-Knuuttila *et al.*, 2006), despite its benefits mentioned earlier.

7.4 Future trends

Dental caries continues to be a nuisance to most of the global population despite major advances in oral hygiene and treatment regimens. The mouth is not a sterile environment, but rather is colonized by bacterial populations as large as that found in other regions of the gastrointestinal tract. As such, there will most likely always be opportunistic pathogens lurking for a crack or crevice in a tooth that is not sufficiently remineralized to inflict their cariogenic assault. Separation technologies are making it possible and economical to isolate and purify bioactive components from milk. The use

of bioactive peptides and probiotics as prophylactic and therapeutic agents will require more well designed clinical trials to be conducted with a large number of patients. A thorough understanding of mechanisms of action validated by repeatable clinical trials will establish bioactives as natural and effective alternatives to chemical and pharmacological treatments.

7.5 Sources of further information and advice

Only a small fraction of the general literature on milk protein and peptide physical and physiological functionalities could be covered in the space available for this chapter. Furthermore, the literature is changing very rapidly as more is understood about milk proteins. The reader is advised to carefully monitor progress in journals dedicated to dairy research.

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Part II

Functional dairy ingredients

Dairy propionibacteria as probiotics

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8.1 Propionibacteria characteristics relevant to beneficial effects

8.1.1 General features of dairy propionibacteria

Propionibacteria were first described by E. von Freudenreich and S. Orla-Jensen at the end of the 19th century as microorganisms involved in the fermentation of lactate into acetate and propionate with the concomitant production of carbon dioxide during the ripening of Emmental cheese. The first pure culture was isolated from such cheese in 1906 (von Freudenreich and Orla-Jensen, 1906) and the genus *Propionibacterium* was proposed by Orla-Jensen because of this fermentation in 1909 (Orla-Jensen, 1909).

Propionibacteria are described as pleomorphic rods, 0.5 to 0.8 μm in diameter and 1 to 5 μm in length, often club-shaped, or coccoid, bifid or even branched. Cells occur singly or in characteristic arrangements in V, Y, or Chinese character configuration. The morphology also varies with the physiological stage and the environment, as illustrated in Fig. 8.1. They are non-motile and non-sporing bacteria, anaerobic to aerotolerant and generally catalase positive, which grow in the temperature range 15 to 40°C and in the pH range 5.1 to 8.5 with an optimum at 30°C and neutral pH. They are heterofermentative and metabolise different carbohydrates (including glucose, galactose, fructose and lactose), various alcohols (including glycerol) and organic acids (including pyruvate and lactate, the preferred substrate) to a mixture of propionate, acetate, succinate and carbon dioxide. This particular central carbon metabolic pathway, the propionic fermentation, involves the Wood-Werkman cycle (Wood, 1981) and requires a multimeric transcarboxylase (methyl malonyl CoA carboxyl transferase, EC 2.1.3.1). This enzyme catalyses

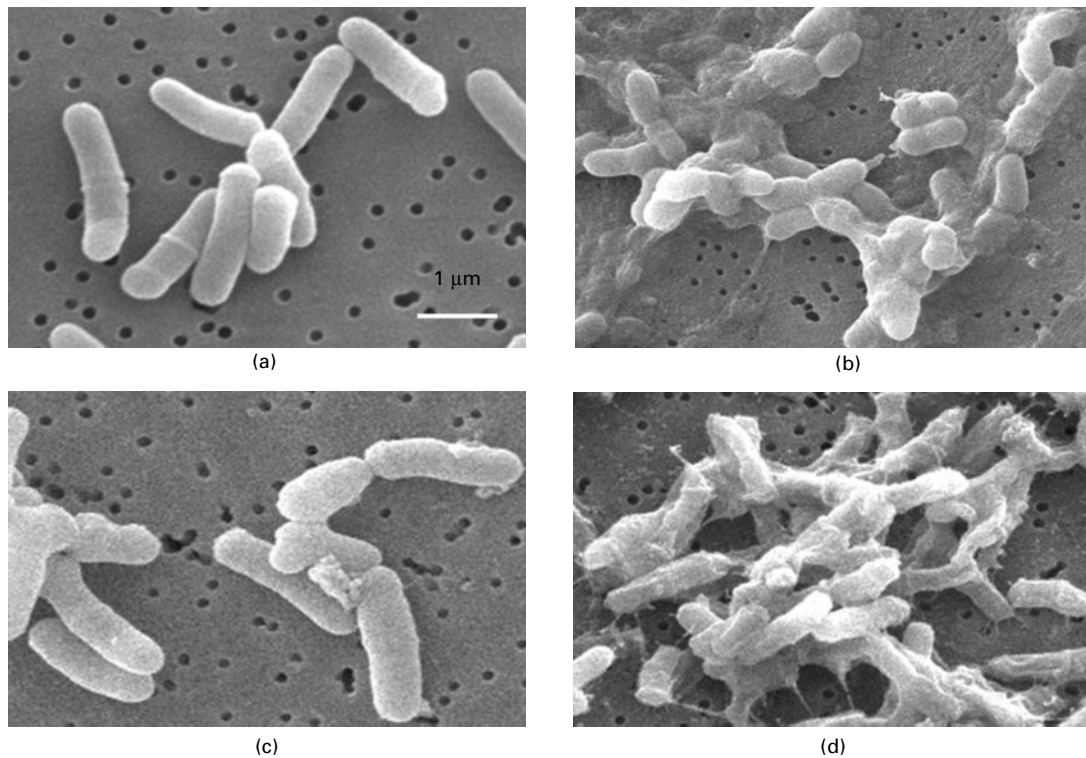


Fig. 8.1 Variable size and morphology of *P. freudenreichii*. Morphology is analysed using scanning electron microscopy for bacteria in exponential phase of growth (a), in stationary phase of growth (b), during acid adaptation at pH 5.0 (c) or bile salts adaptation (0.2 g.L⁻¹, 4 h, d).

the reversible transfer of a carboxyl group from methylmalonyl-CoA to pyruvate to form propionyl-CoA and oxaloacetate (second last step before propionate formation) (Deborde, 2002).

Propionibacteria are firmicutes with a high G+C content and are included in the *Actinomycetale* order. They are thus phylogenetically far from the low G+C firmicutes such as lactic acid bacteria but more related to corynebacteria and mycobacteria. The genus *Propionibacterium* comprises two distinct groups from different habitats (Cummins and Johnson, 1986). One group includes propionibacteria typically found on the skin and referred to as 'cutaneous propionibacteria'. These bacteria, previously described as anaerobic coryneforms, are involved in the pathology of acnes and may cause opportunistic infections. They are thus not considered for probiotic applications, although the immunomodulatory potential of the *P. granulosum* species may be of interest (Isenberg *et al.*, 1995). The other group contains strains isolated from cheese and dairy products, and is described as 'dairy propionibacteria' or 'classical propionibacteria'. This includes the species *P. freudenreichii*, *P. acidipropionici*, *P. jensenii*, *P. thoenii*, *P. microaerophilum* and *P. cyclohexanicum*. The species *P. acidipropionici* and *P. freudenreichii*, which is divided into two subspecies, *freudenreichii* and *shermanii*, are considered for probiotic applications.

Although dairy propionibacteria have been traditionally isolated from dairy products, their natural habitat is the digestive tract of ruminants (Jarvis *et al.*, 1998; Cheong and Brooker, 1999; Rinta-Koski *et al.*, 2001) and they are found in various environments such as soil, fodder, silage, various dairy or vegetable fermented products, dairy plants and also waste waters. Their main application is the ripening of Swiss type cheeses, characterised by round 'eyes' (Noël *et al.*, 1999), in which they are involved in the formation of the characteristic flavour and opening, via the fermentation of lactate to acetate, propionate and CO₂ (Langsrud and Reinbold, 1973b), but also the production of branched-chain fatty acids (Thierry *et al.*, 2002, 2004a, 2004b) and in lipolysis (Thierry *et al.*, 2005). However, their metabolic characteristics and the fact that their use in cheese has achieved a 'generally recognised as safe' (GRAS) status allows other applications mainly in the context of food preservation and health promotion, as developed below.

8.1.2 Production of antimicrobial compounds

The production of antimicrobial compounds by safe and food-grade bacteria traditionally used in food processing constitutes a promising alternative to the use of chemical food preservatives.

Propionic acid and its salts are widely used as antifungal agents in the industry. Food-grade propionate can be produced by propionic fermentation instead of chemical synthesis. The short chain fatty acids produced during food fermentation by dairy propionibacteria, mainly propionate, thus gained increased interest because of inhibitory effects towards undesirable

microorganisms. Propionibacterial cultures are also considered in the context of food protection as biopreservatives. A commercial product, Microgard™, consisting of a pasteurised skim milk fermented by *P. freudenreichii* subsp. *shermanii*, exhibits inhibitory activity associated with organic acids (Daeschel, 1989). It inhibits several Gram-negative bacteria (*Pseudomonas*, *Salmonella* and *Yersinia*) and several fungi, but not Gram-positive bacteria (Al-Zoreky *et al.*, 1991). Propionibacteria were effective at protecting fermented milks and bread sour dough against spoilage by yeast and moulds (Suomalainen and Mayra-Makinen, 1999). The amount of short chain fatty acids and the antifungal activity were shown to vary widely depending on the medium and on the species within propionibacteria (Lind *et al.*, 2005). Finally, co-cultures of lactic acid bacteria and propionibacteria were shown to be more effective in avoiding these food spoilages (Suomalainen and Mayra-Makinen, 1999; Schwenninger and Meile, 2004).

Undesirable flora can also be inhibited by bacteriocins. Bacteriocins are secreted bacterial peptides, which may be covalently modified and have an antimicrobial activity against bacteria closely related to the producer strain (Cotter *et al.*, 2005). However, bacteriocins produced by Gram positive bacteria may have broad spectra including both Gram positive and negative bacteria. Bacteriocins of propionibacteria were reviewed by Holo *et al.* in 2002 (Holo *et al.*, 2002). The first description of bacteriocin production by dairy propionibacteria deals with *P. thoenii* P127, which produces propionicin PLG-1 (Lyon and Glatz, 1991). It is active against *P. thoenii*, *P. jensenii* and *P. acidipropionici*, but not *P. freudenreichii*, and also against several lactic acid bacteria and Gram-negative bacteria, yeasts and moulds. *In vitro*, PLG-1 failed to inhibit growth of common food-borne pathogens of the genus *Bacillus*, *Staphylococcus*, *Clostridium*, *Yersinia* and *Salmonella*. However, it was active in fermented milk against psychrotrophic spoilage or pathogenic organisms including *Listeria monocytogenes*, *Pseudomonas fluorescens*, *Vibrio parahaemolyticus* and *Yersinia enterocolitica* (Lyon and Glatz, 1993; Lyon *et al.*, 1993). Jensenin G is produced by *P. jensenii* P126 (later reclassified as *P. thoenii*), it is active against propionibacteria, lactocci and lactobacilli, while no other bacteria, yeasts or moulds were shown to be inhibited (Grinstead and Barefoot, 1992). However, this bacteriocin was reported to inhibit the outgrowth of clostridial spores (Ekinci and Barefoot, 1999; Holo *et al.*, 2002). Thoeniicin 447, produced by *P. thoenii*, is active against *Propionibacterium acnes* (Van der Merwe *et al.*, 2004) and may thus be useful against this opportunistic pathogen.

A growing number of bacteriocins are still discovered in the different species of dairy propionibacteria (Brede *et al.*, 2004; Van der Merwe *et al.*, 2004). Broad screening of strains with appropriate indicators revealed the production of bacteriocin is widespread within dairy propionibacteria (Miescher, 1999). As an example, propionicins SM1 and SM2, firstly described by S. Miescher *et al.* in *P. jensenii* are encoded by a plasmid which was also detected in *P. acidipropionici* and *P. freudenreichii* (Rehberger and Glatz,

1990; Kiatpapan *et al.*, 2000). Only a limited number of propionibacteria bacteriocins have been fully sequenced and characterised. Moreover, other antimicrobial compounds with either wide or narrow spectra of inhibition still are to identify in propionibacteria (Holo *et al.*, 2002). This clearly opens new perspectives for the improvement of food quality and health.

8.1.3 Production of vitamins

The peculiar central carbon metabolism of propionibacteria is characterised by the transfer and rearrangement of C1-compounds. The Wood-Werkman cycle involves a multimeric transcarboxylase (EC 2.1.3.1) responsible for the formation of propionyl-CoA and oxaloacetate. These reactions are catalysed by enzymes with specific cofactors including vitamin B₁₂ (cobalamin), B₉ (folic acid) and biotin.

B₁₂ and B₉ are two distinct hydrosoluble vitamins, their main source are eggs and dairy products for B₁₂, and cereals and vegetables for B₉. They are both involved in haematopoiesis and in the biosynthesis of methionine, thymidine and purine. Deficiency in B vitamins causes anaemia due to decreased synthesis of nuclei basis and is also involved in neurodegenerative diseases. The daily recommended intake of B₁₂ is 2.4 µg for adults and 2.6 µg for pregnant women while that of B₉ is 300 µg for adults and 400 µg for pregnant women. Indeed, this last is known to prevent neural-tube defects during development.

Dairy propionibacteria have long been used for the industrial production of vitamin B₁₂ by fermentation. *Pseudomonas denitrificans* can also be used in that aim, but propionibacteria allow the production of food-grade vitamin, either in food during fermentation, or as a food additive (Hugenholtz *et al.*, 2002). The industrial production of B₁₂ by *P. freudenreichii* fermentation is a two-stage process which can lead to the production of 330g of B₁₂ from 1 kg of propionibacterial biomass (Deborde, 2002). B₉ is naturally present in milk, but its concentration can be enhanced in fermented milks. The yogurt starter *Streptococcus thermophilus* was shown to produce B₉ which is consumed by lactobacilli (Crittenden *et al.*, 2003). Different strains of dairy propionibacteria produce varying amounts of B₉, which can be higher than that produced by *S. thermophilus* (Hugenholtz *et al.*, 2002). Interestingly, an almost complete excretion of B₉ into the medium was observed for some strains.

8.1.4 Use in fermented food products

The main fermented food product containing propionibacteria is Swiss-type cheese. In addition to ripening, propionibacteria participate in microbiological safety and nutritional quality of cheeses. Propionibacteria being able to ferment a wide variety of substrates, they can acidify different non-dairy food products. Their antimicrobial properties afford increased shelf-life of the fermented products.

Babuchowski *et al.* reported the use of dairy propionibacteria in fermented vegetables such as sauerkraut, red beet juice and vegetable salads. This resulted in increases in B₉, B₁₂, propionic and acetic acid contents, inhibition of harmful and pathogenic microorganisms and the extension of the shelf-life of the products (Babuchowski *et al.*, 1999). This was later confirmed in a pilot study on the fermentation of root vegetables (mainly beetroots and turnips) (Jagerstad *et al.*, 2004).

Dairy propionibacteria were also used in fermented milks where they improve nutritional quality. Suomalainen and Mayra-Maliken reported the use of propionibacteria in conjunction with lactic acid bacteria to avoid spoilage by yeasts, moulds and *Bacillus* species in fermented milk and in bakery products (Suomalainen and Mayra-Makinen, 1999). In another study, *P. freudenreichii* NIZO B2336, which produces large amounts of riboflavin, was incorporated into yoghurts and ingested by rats with were fed a riboflavin-deficient diet (LeBlanc *et al.*, 2006). The resulting fermented milk had enhanced concentration of riboflavin and its ingestion suppressed the symptoms of ariboflavinosis in rats, while the conventional yoghurt without propionibacteria failed to have such health effect. Furthermore, *P. freudenreichii* was shown to convert *in vitro* free linoleic acid into conjugated linoleic acid, mainly the cis-9, trans-11 and trans-9, cis-11 octadecadienoic isomers (Rainio *et al.*, 2001; Jiang *et al.*, 1998b). In addition, *P. freudenreichii* was shown to convert free linoleic acid, added to skim milk by the incorporation of hydrolysed soil oil, during fermented milk production and storage (Xu *et al.*, 2005). The main isomer produced was rumenic acid, the cis-9, trans-11-octadecadienoic acid. Considering the potent beneficial effects of ruminic acid (Wahle *et al.*, 2004), in particular on carcinogenesis (Lavillonniere *et al.*, 2003; Lock *et al.*, 2004), this can constitute another health-promoting potential for dairy propionibacteria. However, enhancement of free rumenic acid in dairy products, as a result of probiotic growth, remains to be demonstrated. Indeed, the CLA content of dairy fat is already elevated and subjected to variations linked to cattle feed or breed (Sieber *et al.*, 2004). Further research in this field is thus necessary to evidence the positive role of probiotics, via CLA production, in dairy or non-dairy fermented products.

8.1.5 Recent use as probiotics

Most of the first publications on dairy propionibacteria dealt with their peculiar metabolism and their use in cheese technology. However, there is a growing interest on their use as probiotics. Indeed, considering that they benefit of the GRAS status in dairy products fermentation, that they adapt to different environments and substrates, and that they produce metabolites with potent health-promoting effects, it is tempting to speculate on their efficacy as probiotics. There is presently much more experimental data on the probiotic applications of bacteria belonging to the *Lactobacillus* and *Bifidobacterium* species than on propionibacteria. However, several reports suggest that they

may have a positive impact on the gut health, function and comfort. These promising characteristics are summarised in Table 8.1 and further described below.

8.2 Stress tolerance and survival

In their natural environments or during industrial processes, bacterial cells are often subjected to a variety of abiotic stresses. In order to survive, bacteria have developed a set of mechanisms leading to protection against severe injuries after an unfavourable environmental factor has been sensed. Stress adaptation implies the complex regulation of gene expression (Segal and Ron, 1998), including overexpression of well known heat shock proteins (HSPs), which seem to be well conserved within the prokaryotes, including actinomycetales, while the corresponding regulation mechanisms are less conserved (Servant and Mazodier, 2001). However, striking differences are observed between bacterial species, and even between strains of the same species, in terms of stress susceptibility (Anastasiou *et al.*, 2006). Propionibacteria are isolated from various environments such as soil, fodder, rumen, various cheeses and dairy plants. The drastic variations in physicochemical parameters between these different niches evidence the ability to adapt and survive unfavorable conditions in these non-sporulating firmicutes.

8.2.1 Technological stress adaptation

During technological processes, *P. freudenreichii* has to cope with injurious stresses linked to either starter production or fermentation process. In the manufacture of Swiss-type cheeses, *P. freudenreichii* copes with thermal treatment (52°C, 30 to 60 min), slightly acidic environment (down to pH 5.2, caused by the lactic acid bacteria starters) and saline stress caused by immersion (48 to 72 h) in saturated brine (Mocquot, 1979). Despite these technological stresses, propionibacteria grow in cheese to a final population around 10⁹ CFU/g, convert the lactic acid to propionic and acetic acids as well as CO₂, leading to the characteristic flavor and the opening of Swiss-type cheeses (Langsrud and Reinbold, 1973b). This suggests particularly efficient adaptive mechanisms in dairy propionibacteria towards variations in temperature, pH, water activity and redox potential.

P. acidipropionici was shown to grow in the absence of osmoprotectants in media containing up to 1.2 M NaCl, while other bacteria of technological interest (*Acetobacter aceti*, *Gluconobacter frateurii*) were totally inhibited by 0.4 M NaCl. Furthermore, the growth parameters were restored by the addition of the exogenous osmoprotectant glycine betaine which accumulation in *P. acidipropionici* increased with NaCl concentration (Kylma *et al.*, 2000). *P. freudenreichii* was inhibited by 0.7 M NaCl in a chemically defined medium,

Table 8.1 Useful characteristics for probiotic applications and beneficial effects reported for dairy propionibacteria

Characteristic/effect	Mechanisms described <i>in vitro</i>	Property demonstrated in animals	Property confirmed in humans
Digestive stress adaptation and survival in the gut	Efficient adaptive response to acid and bile in <i>P. freudenreichii</i> (Jan <i>et al.</i> , 2001; Leverrier <i>et al.</i> , 2003)	<i>P. jensenii</i> survival in rats (Huang <i>et al.</i> , 2003) <i>P. acidipropionici</i> survival in mice (Perez Chaia and Zarate, 2005)	<i>P. freudenreichii</i> survival in humans (Bougle <i>et al.</i> , 1999; Jan <i>et al.</i> , 2002b)
Modulation of the intestinal microbiota	Identification of bifidogenic compounds (Mori <i>et al.</i> , 1997; Isawa <i>et al.</i> , 2002) in <i>P. freudenreichii</i>	Modulation of mice intestinal microbiota by <i>P. acidipropionici</i> (Perez Chaia <i>et al.</i> , 1999)	Enhancement of the human colic bifidobacteria by <i>P. freudenreichii</i> (Satomi <i>et al.</i> , 1999; Hojo <i>et al.</i> , 2002; Bougle <i>et al.</i> , 1999)
Metabolic activity in digestive environment	Enhancement of <i>P. acidipropionici</i> β -galactosidase activity in the presence of bile (Zarate <i>et al.</i> , 2002a)	<i>P. acidipropionici</i> increase β -galactosidase activity and propionate concentration in mice caecum (Perez Chaia and Zarate, 2005)	Modulation of SCFA in human faeces by <i>P. freudenreichii</i> (Jan <i>et al.</i> , 2002b)
Modulation of intestinal motility and absorption	N.D.	Enhanced iron absorption from the rat colon in the presence of <i>P. freudenreichii</i> (Bougle <i>et al.</i> , 2002)	Constipation alleviation by <i>P. freudenreichii</i> (Hojo <i>et al.</i> , 2002)
Adhesion to digestive epithelial cells	Adhesion of <i>P. freudenreichii</i> and <i>P. acidipropionici</i> to cultured or ex vivo isolated intestinal cells (Tuomola <i>et al.</i> , 1999; Zarate <i>et al.</i> , 2002b)	Adhesion of <i>P. acidipropionici</i> to mice ileal epithelium (Zarate <i>et al.</i> , 2002b)	N.D.
Impact on intestinal inflammation	N.D.	Healing by <i>P. freudenreichii</i> and <i>P. acidipropionici</i> of TNBS-induced colitis in rats (Uchida and Mogami, 2005; Michel <i>et al.</i> , 2005)	N.D.

Table 8.1 Continued

Characteristic/effect	Mechanisms described <i>in vitro</i>	Property demonstrated in animals	Property confirmed in humans
Modulation of the immune system	N.D.	Stimulation of mice phagocytosis by <i>P. acidipropionici</i> and lymphocyte proliferation by <i>P. freudenreichii</i> (Perez Chaia <i>et al.</i> , 1995; Kirjavainen <i>et al.</i> , 1999)	N.D.
Impact on digestive cancer	Binding of genotoxics (Lee <i>et al.</i> , 2003), induction of apoptosis (Jan <i>et al.</i> , 2002a), antimutagenic properties (Vorobjeva <i>et al.</i> , 1995)	Decreased uptake of aflatoxin from the chicken intestine (El Nezami <i>et al.</i> , 2000) Decreased β -glucuronidase activity in mice caecum (Perez Chaia <i>et al.</i> , 1999)	N.D.

N.D.: not determined

but this inhibition was suppressed in rich medium or in the presence of exogenous osmoprotectants including glycine betaine, dimethylsulfonioacetate and dimethylsulfoniopropionate (Boyaval *et al.*, 1999). The major accumulated osmolytes are glutamate and trehalose, which *de novo* synthesis is enhanced by osmolarity, in the absence of osmoprotectants, while glycine betaine and proline are accumulated in rich medium (Rolin *et al.*, 1995; Cardoso *et al.*, 2004).

Thermal stress adaptation was also described in dairy propionibacteria (Leverrier *et al.*, 2004; Anastasiou *et al.*, 2006). Surprisingly, two distinct phenotypes were observed in strains of *P. freudenreichii* isolated from a Gruyère-type cheese (made without added propionic starters). A subset of these, as the CIP 103027 type strain, suffers a severe decrease in viability at 55°C, while this temperature has a very limited effect on viability in the other subset. A temperature above 60°C is necessary to kill these latter strains. Moreover, a mild thermal treatment (42°C) triggers a very efficient thermoprotection in the sensitive strains (Anastasiou *et al.*, 2006). The corresponding adaptive mechanisms were studied in *P. freudenreichii*. Thermal adaptation involves overexpression of classical heat shock proteins including chaperones and ATP-dependent proteases involved in protein turnover, Single-strand binding protein known to take part in SOS response, enzymes involved in the metabolism of cell wall and superoxide dismutase involved in reactive oxidative stress remediation (Leverrier *et al.*, 2004). In the thermotolerant strains, a distinct subset of proteins are overexpressed, whatever the temperature, in addition to HSPs. This includes enzymes involved in propionic fermentation, aminoacids metabolism, oxidative stress remediation and nucleotide phosphorylation (Anastasiou *et al.*, 2006). Involvement of guanosine pentaphosphate in *P. freudenreichii* thermoprotection suggests enhanced biosynthesis of (p)ppGpp, a stringent mediator that would protect cellular components from damages caused by heat stress. Interestingly, thermal adaptation in *P. freudenreichii* confers cross-protection towards acid and bile salts stresses, while osmotic adaptation does not (Leverrier *et al.*, 2003). Finally, it should be noted that the ability of dairy propionibacteria to adapt variations in temperature and water activity allows efficient drying and storage of powders of dried bacteria. Taken together, these data indicate that dairy propionibacteria possess diverse and efficient strain-dependent mechanisms allowing their adaptation to the main technological stresses. This is consistent with their occurrence in the stressing environment of fermented products and suggests that the incorporation of propionibacteria in various food vectors, fermented or not, will not be limited by survival during process and storage.

8.2.2 Digestive stress adaptation

Dairy propionibacteria are not considered as usual commensal inhabitants of the human digestive tract, by contrast with bifidobacteria. It should be noticed that cutaneous species of propionibacteria are frequently isolated from the

digestive tract of animals and humans (Salanitro *et al.*, 1977; Macfarlane *et al.*, 1986). However, dairy propionibacteria are natural inhabitants that make up 1.4% of the ruminal microflora and produce propionic acid in the rumen (Oshio *et al.*, 1987), a major precursor for glucose production through hepatic gluconeogenesis (Sauer *et al.*, 1989). Tolerance towards digestive stresses thus seems a characteristic of some species of dairy propionibacteria and strains isolated from the rumen were thus proposed as candidate probiotics (Rinta-Koski *et al.*, 2001).

Dairy propionibacteria were less frequently isolated from human faecal samples. In this case, their presence was attributed to the consumption of fermented products in which they are usually found. Accordingly, their constitutive tolerance towards the digestive stresses encountered in the digestive tract, including acidity and the presence of bile salts, is low. However, particularly efficient adaptive responses were evidenced in certain strains of *P. freudenreichii*, following moderate doses of stress. Hence, adapted propionibacteria are able to survive long exposure to pH values as low as 2 (Jan *et al.*, 2000, 2001) and to bile salts concentrations above those reported in the content of the human colon (Leverrier *et al.*, 2003). The mechanisms leading to stress adaptation were investigated using proteomics in *P. freudenreichii* (Leverrier *et al.*, 2004). Distinct mechanisms leading to stress tolerance were evidenced, they include overexpression of a particular subset of stress proteins. Acid tolerance response is triggered upon exposure to pH values between 3.5 and 6.0 with an optimal protection conferred between pH 4.0 and 5.0 (Jan *et al.*, 2000). It depends on the over-expression of a subset of inducible proteins, which include enzymes involved in DNA synthesis and repair, enzymes of the central carbon metabolism including the transcarboxylase cycle, specific to propionic fermentation in propionibacteria, proteins involved in polypeptide metabolism (ClpB, ClpC) and the universal chaperonins GroEL and GroES (Jan *et al.*, 2001; Leverrier *et al.*, 2004). Bile salts induced proteins involved in stress sensing and signal transduction, enzymes involved in oxidative stress remediation and detoxification (superoxide dismutase, cysteine synthase, ABC transporters) (Leverrier *et al.*, 2003, 2004). It should be noticed that for acid and bile salts stresses, the levels of both constitutive and acquired tolerances were highly variable among strains within the *P. freudenreichii* species.

8.2.3 Impact of probiotic vector on tolerance

For most of the claimed probiotic effects, the ingested microorganism has to reach the colon alive and in substantial amounts. Digestive stress survival is thus a limiting step and the product in which the probiotic is incorporated definitely impacts on its toughness. Probiotics may be incorporated in fermented milks, the most popular vector, in capsules or even in cheeses (Stanton *et al.*, 1998), ice creams (Hekmat and McMahon, 1992), powders and buttermilks (Rodas *et al.*, 2002). The impact of the probiotic vector on acid and bile salts

stress survival was thus investigated *in vitro* for *P. freudenreichii*. Acid stress tolerance is higher for propionibacteria included in cheese than for free cultured ones, at the same acidic challenge pH 2.0 (Jan *et al.*, 2000). The same strain was shown to exhibit a much higher tolerance towards acid challenge, bile salts challenge, or the succession thereof, after inclusion in different food matrices (Leverrier *et al.*, 2005). Significant protection was afforded by inclusion in alginate beads, while fermented milk was determined as the best probiotic vector to protect propionibacteria from these challenges. In addition to a buffering effect of the food matrix, the technological process may also induce the adaptive responses described above (see Section 8.2.2). Indeed, propionibacteria and lactic acid bacteria were shown to express elevated amounts of stress proteins during Emmental cheese ripening, suggesting induction of the corresponding stress responses (Gagnaire *et al.*, 2004).

8.2.4 Survival *in vivo*

The level of stress to which adapted propionibacteria can survive is elevated and this suggest that they can adapt and survive within the digestive tract. Indeed, *in vivo* studies confirmed this hypothesis.

P. jensenii 702 was administered to male Wistar rats ($n = 7$) under the form of propionibacterial cultures, washed and resuspended in drinking water at a daily dose of 5×10^{10} CFU/rat/day (Huang *et al.*, 2003). While no dairy propionibacteria were detected in the rats' faeces before providing the probiotic, their population reached 10^8 CFU/g after five weeks of treatment. No adverse effect on the rats' health (general health status, live weight gain, visceral organ size, and beta-glucuronidase) and no propionibacteria translocation occurred as a result of this treatment.

P. acidipropionici CRL1198 (10^9 CFU/day) was given to BALB/c mice ($n = 5$) under the form of propionibacterial cultures, washed and resuspended in sterile skimmed milk (Perez Chaia and Zarate, 2005). The population of viable propionibacteria, expressed in log CFU per gram, in the caecal content after seven days of treatment was 9.5 ± 0.7 . The most promising result, in this study, is that the presence of *P. acidipropionici* CRL1198 in the mouse caecum resulted in a higher beta-galactosidase activity and a higher production of propionate.

In a human study, *P. freudenreichii* SI 41 and SI 26 (5×10^{10} CFU of the two mixed freeze-dried strains per day) were given to male adult students ($n = 17$, age 20–29 years) (Bougle *et al.*, 1999). This daily dose is equivalent to approximately 100 g of Emmental cheese per day. These subjects excluded fermented milks and Swiss-type cheese from their diet two weeks before and during the study. While basal counts of propionibacteria in faeces was below $5 \log$ CFU/ml stools, it reached in 15 subjects 6.37 ± 0.89 after two weeks of supplementation, but remained constant in two subjects. In a later study, *P. freudenreichii* SI 41 was given to seven healthy volunteers (age 25–35

years) during 3 periods (1) in classical gelatine capsules at a low dose (5×10^9 CFU freeze-dried bacteria per day), (2) in the same capsules at a higher dose (5×10^{10} CFU per day), or 3) in acid-resistant capsules at 5×10^9 CFU per day (Jan *et al.*, 2002b). The population of live propionibacteria recovered from faecal samples after 2 weeks of consumption was (1) 5.50 ± 1.11 ; (2) 6.25 ± 0.66 and (3) 6.12 ± 0.59 . This study stressed the importance of the ingested dose and of gastro-protection. The acid-resistant capsules restored a propionibacterial viability comparable to that afforded by a ten times higher dose, suggesting that acid stress is the bottleneck of *P. freudenreichii* survival *in vivo*.

It is generally accepted that the minimal concentration in the content of the colon should be at least 10^6 CFU per gram for a probiotic to exert a beneficial effect on colon ecology and host physiology (Collins and Gibson, 1999). The dairy propionibacteria populations reached in these studies are consistent and suggest that they may have an impact on the gut ecosystem and metabolism. Indeed, their presence was reportedly related to an increase in propionate concentration in mice (Perez Chaia and Zarate, 2005) and in humans (Jan *et al.*, 2002b). Further works, *in vitro*, in animals and in humans are thus necessary to further specify the impact of propionibacteria and of their metabolites on the intestinal functions.

8.3 Bacterial interactions

8.3.1 With lactic acid bacteria

During the manufacture of Swiss-type cheeses, propionic fermentation is allowed by the ability of dairy propionibacteria to metabolise lactate produced by lactic acid bacteria during lactose fermentation, while no residual lactose or galactose is available in the cheese at the beginning of ripening (Mocquot, 1979; Thierry *et al.*, 1999). Indeed, stimulation of propionibacterial growth by lactic acid bacteria was reported (Liu and Moon, 1982; Marcoux *et al.*, 1992; Piveteau, 1999). More precisely, the growth of propionibacteria in cheese is stimulated by thermophilic lactobacilli (*L. helveticus*, *L. delbrueckii* subsp. *lactis*, *L. delbrueckii* subsp. *bulgaricus*) and streptococci (Baer, 1995; Langsrud and Reinbold, 1973a; Piveteau *et al.*, 1995; Thierry *et al.*, 1999). On the contrary, it is inhibited by facultative heterofermentative lactobacilli (*L. paracasei*, *L. casei*, *L. plantarum* and *L. rhamnosus*) (Baer and Ryba, 1999; Jimeno *et al.*, 1995). Growth of propionibacteria on media previously fermented by lactic acid bacteria allowed valorisation of industrial by-products including spent media (Marcoux *et al.*, 1992; Gardner and Champagne, 2005) to produce propionic acid, starter cultures or probiotics.

8.3.2 With bifidobacteria

Another important bacterial interaction in which dairy propionibacteria are involved is the stimulation of bifidobacteria growth. The former release in

the extracellular space compounds responsible for the stimulation of the latter. The probiotic implication of this is described below (see Section 8.4.1). Co-cultures of propionibacteria and bifidobacteria were used to produce a mixture of antimicrobial organic acids from lactose, thus allowing another valorisation of industrial by-products useful in order to maintain food safety (Taniguchi *et al.*, 1998).

All these bacterial interactions should be taken into account for the development of probiotic products containing live dairy propionibacteria.

8.4 Propionibacteria and the gut

8.4.1 Modulation of the microbiota

One of the mechanisms by which probiotics may confer a health benefit on the host is the modulation of the intestinal microbiota (Ouwehand *et al.*, 2002b). Accordingly, food supplements such as prebiotics, probiotics and symbiotics are generally developed to improve the population and/or the activity of lactobacilli and bifidobacteria within the gut microbiota. One of the most promising properties of dairy propionibacteria, in this context, is their bifidogenic effect. Some strains of *P. freudenreichii* were shown to enhance the growth of bifidobacteria *in vitro* and their population in the gut. In this respect, propionibacterial metabolites would best fit to the first definition of probiotics being 'growth promoting factors produced by microorganisms' (Lilly and Stillwell, 1965).

Indeed, a bifidogenic effect was observed *in vitro* for cell-free extracts of *P. freudenreichii* (Kaneko *et al.*, 1994) and is due to the presence of 2-amino-3-carboxy-1,4-naphthoquinone (Mori *et al.*, 1997) and 1,4-dihydroxy-2-naphthoic acid (Isawa *et al.*, 2002). Moreover, independent studies showed that ingestion of *P. freudenreichii*, under the form of whey cultures, either heat-inactivated (Satomi *et al.*, 1999) or not (Hojo *et al.*, 2002), or of freeze-dried live bacteria (Bougle *et al.*, 2002), resulted in a higher faecal bifidobacteria concentration in humans. This was linked to an increased number of defecations in constipated female volunteers (Hojo *et al.*, 2002).

8.4.2 Modulation of enzymatic activities

The best and first recognised effect of probiotics is the alleviation of lactose intolerance by lactic acid bacteria, mainly *Streptococcus thermophilus* and *Lactobacillus bulgaricus* used as yoghurt starters. The genetically programmed drop in human enterocyte lactase activity in childhood (Wang *et al.*, 1998), as well disorders leading to small intestinal mucosa damages or increase of the gastrointestinal transit time (Labayen *et al.*, 2001), trigger lactose malabsorption responsible for gastrointestinal symptoms such as bloating, flatulence, abdominal pain and diarrhoea (Shaw and Davies, 1999). Lactic acid bacteria have proved useful in this context to improve lactose

absorption via their lactase activity (de Vrese *et al.*, 2001; Montalto *et al.*, 2006).

Among the carbon sources used by dairy propionibacteria, lactose from dairy origin can be used for the production of propionic acid (Jin and Yang, 1998). Utilisation of lactose requires the expression of lactase, or beta-galactosidase, by the bacterium, in order to convert the disaccharide lactose into the monosaccharides galactose and glucose.

Indeed, the genome sequence of *P. freudenreichii* CIP103027 suggests the existence of a beta-galactosidase (118 kDa, 41% identical to *Actinobacillus pleuropneumoniae* betagalactosidase, G. Jan, unpublished data) and the corresponding activity was evidenced in the species *P. freudenreichii* and *P. acidipropionici* (Zarate *et al.*, 2000b). *In vitro*, this activity was shown to be increased in the extracellular medium by the presence of raw bile or bile salts, probably via permeabilisation of propionibacteria (Zarate *et al.*, 2000b). The impact of physicochemical parameters on this activity suggest that propionibacterial beta-galactosidase may be active within the gut (Zarate *et al.*, 2000a, 2002a).

Accordingly, feeding mice with *P. acidipropionici*, either as pure cultures in milk or in conjunction with *S. thermophilus* and *L. bulgaricus* in Swiss-type cheeses, resulted in a significant increase in beta-galactosidase activity within the small bowel and the caecum of mice, compared to sterile milk (Perez Chaia and Zarate, 2005).

Altogether, these data suggest that, as yoghurt starters, dairy propionibacteria, in fermented dairy products such as probiotic cheeses, may help treating lactose intolerance although clinical studies are needed to confirm this. Another noticeable modulation of intestinal activity is the ability of *P. acidipropionici* to lower mice caecal beta-glucuronidase activity, this enzyme being involved in the generation of carcinogenic compounds. This is described in Section 8.6.

8.4.3 Intestinal motility and absorption

During the different clinical studies dealing with dairy propionibacteria intake, an effect on the intestinal functions, including motility, was sought. The hypothesis of a possible impact of propionibacteria on digestive motility was based on such effect reported for other short chain fatty acids-producing bacteria (Cherbut, 2003).

The increase in both propionibacteria and bifidobacteria was accompanied by an increased number of defecations in constipated female volunteers, as a result of *P. freudenreichii* ingestion (Hojo *et al.*, 2002). This result confirms another study in which a combination of *P. freudenreichii* and *L. rhamnosus* increased defecation frequency in elderly subjects (Ouweland *et al.*, 2002a). In healthy volunteers, *P. freudenreichii* ingestion was accompanied by a slower left colon transit yet unchanged right colon and rectosigmoid ones (Bougle *et al.*, 1999).

Short chain fatty acids being associated with gut functions such as absorption of cations, the impact of *P. freudenreichii* on iron absorption was investigated *ex vivo* using isolated rat intestine sections in an Ussing chamber model. The presence of propionibacteria or their metabolites enhanced iron absorption from the proximal colon (Bougle *et al.*, 2002).

Dairy propionibacteria may thus modulate intestinal functions by mechanisms still unclear but probably via the local production of their metabolites.

8.4.4 Adhesion to colonic epithelium

Adhesion to the intestinal mucosa is generally recognised as an important feature of probiotics and constitutes one of the main selection criteria (Lee *et al.*, 2003b).

Epithelial intestinal cells being covered with mucus, an *in vitro* model was developed in order to investigate adhesion of both pathogenic and probiotic bacteria to small intestinal mucus, based on human ileostomy glycoproteins (Tuomola *et al.*, 1999). Thus, *P. freudenreichii* was shown to adhere to glycoproteins, to a lesser extent than *Lactobacillus rhamnosus* or *Escherichia coli*, while *Lactococcus lactis* failed to adhere. All these adherent strains were previously shown to adhere to Caco-2 cells (Tuomola and Salminen, 1998). Surprisingly, heat inactivation caused an increased adherence of *P. freudenreichii* yet a decreased of *L. rhamnosus* (Ouwehand *et al.*, 2000). This suggests that metabolic activity is not required for *P. freudenreichii* adhesion and a major role of cell envelope constituents. Propionibacteria adhesion to intestinal mucus was later shown to be instantaneous and favoured by prior adhesion of other existing probiotics (Ouwehand *et al.*, 2002c). Further, this high affinity is increased by propionibacteria pre-treatment, either at low pH or in the presence of bile (Thiel *et al.*, 2004). Interestingly, adherent *Staphylococcus aureus* was displaced from human glycoproteins by *P. freudenreichii* and other probiotics (Vesterlund *et al.*, 2006).

In another model, adhesion of propionibacteria to mice ileal epithelial cells was investigated both *in vitro* (exfoliated ileal epithelial cells) and *in vivo* (bacteriological analysis of intestinal walls of mice receiving propionibacteria in milk). Dairy propionibacteria (6 strains) including *P. acidipropionici*, *P. freudenreichii* and *P. jensenii* adhered to ileal epithelial cells to various extents (Zarate *et al.*, 2002b). This was not correlated with the ability to hemagglutinate, to autoaggregate (uncommon properties among dairy propionibacteria) or with cell surface hydrophobicity, by contrast with the correlation reported for other probiotics (Del Re *et al.*, 2000; Kos *et al.*, 2003). In this model, adhesion of *P. acidipropionici* depended on the presence of divalent cations, was not affected by bile and pancreatic secretions, but decreased by pronase and metaperiodate (Zarate *et al.*, 2002c).

These different studies suggest the existence of complex interactions between propionibacteria and intestinal cells and mucus, leading to efficient adhesion

which would explain the slow decrease in propionibacteria faecal population after stopping supplementation.

8.4.5 Colonic inflammation

Inflammatory bowel diseases are severe chronic affections characterised by a recurrent inflammation. Some of the microorganisms present within the gut microbiota may be involved in this yet not fully understood pathogenesis, via a pro-inflammatory effect (Marteau *et al.*, 2003). Indeed, an *in vivo* model based on chemically induced colitis (using dextran sodium sulfate, DSS or trinitrobenzene sulfonic acid, TNBS) in mice being developed, this colitis was shown to be reduced by antibiotics (Hans *et al.*, 2000). In various models of induced colitis in rodents, a protective effect of different probiotics was reported, suggesting that probiotic strains may counteract the deleterious effects of resident microorganisms (Madsen *et al.*, 1999; Marteau *et al.*, 2003; Dieleman *et al.*, 2003; Osman *et al.*, 2004). Based on these observations, a bifidogenic strain of *P. freudenreichii* cultured on milk whey, was tested in TNBS-induced colitis in rats (Uchida and Mogami, 2005). This probiotic preparation significantly accelerated the healing of the colitis in a dose-dependent manner and the healing effect was attributed to propionate. In another study, the propionibacterial bifidogenic compound 1,4-dihydroxy-2-naphthoic acid (see Section 8.4.1) was tested in DSS-induced colitis in mice (Okada *et al.*, 2006). 1,4-dihydroxy-2-naphthoic acid improved survival rate, histological damage score, suppressed lymphocyte infiltration and restored the *Lactobacillus* and *Enterobacteriaceae* populations induced by DSS. *P. acidipropionici* was administered by colonic infusion in rats with TNBS induced colitis. A positive effect on food consumption, body weight, gross score for inflammation and myeloperoxidase activity was observed (Michel *et al.*, 2005).

8.5 Propionibacteria and the immune system

The impact of propionibacteria on the immune system was mainly studied regarding the cutaneous species, which display marked immunomodulatory properties (Roszkowski *et al.*, 1990). Indeed, *P. avidum* KP-40, administered parenterally to mice, increase significantly mice thymocyte proliferation and peripheral blood lymphocytes and monocytes counts (Isenberg *et al.*, 1995). Oral supplementation by the same strain furthermore counteracts the drop in lymphocytes counts triggered by anaerobic exercise in young male healthy sportives (Pottkamper *et al.*, 1996). Such properties were thus sought in the dairy group of propionibacteria. A pioneer study in this field was undertaken with the species *P. acidipropionici*, orally administered to mice. The treatment resulted in an enhanced phagocytic activity of peritoneal macrophages activity, assessed using killed salmonella in a phagocytosis assay (Perez Chaia *et al.*,

1995). This is consistent with the activation of immunocompetent cells observed using other probiotics, when administered orally. *P. freudenreichii*, in a similar study, triggered a decrease in spontaneous basal proliferation activity of mice splenic lymphocytes (assessed *ex vivo*), yet an increase in both T-cell proliferation triggered by concanavalin A- and B-cell proliferation triggered by bacterial lipopolysaccharide (Kirjavainen *et al.*, 1999). Another dairy species, *P. jensenii*, was orally administered to mice in order to evidence its immunostimulating effect. It was compared to cholera toxin during co-administration with soluble tuberculosis protein antigen to mice (Adams *et al.*, 2005). The spleen lymphocytes proliferation observed following stimulation was significantly higher with *P. jensenii* than with the adjuvant cholera toxin.

These studies evidence some immunomodulatory properties in dairy propionibacteria. They suggest that these probiotics may be useful in therapeutical strategies and could replace the cutaneous propionibacteria used for immunomodulation in clinical trials (Kirjavainen *et al.*, 1999). Furthermore, propionibacteria may have applications as living vaccine vector because of their immunostimulating properties (Adams *et al.*, 2005).

8.6 Propionibacteria and cancer

Among the beneficial effects that have been attributed to probiotics, the prevention of cancer, with a main focus on colon cancer, constitutes the most promising and probably the most controversial potential. As stated by J. Rafter, there is no direct evidence for cancer suppression in humans as a result of probiotic consumption (Rafter, 2003). However, there is wealth of indirect evidence, based on experimental work, both *in vivo* and *in vitro*, of anticancer properties of some probiotics. Probiotics may interfere with the development of cancer via binding and degradation of carcinogens, production of anti-mutagenic compounds, modulation of the intestinal microbiota and/or metabolic activities or by enhancing the host's immune response.

8.6.1 Binding of aflatoxin

Aflatoxins, mycotoxins produced by fungi, are unavoidable food contaminants recognised as human hepatocarcinogens (Motola-Kuba *et al.*, 2006). Efforts have thus been focused on their removal from food components or their binding by probiotics within food and digestive tract. *P. freudenreichii* JS was shown to bind aflatoxin within the lumen of chicken intestine. Furthermore, a reduction in the amount of aflatoxin accumulation in the intestinal tissue was observed in the presence of this probiotic, as for *Lactobacillus rhamnosus* GG (El Nezami *et al.*, 2000). *In vitro* investigation of this adsorption evidenced that heat-inactivated bacteria are more efficient than living bacteria and that bacterial surface hydrophobicity is not correlated with adsorption capacities (Lee *et al.*, 2003a). Further, this binding capacity was shown to be altered by

the presence of intestinal mucus *in vitro*. The authors hypothesise a competition towards the same intestinal binding sites for mucus and aflatoxin (Gratz *et al.*, 2004, 2005). In a human study, a mixture of both *P. freudenreichii* JS and *L. rhamnosus* GG reduced the urinary excretion of aflatoxin B₁-N⁷-guanine, strongly suggesting a reduction in aflatoxin bioavailability (El-Nezami *et al.*, 2006). Thus, some strains of propionibacteria may be useful to reduce absorption and increase excretion of dietary aflatoxin from the human digestive tract.

8.6.2 Induction of apoptosis

Several dairy propionibacteria species were shown to induce apoptosis of two human colorectal cancer cell lines *in vitro* (Jan *et al.*, 2002a). This effect was attributed to the secretion by propionibacteria of short chain fatty acids, acetate and propionate. These acids were shown to bind to cancer cells mitochondria and to trigger opening of a the mitochondrial pore complex PTPC, resulting in mitochondria depolarisation, typical signs of apoptosis including the generation of reactive oxygen species, caspase-3 processing, nuclear fragmentation and finally cell death by apoptosis. Apoptosis induction is illustrated in Fig. 8.2. Propionibacteria adhere to cultured human colorectal cancer cells and cause drastic modifications including nuclear condensation and mitochondria swelling. This effect is consistent with the effect of another short chain fatty acid, butyrate, reportedly trophic towards normal colonocytes (Roediger, 1982) and toxic towards colon cancer cells (Hague *et al.*, 1995). However, the beneficial impact of short chain fatty acids on the development of cancer cells *in vivo* remains to be fully proven (Lupton, 2004).

8.6.3 Antimutagenic properties

The proposed anti-cancer properties of some probiotics include an antimutagenic effect which protects or rescues cells exposed to DNA damaging agents. An *in vitro* method widely used to evidence this property is the ability for probiotic antimutagenic compounds to prevent damages caused to bacteria (mainly *Salmonella enterica*) using the Ames test (Pool-Zobel *et al.*, 1993). Using this test, dairy *P. freudenreichii* was shown to produce compounds able to lower mutagenesis induced by various mutagens such as sodium azide, N-methyl-N'-nitro-N-nitrosoguanidine, 9-aminoacridine (Vorobjeva *et al.*, 1991), 4-nitro-quinoline-1-oxide (4NQO) and by UV light (Vorobjeva *et al.*, 1996, 2001). This active proteic compound is recovered either from culture supernatants or from cell sonicates, suggesting that the protein(s) involved is (are) released either by secretion or by cell lysis. Interestingly, the antimutagenic activity can be enhanced by pre-treating propionibacteria with small amounts of 4NQO, conferring higher protection towards both 4NQO and UV light. This was concomitant with a significant increase in glutathione-S-transferase (GST) activity within propionibacteria cells

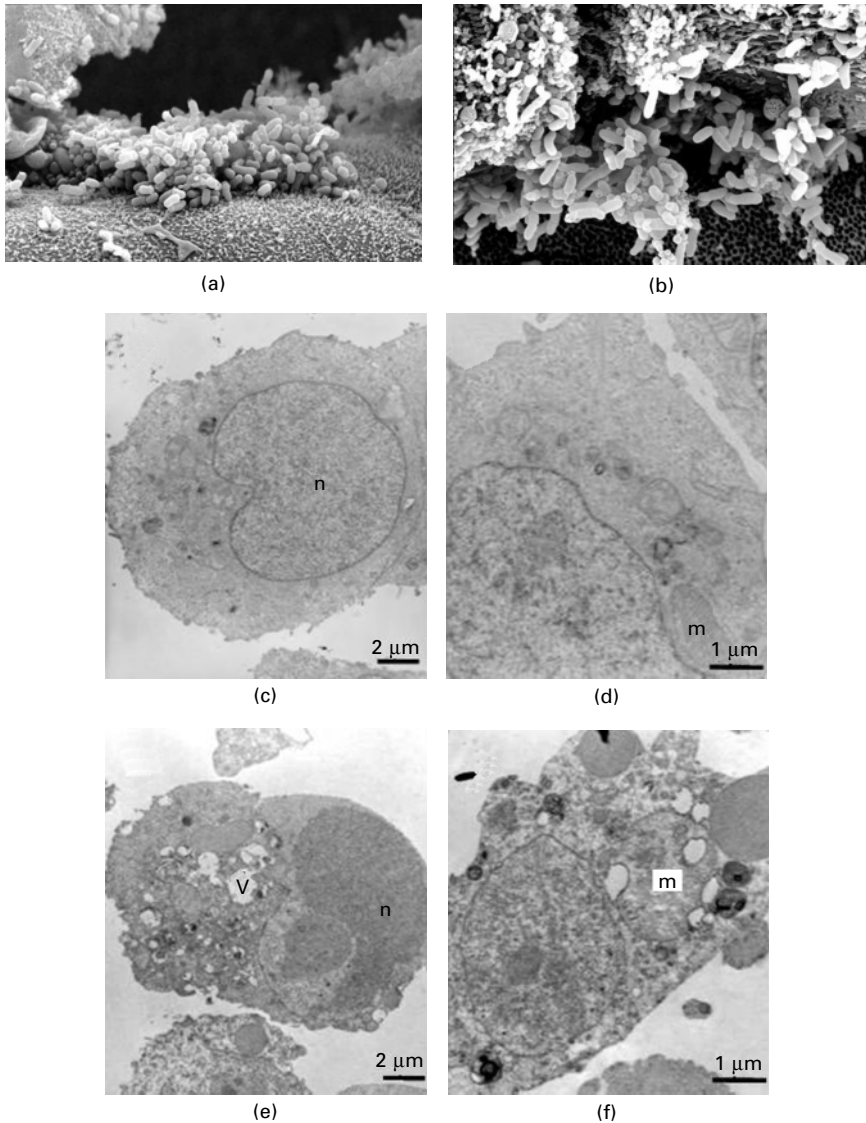


Fig. 8.2 Microscopy analysis of interactions between propionibacteria and cultured human colon cancer cells. Caco-2 cells were co-cultured during 12 (a) and 48 hours (b) with *P. freudenreichii* and examined using scanning electron microscopy (magnification $\times 5000$).

The effect of *P. freudenreichii* metabolites on cellular morphology of HT-29 cells treated for 48 hours was analysed using transmission electron microscopy (c)–(f).

Proliferating untreated cells (c), (d) displayed the characteristic morphology of intestine epithelial cells: regularly dispersed chromatin in nuclei (n), numerous mitochondria (m) in the perinuclear space and high nucleoplasmic ratio. Contrastingly, propionibacterial metabolites caused typical signs of apoptosis (e), (f): chromatin condensation and margination (n), swelling and vacuolated aspect of mitochondria (m), cytoplasm shrinkage, and cytoplasm vacuolisation (V).

(Vorobjeva *et al.*, 1996). It is noteworthy that glutathione and GST play a protective role in mutagenesis and carcinogenesis (Ketterer, 1988; Wiencke *et al.*, 1995). It was first shown that a cell-free extract obtained from *P. freudenreichii* exerted a protective activity on UV-inactivated *Escherichia coli* (Vorobjeva *et al.*, 1993). A proteic compound comprised in this extract was further shown to reactivate both prokaryotes and eukaryotes (Vorobjeva, 2000). After purification, it was identified as a cysteine synthase which expression is induced by various stresses in *P. freudenreichii* (Vorobjeva *et al.*, 2004).

8.6.4 Modulation of genotoxic enzymatic activities

Bacterial enzymes, including azoreductase and beta-glucuronidase, are involved in the generation of carcinogenic compounds within the gut (Rowland, 1992). Consumption of probiotics and/or prebiotics has been shown to reduce beta-glucuronidase and carcinogen-induced aberrant crypt foci in rats (Rowland *et al.*, 1998). The impact of probiotics on these activities has thus been used as a criterion for probiotic screening.

Accordingly, the impact of dairy propionibacteria, ingested as bacterial suspensions in sterile milk, on caecal beta-glucuronidase activity was investigated in mice. While two strains of *P. freudenreichii* had no effect, two strains of *P. acidipropionici* reduced significantly beta-glucuronidase activity (Perez Chaia *et al.*, 1999). Interestingly, *P. acidipropionici* was able to counteract the increase in beta-glucuronidase activity induced by supplementing mice diet with cooked red meat. Another study on rats failed to evidence an effect of *P. jensenii* on faecal beta-glucuronidase activity (Huang *et al.*, 2003), confirming that this property is species-dependent. Dairy propionibacteria may thus have a beneficial effect by preventing elevated levels of beta-glucuronidase activity linked to meat-rich diets. However, wide strain screening, animal and clinical studies are still necessary to confirm these preliminary results.

Lastly, it should be indicated here that immunostimulation by the cutaneous *Propionibacterium avidum* KP-40 was reported to limit the dissemination of metastasis in mice. Furthermore, pre-operative immunostimulation during a prospective randomised trial in colorectal carcinoma patients evidenced positive effects on survival time, local tumour recurrence and distant metastasis, only if immunostimulation occurred during early stages (Isenberg *et al.*, 1995). Similar immunostimulation by dairy species should be investigated.

8.7 Future trends

Several probiotic abilities were claimed for dairy propionibacteria. Some strains were shown to possess efficient adaptive mechanisms allowing survival within the gut. Beneficial modulations of the gut motility, absorption, microbiota

and of the corresponding enzymatic activities were described, suggesting a beneficial impact on digestion and on carcinogenesis. The *in situ* production of short chain fatty acid without the possible side effects of butyrogenic oligosaccharides, linked to gas production (Scholtens *et al.*, 2006), also constitutes a promising application. However, it should be kept in mind that each probiotic ability was described for a still limited number of strains, while the corresponding characteristics seem to be highly variable depending on the species within the *Propionibacterium* genus and on the strain within each species.

Other promising healthful properties were also described for dairy propionibacteria. The production of B vitamins may help in reaching the daily recommended intake by using fermented products. The conversion of free linoleic acid to extracellular conjugated linoleic acid (CLA) was described for certain strains of *P. freudenreichii* (Jiang *et al.*, 1998a), allowing CLA production by fermentation (Rainio *et al.*, 2001, 2002). Considering the positive influence of CLA on body fat gain, carcinogenesis, insulin resistance, and lipid peroxidation (Wahle *et al.*, 2004), this may constitute another healthful potential. The production of such nutraceuticals, however, remains to be demonstrated during the various industrial processes involving propionibacteria (Xu *et al.*, 2005; Gnadig *et al.*, 2004) or during transit within the digestive tract.

The technological abilities of dairy propionibacteria used in the industry constitute another key advantage. Considering the industrial strains of *P. freudenreichii*, there is a long history of their use in dairy technology. They possess a remarkable tolerance towards stresses imposed during freeze-drying, spray-drying, storage of the starter, reconstitution in milk and cheese-making. Their growth during fermentation of various vegetable fermented products including sauerkraut (Babuchowski *et al.*, 1999), olives (Koussemon *et al.*, 2001) but also silage (Merry and Davies, 1999) evidence a great robustness and adaptation to various environments.

However, a gap remains between, on one hand, knowledge and know-how in the food technology of propionibacteria and, on the other hand, scientific work on their probiotic applications. Combining the expertise in these distinct fields of research should undoubtedly allow filling this gap. A broader screening of strains, taking into accounts both technological and probiotic criteria would allow identifying efficient probiotics. Moreover, the existing probiotic products are still limited. Propionibacteria being able to ferment a variety of food products, different vectors should be developed and tested. In this respect, not only the stability in the product, but also the impact of pre-treatments undergone during the process on tolerance towards digestive stress and functionality is a determining factor.

Getting the proofs of probiotic efficacy, using the good tools, is another key challenge in this field. Working on selected strains and vectors, evidence of survival and activity within the digestive tract should be made. In this context, sequencing of propionibacteria genomes, comparison between species

and strains will allow understanding of the molecular bases and developing molecular tools to evidence metabolic activity *in vivo*, including during clinical trials. This will in turn allow determining a list of reasonable positive effects based on experimental evidence and appropriate products.

8.8 References

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9

Synbiotics: combining the benefits of pre- and probiotics

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9.1 Introduction

The gastrointestinal tract is populated by a myriad of microbes that outnumber the eukaryotic cells in our body by 10:1 (Savage 1986), on a genetic level, the intestinal microbiome outnumbers the human genome by a factor 100 (Bäckhed *et al.* 2005) (analysis of the microbiota will be described in more detail in Chapter 15). It is therefore not surprising that the intestinal microbiota has a pronounced influence on our health and well-being, an influence that extends well beyond the intestine. Nor is it surprising that an important target of functional food ingredients is the intestinal microbiota.

The main products on the market designed to positively influence the composition and activity of the intestinal microbiota are probiotics and prebiotics. The properties of probiotics are discussed in more detail in later chapters. But, in short, probiotics have been defined by an expert group of the FAO/WHO (2002) as: ‘live microorganisms which, when administered in adequate amounts, confer a health benefit on the host’. Prebiotics have been defined as ‘non-digestible food ingredients that, when consumed in sufficient amounts, selectively stimulate the growth and/or activity of one or a limited number of microbes in the colon resulting in documented health benefits’ (Ouwehand *et al.* 2006), modified after (Gibson and Roberfroid 1995). With probiotics, there is an emphasis on viability and the dose consumed. Prebiotics, on the other hand, positively influence selected microbes. Since both types of ingredients have the intestine as functional target, it is obvious that the two can be mixed and may reinforce each other’s effect. Thereby creating a synbiotic: ‘a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial

dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or activating the metabolism of one or a limited number of health promoting bacteria, and thus improving host welfare' (Gibson and Roberfroid 1995). Synbiotics are, however, much more than just a mixture of pre- and probiotics. As the name suggests, a synergy must exist between the two components and hence not just any mixture will be a synbiotic.

9.2 Developing new synbiotics and testing their efficacy

The development of new synbiotics typically requires a long-term screening process. During the screening, combinations of selected prebiotic substances and probiotic strains are tested both *in vitro* and *in vivo* in order to find the most active and synergistic pairs. Figure 9.1 portrays a typical product development pipeline with several interesting candidates in the beginning, and the necessity of the use of *in vitro* methods in reducing the number of studied substances prior to both expensive and time consuming *in vivo* tests can clearly be seen. Naturally, the *in vitro* tests alone can not be used to determine the *in vivo* effectiveness of any prebiotic substance or probiotic strain, but will aid in the decision process. In the end, both animal and, especially, human tests are needed to substantiate efficacy.

9.2.1 *In vitro* pure culture

Typically the first step in large scale synbiotic screening process is an extensive series of *in vitro* pure culture cultivations (e.g. Crittenden *et al.* 2001). All the selected combinations of pre- and probiotics are grown under optimal or near-optimal cultivation conditions, with the test prebiotic being the major carbon source for the probiotic strain. The evaluation of the optimum usage of the prebiotic substances by the probiotic bacteria and the comparison of the bacterial growth rates allows a first selection of the most promising synbiotic pairs for further analyses.

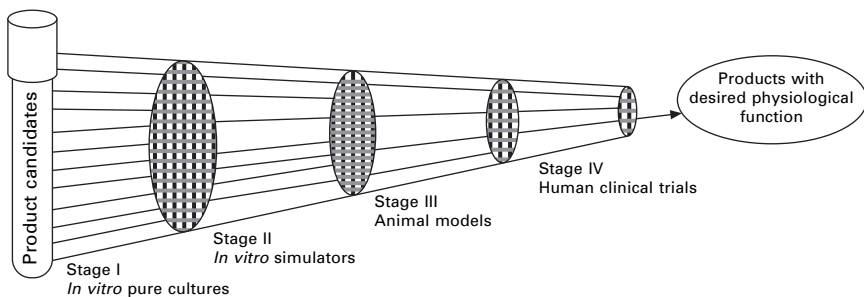


Fig. 9.1 Typical product development pipeline with four stages, each reducing the number of studied candidates.

9.2.2 Faecal batch culture fermentations

To simulate the conditions in the colon in a simple way, synbiotic preparations can be incubated with faecal slurries. These batch fermentations can be simple bottles or containers with some kind of pH control. For example, Tzortzis *et al.* (2004) used pH-controlled anaerobic batch cultures to simulate the changes in canine fecal microflora, when fermented with a synbiotic combination of *Lactobacillus reuteri* of canine origin and α -galacto-oligosaccharide synthesised with an enzyme extract from the *L. reuteri*-strain. The synbiotic combination increased the concentrations of bifidobacteria and lactobacilli compared either to α -galacto-oligosaccharide alone or to commercial prebiotics (fructo-oligosaccharide, melibiose and raffinose).

9.2.3 *In vitro* simulators

The probiotic strains have to be able to survive through the gastric digestive tract, while the prebiotic substances should not be degraded or absorbed in order to promote a synbiotic effect in the colon. Thus, both the components have to withstand the severely acidic (pH 1–2) conditions in the stomach and the degrading effects of both bile and digestive enzymes during passage through the small intestine. The survival of the components from mouth to colon is difficult to study in human tests due to both ethical and technological problems in the sample collection, although ileostomy patients could be used for this kind of research (Tidehag *et al.* 1995). Therefore models that simulate the gastric passage are needed. These models also serve as an essential candidate-reducing stage in the product development pipeline before animal tests (Fig. 9.1).

Crittenden *et al.* (2001) presented a simple gastric model that was used in the synbiotic research. It consists of tubes that simulate first the acidic stomach and subsequently the more neutral duodenum with bile. They screened 40 *Bifidobacterium* strains for their ability to both ferment resistant starch and to survive the passage through the upper gastrointestinal tract. Only one strain, *B. lactis* B94, fulfilled both criteria and proved to be a potential probiotic strain for synbiotic yoghurt.

A more sophisticated model, the SHIME-reactor, simulating the whole gastrointestinal tract from mouth to rectum in six individual computer-controlled vessels, has been used to study the effects of synbiotic compounds on the human intestinal microbes (Gmeiner *et al.* 2000). *Lactobacillus acidophilus* 74-2 was administered to the second vessel (duodenum/jejunum) of the reactor in a milk-based product with fructo-oligosaccharides for seven days. The changes in the intestinal microflora were monitored in vessels 4, 5 and 6, simulating the different sections of the human colon. The administration of the synbiotic combination was found to stimulate the growth of bifidobacteria and also induced an increase in the butyrate concentrations in all three vessels. These results suggest that the synbiotic had a positive impact on the simulated environment of the colonic microflora, changing it to more supportive

towards the beneficial microbes and suppressing the growth of the harmful microbes.

9.2.4 Animal models

To investigate the complex interactions between host, gut microbes and synbiotics in a more realistic way than the *in vitro* models, pigs (Brown *et al.* 1997), dogs (Swanson *et al.* 2002), rats (Roller *et al.* 2004; Rowland *et al.* 1998; Rowland and Tanaka 1993) and mice (Asahara *et al.* 2001) are used as models for human gastrointestinal functions. However, conventional animal models may be of little relevance for the assessment of synbiotic effects on human microbiota and intestinal environment. To overcome this problem, in part, germ-free rats and mice have been associated with a human microbiota, this approach makes a valuable contribution to study the effects of synbiotics.

As the indigenous microbiota of rats and humans differ considerably in their composition and activity (Sembries *et al.* 2003), synbiotic combinations have been studied in gnotobiotic rats (Rowland and Tanaka 1993) and rats associated with human fecal microbiota. Synbiotic effects of *L. rhamnosus* (2×10^{13} cfu/kg diet) and cellobiose (10%) on serum lipids have been reported in conventional rats (Umeki *et al.* 2004). The effect of synbiotics on gastrointestinal infections has been investigated as discussed in more detail in Section 9.4.1 (Asahara *et al.* 2001; Qiao *et al.* 2002).

Spontaneous colorectal cancer is not typical in animals, but it can be induced with carcinogens such as 1,2-dimethylhydrazine or azoxymethane (Martin *et al.* 1981). In the azoxymethane-induced rat model the combination of inulin (5%) and *B. longum* (4×10^8 /g diet) was shown to inhibit carcinomas in a synergistic manner (Rowland *et al.* 1998). A combination of bifidobacteria (10^8 cfu/d) and oligofructose (2%) was found to reduce carcinomas in 1,2-dimethylhydrazine-treated rats (Gallagher and Khil 1999). Experimental carcinogenesis can also be studied using transgenic mice (Taketo 2006). An APC-mutant mouse is a colon cancer model mimicking the development of adenomatous polyps in humans where the polyps will eventually develop into colon cancer. The major drawback of these mutants as models of human colon cancer is that their tumours occur predominantly in the small intestine, not the colon.

9.2.5 Human studies

After the functionality and synergistic features of the synbiotic combination have been evaluated using *in vitro* methods and possibly animal tests, the product candidates move to the last stage of the product development pipeline. In general human clinical trials need to follow certain guidelines:

- The trial protocol needs to be ethically acceptable by the local administrative committee.

- The randomisation of the trial group participants and the results have to be kept double-blinded from both organisers and participants until the trial is completed.
- The participants have to be selected so that their current health status suits the focus of the trial.
- Sufficient participants must be gathered in order to fulfil statistical criteria, otherwise a cross-over study is needed to reduce the bias caused by a small study group.
- A full comparison of the effects of all the studied substances is needed, i.e. four study arms are needed: control, synbiotic, prebiotic and probiotic.

The first three above-mentioned categories have been followed well in the synbiotic clinical trials published so far, but unfortunately the two latter categories are not so well taken into account. This problem is emphasised in Table 9.1, presenting ten human clinical trials conducted on healthy persons, of which six trials have only 30 participants or less and of which only two have separate groups for pre-, pro- and synbiotics and control. As these trials are final evaluation points for the synbiotic candidates, more trials with separate groups for the given prebiotic and probiotic(s) alone along with the synbiotic group are needed in order to fully validate the effects of these combinations.

9.3 Safety assessments of synbiotics

Besides efficacy, safety of a functional food ingredient is of main importance. Since synbiotics are a combination of prebiotics and probiotics, we will first address the safety considerations for these two components separately. Subsequently the specific safety considerations for their combination in synbiotics will be discussed.

9.3.1 Safety of prebiotics

Prebiotics have long been part of the human diet. Fructosyl-type ingredients are natural components of edible plants (van Loo *et al.* 1995). Furthermore, some novel prebiotics such as polydextrose have been used as food ingredients for decades.

A practical concern with the use of prebiotics is their tolerability, which varies considerably between different prebiotic compounds. In addition, sensitivity to side effects varies among members of the general population. Typically, excessive consumption of prebiotics can lead to gastrointestinal symptoms such as bloating and laxation. Intestinal tolerance to non-digestible compounds depends mainly on its osmotic and fermentation effects. Osmotic diarrhoea is caused especially by a high luminal concentration of low molecular weight compounds, such as polyols. On the other hand, highly fermentable

Table 9.1 Selected *in vivo* human clinical trials performed on healthy participants

Intervention	Participants	Study groups	Main results	Reference
<i>B. bifidum</i> and <i>B. lactis</i> (3.5×10^{10} cfu/d) + oligofructose (6 g/day)	18 healthy elderly females	control vs. synbiotic	Increase in the numbers of bifidobacteria in the synbiotic group	(Bartosch <i>et al.</i> 2005)
<i>Bifidobacterium</i> spp. + inulin (12 g/d) for 12d	12 healthy adults	probiotic + synbiotic vs. baseline ¹	Decrease in the β -glucuronidase activity in the probiotic group	(Bouhnik <i>et al.</i> 1996)
<i>L. plantarum</i> (10^{10} cfu/d) + oat fibres (0.16 g/d) for 6 weeks	30 healthy adults with slightly elevated cholesterol levels	control vs. synbiotic	Decrease in total cholesterol, LDL-levels and fibrinogen levels in the synbiotic group	(Bukowska <i>et al.</i> 1998)
<i>L. paracasei</i> (10^9 cfu/d) + FOS ² (6 g/d) for 1 year	60 elderly persons	control vs. synbiotic	Decrease in respiratory tract infections in the synbiotic group	(Bunout <i>et al.</i> 2004)
<i>L. acidophilus</i> and <i>Bifidobacterium</i> spp. (both 3×10^7 cfu/g) +FOS (0.5 g/L) for 4 months	626 healthy malnourished children aged 1–6 years	control vs. synbiotic	Decrease in number of sick days and constipation in children aged 3–5 years in the synbiotic group with at least one period of illness	(Fisberg <i>et al.</i> 2002)
Two <i>L. paracasei</i> -strains (5×10^9 cfu/bag), <i>L. gasseri</i> (0.5×10^9 cfu/bag) + inuline, oligosaccharides (6 g/bag); 3 bags/d for 15d	12 healthy adults	synbiotic vs. baseline	Increase in numbers of <i>L. paracasei</i> -like lactobacilli	(Morelli <i>et al.</i> 2003)
<i>L. acidophilus</i> strains (10^7 – 10^8 cfu/g of fermented milk) + FOS (2.5% of fermented milk); daily intake of milk 375 ml; for 3 weeks	30 healthy adults with slightly elevated cholesterol levels	control vs. synbiotic	Decrease in total cholesterol, LDL-levels and in LDL/HDL-ratio in the synbiotic group	(Schaafsma <i>et al.</i> 1998)

Table 9.1 Continued

Intervention	Participants	Study groups	Main results	Reference
<i>L. acidophilus</i> and <i>Bifidobacterium</i> spp. (both 10^9 cfu/g of formula) +FOS (3.5 g/L of formula) during a antibiotic therapy	84 children with bacterial infections	control vs. synbiotic	No difference between the groups	(Schrezenmeir <i>et al.</i> 2004)
<i>L. acidophilus</i> (2×10^9 cfu/d) + FOS (6 g/d)	62 healthy adults	prebiotic + probiotic + synbiotic vs. control	Decrease in faecal total phenol in the synbiotic group	(Swanson <i>et al.</i> 2002)
<i>B. breve</i> (3×10^6 cfu/d) + TOS ³ (3 g/d the 1st week, 10 g/d the 2nd week) for 2–3 weeks	16 healthy adults	prebiotic + probiotic + synbiotic vs. baseline	Decrease in faecal ammonia and urinary hydrogen sulphide in the synbiotic group	(Tanaka <i>et al.</i> 1983)

¹A period of evaluation before administration of the studied substances

²FOS: fructo-oligosaccharide

³Transgalactosylated oligosaccharide

prebiotics may lead to the production of gases which cause abdominal distension and produce flatus. The excessive fermentation in the proximal colon may also decrease the carbohydrates available for microbes in the distal colon. Hence, the microbes may turn to protein fermentation in the more distal parts of the colon and produce harmful metabolites such as indoles, phenoles and ammonia.

9.3.2 Safety of probiotics

The most common species of microbes used as probiotics, and hence in synbiotics, are members of the genera *Bifidobacterium* and *Lactobacillus*. Probiotics from other genera are also used, but are less common or may not be relevant to dairy applications and will therefore not be considered here. One of the main safety considerations for probiotics is considered to be the presence or ability to transfer antibiotic resistance (Anadon *et al.* 2006); this will be discussed in Chapter 20.

Most members of the genera *Lactobacillus* and *Bifidobacterium* are generally regarded as safe; this is mainly based upon empirical observations (Ouweland and Salminen 2003):

- Lactobacilli have a long history of safe use, especially in fermented dairy products.
- Bifidobacteria, and to a lesser extent lactobacilli are a major component of the normal human intestinal microbiota.

- Despite their high levels in the intestine, lactobacilli and bifidobacteria are extremely rarely associated with disease.

All microbes that have, theoretically, the ability to grow under the conditions that are found in the human body, can, in principle, colonise sites in the human body and can thus be pathogens. Bacteria with such ability include lactobacilli and bifidobacteria. However, members of these two genera are, in general, not considered pathogens. The frequency of bacteraemia caused by these organisms is extremely low; for lactobacilli 0.2% of all positive blood cultures and for bifidobacteria even less (Salminen *et al.* 2002). The *Lactobacillus* bacteraemia does not appear to be related to the consumption of probiotics by the general healthy population, but is mainly associated to subjects with severe underlying diseases (Salminen *et al.* 2004). Cases of infections by probiotic lactobacilli have been reported, but these are even more rare than infections by endogenous lactobacilli and also these occur in subjects with severe underlying disease, e.g. (de Groote *et al.* 2005; Land *et al.* 2005). There does, therefore, not appear to be a concern for the general healthy population. For subjects with severe underlying diseases, the risks and benefits from probiotic use should be considered.

9.3.3 Specific safety considerations for synbiotics

Because the prebiotic part of a synbiotic combination by definition is supposed to improve the transient colonisation of the probiotic part, there could theoretically be a risk to also enhance the influence of any potential risk factors expressed by the probiotic. Such studies are rare. Anderson and co-workers (2004) did not observe any change in translocation of members of the endogenous microbiota and no translocation by the probiotics fed (*B. lactis* Bb-12 and *L. acidophilus* La-5) in elective surgical patients, indicating that the prebiotic (inulin), although supporting the probiotics, does not cause any translocation of the strains. This suggests that a concern for such an increased risk is not warranted. In a study with rats by Challa and co-workers (1997), a synbiotic consisting of *B. longum* BB536 and lactulose, induced significantly more diarrhoeal symptoms in the prebiotic and synbiotic groups compared to control. This indicates that the prebiotic component, lactulose, was responsible for the symptoms rather than the synbiotic.

The safety considerations for synbiotics seem, therefore, not to differ from the safety considerations for the components constituting the synbiotic; i.e. the probiotic and prebiotic, although more structured investigations on this issue would be welcome.

9.4 Functional benefits of synbiotics

Improving gastrointestinal health is a natural health focus area for the use of synbiotics in foods. Different gastrointestinal (GI) compartments and types

of diseases have been studied. Intervention studies with combinations of prebiotics and probiotics have so far focused on infectious diseases of the upper GI tract, and chronic diseases of the lower GI tract. Table 9.2 summarises examples of human clinical studies on GI health. Due to high costs and/or long duration of the clinical studies the actual synergy of pre- and probiotics is often not evaluated, and evaluation of efficacy is based on comparison of the control/placebo treatment with the pre- probiotic treatment. Although the table attempts to summarise studies conducted up to date, generalisation of efficacy of synbiotics for certain diseases is difficult since only limited combinations of pre- and probiotics have been considered so far. Finally, interventions of some non-gastrointestinal diseases are discussed.

9.4.1 Gastrointestinal infections

The effect of synbiotics on gastrointestinal infections has, to date, received little attention. A study by Sazawal and colleagues (2004) indicated that consumption of a combination of GOS and *B. lactis* HN019 resulted in a non-significant reduction in diarrhoea incidence and a significant reduction in the incidence of dysentery.

More work has been done on the effect of synbiotics in animal challenge models. In a rotavirus challenge, mice were treated with *B. bifidum* and *B. infantis* in combination with either FOS, arabino-galactan or iso-malto-dextrins, or probiotics alone, for seven weeks. The authors reported no substantial differences between the probiotic and the three synbiotics (Qiao *et al.* 2002), suggesting that most of the effect may have been mediated through the bifidobacteria. In an oral challenge of mice with *Salmonella enterica* serovar Typhimurium (Asahara *et al.* 2001), the tested synbiotic, a combination of *B. breve* and GOS, and the probiotic alone significantly reduced the translocation and faecal excretion of *Salmonella*. Compared to control, treatment with GOS alone had no effect. Here, too, the protective effect appears to be mediated through the probiotic rather than the prebiotic and no synergistic effect could be observed.

Susceptibility to infections is increased in patients undergoing abdominal surgery, transplantation, or otherwise critically ill (Table 9.2). The efficacy of synbiotics for reducing incidence of infections has been evaluated in several studies, and combinations of mixtures of both pre- and probiotics have been used both pre- and post-operatively for a week or two at most. In many cases even a short prophylactic treatment was protective; however, it is not clear why some of the studies failed. Thus, further studies are needed before recommendations can be made.

In conclusion, the use of synbiotics appears effective in reducing susceptibility for infections. However, the mediating mechanisms especially for infections residing elsewhere than in the GI tract remain totally unresolved.

Table 9.2 Summary of human clinical trials with synbiotics on gastrointestinal health

Type of GI disease	Intervention	Main outcome [‡]	References
Infectious diseases	<i>B. lactis</i> (10^7 – 10^8 CFU/day) + GOS	☺	(Sazawal <i>et al.</i> 2004)
Critically ill (incidence of infections)	<i>L. plantarum</i> (10^{10} CFU/day) + oat fibre	☹	(McNaught <i>et al.</i> 2005)
	<i>L. acidophilus</i> , <i>B. lactis</i> , <i>S. thermophilus</i> , <i>L. bulgaricus</i> (12×10^9 CFU/day) + FOS (15 g/day)	☹	(Jain <i>et al.</i> 2004)
	<i>L. plantarum</i> (2×10^9 CFU/day) + oat fibre	☺	(Olah <i>et al.</i> 2002)
Elective surgery (incidence of infections)	<i>L. plantarum</i> (2 – 8×10^9 CFU/day) + oat fibre (abdominal surgery)	☹	(McNaught <i>et al.</i> 2002)
	<i>L. plantarum</i> (2×10^9 CFU/day) + oat fibre (abdominal surgery)	☺	(Rayes <i>et al.</i> 2002a)
	<i>L. plantarum</i> (2×10^9 CFU/day) + oat fibre (transplantations)	☺	(Rayes <i>et al.</i> 2002b)
	<i>L. casei</i> , <i>B. breve</i> (3×10^8 CFU/day) + GOS (12 g/day) (abdominal surgery)	☺	
	<i>L. acidophilus</i> , <i>B. lactis</i> , <i>S. thermophilus</i> , <i>L. bulgaricus</i> (12×10^9 CFU/day) + FOS (32 g/day) (abdominal surgery)	☹	(Anderson <i>et al.</i> 2004)
	<i>L. paracasei</i> , <i>Pediococcus pentosaceus</i> , <i>Leuconostoc mesenteroides</i> (2×10^{10} CFU/day) + mixture of prebiotics* (5 g/day)	☺	(Rayes <i>et al.</i> 2005)
Inflammatory bowel disease (reduction of inflammation)	<i>B. longum</i> (2×10^{11} CFU/day) + Inulin (12 g/day)	☺	(Kong <i>et al.</i> 2004)
	<i>B. longum</i> (4×10^{11} CFU/day) + Inulin-FOS (12 g/day)	☺	(Furrie <i>et al.</i> 2005)
	<i>Lactobacilli</i> (4×10^{10} CFU/day) + mixture of prebiotics* (10 g/day)	☺	
Irritable bowel syndrome (reduction of clinical symptoms)	<i>B. lactis</i> + <i>L. acidophilus</i> + FOS	☹	(Barker <i>et al.</i> 2003)
Short bowel syndrome (reduction of inflammation)	<i>B. breve</i> + <i>L. casei</i> (10^9 CFU/day) + GOS (3 g/day)	☹	(Kanamori <i>et al.</i> 2004)
Colon cancer	<i>L. rhamnosus</i> (10^{10} CFU/day) + <i>B. lactis</i> (10^{10} CFU/day) + Inulin-FOS (10 g/day)	☺	(van Loo <i>et al.</i> 2005)

[‡]☺ improvement in GI-health parameters, ☹ no improvement in GI-health parameters,

☺ no consistent effect

*beta-glucan, inulin, pectin, resistant starch (10 g/day)

9.4.2 Non-infectious gastrointestinal diseases

Of other gastrointestinal disorders than infectious diseases, the main emphasis of pre- and probiotic research has been on inflammatory and functional bowel diseases, and colon cancer.

Inflammatory bowel disease (IBD) is a severe chronic disease that has during recent years become more common with also an earlier onset of the disease peaking before the age of 20 years (Targan and Karp 2005). The disease can be divided into two categories depending on the type and location of inflammation of the GI tract; namely Crohn's disease that affects mainly the upper GI tract, and ulcerative colitis that affects mainly the lower GI tract. The cause for these diseases is not known but the breakdown of the balance of intestinal immune responses and intestinal microbiota is thought to play a key role. Neither is any cure known. Patients are usually treated with immunosuppressive medication and when not responsive to medication by surgical removal of the inflamed areas. Both patients with Crohn's disease and ulcerative colitis have been studied, and a combination of either lactobacilli (Crohn's disease) or bifidobacteria (ulcerative colitis), (Furrie *et al.* 2005; Kong *et al.* 2004) with a mixture of fibres or inulin/FOS have been used (Table 9.2). The length of the study has varied from one month up to six months. Consistent to the reports on anti-inflammatory effects of bifidobacteria in *in vitro* studies, the use of bifidobacteria appeared to reduce expression of selected inflammatory markers also *in vivo* in both of the clinical studies (Furrie *et al.* 2005; Kong *et al.* 2004; Nurmi *et al.* 2005) However, in all of the three studies no significant effect on duration of remission or clinical scoring was observed. Perhaps longer studies are needed to show clinical efficacy.

Irritable bowel syndrome (IBS) is a functional disorder characterised by severe abdominal pain, flatulence, bloating, and either constipation or diarrhoea or a combination of those, with no or mild inflammatory symptoms (Camilleri 2006). Although it is not a life-threatening condition it is quite incapacitating. Similar to IBD, the etiology of IBS is not known, but causative alterations in the intestinal microbiota, and gastrointestinal infections are intensively studied. Barker *et al.* (2003) used a combination of lactobacilli and bifidobacteria with FOS in a randomised double blind trial to study effects of synbiotics on IBS patients. No clinical improvement due to intervention was demonstrated. However, symptoms of both the placebo and the treatment group improved during the study period, making the assessment of efficacy difficult.

A number of paediatric patients with short bowel syndrome have been studied (Kanamori *et al.* 2001, 2004). Enterocolitis was not consistently reduced. However, other clinical benefits, such as maintenance of normal microbiota during frequent antibiotic treatments and improved nutrition, were described.

Efficacy for synbiotics for colon cancer has been mainly studied in animals (see Section 9.2), and the results of those are largely promising. In humans, the development of colon cancer is a slow process, but the diet has been

suggested as either protective or contributing factor in many studies (Lim *et al.* 2005). How the effects are conveyed is not known, but again the role of the intestinal microbiota has been implicated. Based on the limited data available on synbiotics, the positive outcome in animal trials appears to be mediated by the prebiotic component rather than the combination of a prebiotics with probiotics. However, currently only a limited number of studies with proper controls can be used for evaluating the efficacy of the synbiotics. There is only one clinical study attempting to evaluate the efficacy of synbiotics for reducing risk of colon cancer (van Loo *et al.* 2005). Significant improvement of the microbial composition was described by a combination of *L. rhamnosus* and *B. lactis* with inulin-FOS but the effect on colon cancer risk awaits final analyses of the trial.

9.4.3 Non-gastrointestinal diseases

Hepatic encephalopathy (HE) is a complicated metabolic disorder, the pathophysiology of which is not fully understood. The accumulation of unmetabolised ammonia in the blood has been considered to have an important role in pathogenesis of HE as reviewed recently by Faint (2006). Circulating ammonia is produced in the colon by intestinal microbes through the degradation of amines, amino acids, purines, and urea. Acidification of the intestinal lumen modifies general bacterial metabolism, and becomes less favourable to urease-producing bacteria and favours acid-resistant, low-urease-producing species such as lactobacilli and bifidobacteria (Goldin *et al.* 1992; Riordan and Williams 1997).

Both lactulose and lactitol are known to decrease intestinal ammonia production by decreasing the colonic pH (Vince and Burrige 1980). Acidification also increases the relative amount of non-diffusible ammonia ion reducing the absorption of ammonia across the epithelium (Bircher *et al.* 1971; Castell and Moore 1971). The absorption of ammonia across the gut epithelium is also decreased by shortening the residence time of intestinal contents in the intestinal tract by non-absorbable disaccharides such as lactitol (Watanabe *et al.* 1995). In addition to disaccharides and antibiotics, probiotics have also been suggested as therapy for the treatment of HE (Solga and Diehl 2003).

Liu and coworkers (2004) showed that synbiotics or fermentable fibre can be used as an alternative treatment to lactulose in minimal hepatic encephalopathy patients with cirrhosis. Treatment with a combination of prebiotics (β -glucan, inulin, pectin and resistant starch, 2.5 g each) and non-urease producing probiotics (*Pediococcus pentosus*, *Leuconostoc mesenteroides*, *Lactobacillus paracasei* and *Lactobacillus plantarum*, 10^{10} CFU each) modified faecal microbiota composition. *E. coli*, *Fusobacterium* spp. and *Staphylococcus aureus* levels were decreased, while *Lactobacillus* species were increased and this was accompanied by decreased blood ammonia levels. As a similar effect was seen with prebiotic combination alone, it is

therefore likely that the prebiotic is the major effective component. The results, however, show that treatment of HE with different prebiotics and synbiotics may have potential besides the traditional therapy with lactulose and lactitol. The authors also suggested that oral supplementation of synbiotics may reduce translocation of pathogenic gut flora in patients with cirrhosis (Liu *et al.* 2004).

9.5 Using synbiotics in functional dairy products

Dairy products are a rich source of many nutrients; most noticeably calcium and vitamin D. In addition to this, certain fermented dairy products, like cheese, are a valuable source of vitamin K (Vermeer *et al.* 2003). Furthermore, fermented dairy products form an excellent matrix for supplementation with functional ingredients. This has made, in particular fermented, dairy products the leading functional foods and is reflected in the common inclusion of prebiotics and probiotics, and their combinations, in dairy products.

9.5.1 Potential consumer products

Probiotics are commonly added to fermented dairy products and their quality, in terms of viable counts and taxonomy, has improved over the years (Hamilton-Miller and Smith 1996; Temmerman *et al.* 2006). A number of products containing both prebiotics and probiotics are currently on the market. However, the synbiotic interaction between the two components has, in general, not been investigated and as mentioned above, merely mixing the two does not guarantee synbiotic activity. In the area of establishing health benefits, considerable work therefore remains to be done. Fermented dairy products remain, nevertheless, the most likely consumer products for supplementation with synbiotics. Although outside the scope of this book, it is likely that synbiotics will also find application in other than dairy-related products, both liquid products with high water activity and products with low water activity. In particular, in the latter the prebiotic component may fulfil several functions; prebiotic substrate, bulking agent (to provide a low calorie product), and protecting the probiotic. In this non-dairy category, synbiotics may also find application in foods for companion animals.

9.5.2 Technological applications

In addition to providing a source of fermentable energy to the probiotic component of the synbiotic, prebiotics have been suggested to also play a protective role within a synbiotic. They may provide a stabilised food matrix and favourably influence the water activity of dry products. Adding prebiotics to probiotic yoghurts has indeed been observed to improve the storage stability of the probiotic (Capela *et al.* 2006).

Prebiotics have also been investigated as protectants of a probiotic (*Lactobacillus rhamnosus* GG) during spray drying. Although the tested prebiotics, polydextrose and oligofructose, provided the same protection during the spray drying as reconstituted skim milk, oligofructose provided less storage stability at 25°C and at 37°C also the storage stability of polydextrose fell short compared to reconstituted skim milk (Ananta *et al.* 2005). Capela and co-workers (2006), however, observed reduced survival of probiotics upon freeze-drying of synbiotic yoghurt.

Alternatively, the protection of a probiotic by a prebiotic could be used in encapsulation techniques. Many probiotic encapsulation techniques rely on the use of carbohydrates as either carrier and/or coating material (Crittenden *et al.* 2001). E.g. resistant starch has been used as a carrier, but could at the same time provide targeted prebiotic benefits. In particular certain strains of bifidobacteria have been observed to have an affinity for resistant starch (Crittenden *et al.* 2001; O’Riordan *et al.* 2001). This affinity brings the prebiotic in very close proximity of the targeted probiotic and may enhance the efficacy and possibly even the dose of prebiotic required.

9.6 Future trends

The majority of the clinical trials conducted to date with synbiotic candidates do not fulfil the stringent criteria set to determine the efficacy of mixtures, active components. Therefore, much of the current data is inconclusive, and it is difficult to attribute the observed effects to a synergy of both components. However, positive results of the health-benefits of both prebiotics and probiotics administered separately are mounting. This gives a promise in finding the true synergistic interactions of synbiotics in both treatment and prevention of disease and in maintaining general well-being.

9.7 Sources of further information and advice

9.7.1 Reference book on intestinal microbiota

Ouwehand, A.C. and Vaughan, E. (2006) *Gastrointestinal microbiology*. Taylor and Francis, Inc., New York.

9.7.2 Reference books on probiotics and prebiotics

Mitchell, H. (2006) *Sweeteners and Sugar Alternatives in Food Technology*. Blackwell Publishing, Oxfordshire, UK.

Tannock, G.W. (2005) *Probiotics and prebiotics: Scientific aspects*. Caister Academic Press, Norfolk, UK.

Salminen, S., von Wright, A. and Ouwehand, A.C. (2004) *Lactic acid bacteria: microbiology and functional aspects*, 3rd edition. Marcel Dekker, Inc., New York.

9.7.3 Websites

www.syncan.be

www.eumicrofunction.be

<http://virtual.vtt.fi/virtual/proeuhealth/index.htm>

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10

Hypoallergenic hydrolysates for the prevention and treatment of cow's milk allergy

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10.1 Introduction

Breast-feeding is the most suitable form of nutrition for neonates due to the specific content of nutrients and growth and defence factors in human milk (Hanson, 1988; Hattevig *et al.*, 1990; Neville *et al.*, 1991; Wold and Adlerberth, 1998). Mother's milk usually contains low levels of food allergens from the mother's diet, including β -lactoglobulin (β -Lg), one of the main allergens in cow's milk (Huang *et al.*, 1985; Otani, 1987; Okamoto *et al.*, 1991; Selo *et al.*, 1998). Recent theories now propose that favourable bifidogenic-dominant gut flora of breast-fed infants and the low allergen load of mother's milk may promote the induction of oral tolerance towards food proteins (Heine, 1999; Kirjavainen *et al.*, 1999).

When exclusive breast-feeding during the first months of life is not possible, mother's milk must be replaced or supplemented with infant formulas, which are often based on unhydrolysed cows's milk. In predisposed infants, the result may be sensitisation and immunological response to cows's milk proteins (CMP) (Holt and Jones, 2000). This syndrome is known as cow's milk allergy (CMA), that it is mainly an IgE mediated hypersensitivity reaction where multiple sensitisation to different CMP is observed in about 75% of milk allergic patients (Fritsché, 2003). It should be clearly distinguished from non-immunological adverse reactions such as lactose intolerance, which is due to lactose deficiency occurring in large sections of the general population. The three-dimensional structure of proteins seems to be an important feature in cow's milk protein allergenicity, although no definite relationship can be established between protein structure and allergenicity.

Numerous allergenic structures (epitopes) have been identified in the

proteins' molecules. Besides the conformational epitopes, IgE-binding studies show the presence of sequential epitopes, located in strongly hydrophobic regions and/or within the protein molecule. These linear epitopes are peptides as short as 12–14 amino-acid residues (about 1500 Da molecular weight) and they have been demonstrated to account for a significant part of the allergenicity of the whole protein molecule in some patients. The protein structure and the location of IgE-binding epitopes, particularly the evidence of importance of sequential epitopes, have implications for the effects of technological/physiological processing on milk allergenicity.

The prevalence of CMA ranges from 0.5% to 7.5% in children in the first half year of life (Høst *et al.*, 1988), although CMA generally resolves by three years of age. However, it may be a precursor to other, and sometimes more serious, atopic diseases, asthma, and respiratory allergies (Kulig *et al.*, 1998; Exl *et al.*, 2000). The prevention of CMP sensitisation, early allergy and atopic dermatitis is therefore an important strategy in limiting atopic disease in later childhood and adulthood, and research efforts have been put into prevention strategies (Exl, 2001). Because infants may absorb more food allergen during their first year than later in life, the best strategy to prevent food sensitisation is to avoid the relevant allergenic proteins (Dannaes *et al.*, 1978; Chandra *et al.*, 1985; Rowntree *et al.*, 1985; Hattevig *et al.*, 1990; Bousquet and Michel, 1991), with exclusive breast-feeding as a first option. Maternal elimination of cow's milk may be required, although the decision is based on the persistence (or not) of clinical symptoms during exclusive breast-feeding. The second option is the use of hypoallergenic (HA) cow's milk based formulas during the first six months of life, mainly in infants from atopic families (at risk infants) that are partially breast-fed or not breast-fed at all. Their broad classification into extensively (eHF) and partially/moderately hydrolysed formulas (pHF) is based on degree of proteolysis (Aggett *et al.*, 1993; Businco *et al.*, 1993), but there is no unanimous agreement on this classification. Although molecular weight is an important classifier, studies have shown that products with hydrolysates of comparable molecular weight may have different preventive or treatment effects so molecular weight may be of less predictive value than suggested (Halken *et al.*, 2001).

Depending on primary or secondary prevention of CMA, the approach with HA formulas is different. When infants are already sensitised to CMP, low amounts of cow's milk may trigger an allergic reaction, and it has been stressed by both the European Society of Paediatric Allergology and Clinical Immunology (ESPACI, 1993) and European Society of Paediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) that only the eHF or amino acids formulas should be used for infants with established CMA and, the pHF with confirmed reduced allergenicity for primary prevention of CMA in non-sensitised at risk infants (Høst *et al.*, 1999). A statement from the American Academic of Paediatric (AAP) on the topic of HA infant formulas indicates for the first time that pHF may be of benefit in long-term protection

to CMA. Such formulas are sufficiently reduced in allergenicity that they do not sensitise at risk infants but still contain enough immunogenicity to deviate the immune system from hypo sensitivity against cow's milk proteins (Fritsché, 2003). Thus, an optimised pHF should contain sufficient allergens to induce oral tolerance, but a sufficiently low allergen content to minimise sensitisation. Such formulas should also support the establishment of bifidogenic gut flora, which depends on a low content of phosphate and iron, sufficient lactose, and small amounts of protein. They must also be highly nutritious with adequate bioavailability, and have low allergenicity (Exl, 2001; Fritsché, 2003). Although legislation is scarce, all CMP hydrolysates for nutrition of allergic patients must fulfill a series of conditions that would guarantee the suitability of their consumption by these patients (Kleinman *et al.*, 1991; Kleinman, 1992). These requirements include demonstration of hypoantigenicity by physicochemical and immunochemical tests, tolerance by at least 90% of infants or children with confirmed dairy proteins allergy with 95% confidence, when given in prospective randomised, double blind, placebo-controlled trials, as well as positive results in weight gain and nitrogen balance. Formulas that fulfill these criteria may be said to prevent or delay allergic reactions to milk and can be called hypoallergenic.

In the present chapter, methods for the characterisation and safety evaluation of HA formulas, as well as the potential of high pressure (HP) and microwave irradiation (MWI) to produce protein hydrolysates with reduced antigenicity will be discussed. Initially some general aspects of infant formulas are briefly examined.

10.2 Determining the safety and efficacy of hypoallergenic infant formulas

Hypoallergenic protein hydrolysates to be used as an ingredient in infant formulas of high nutritional and therapeutic value should be rich in low molecular weight peptides, especially di- and tripeptides, with the least quantity possible of free amino acids. Sometimes it is difficult to know the composition of hydrolysates because of a large number of possible constituents, due mainly to potential degrees of polymerisation of the peptides, and several analyses may be done to assess their suitability. In addition to degree of hydrolysis, *in vitro* characterisation of peptide size and determination of allergenicity are valuable for quality control of the products and assurance of batch to batch consistency as well as for labelling. However, on the basis of the current knowledge, such data do not predict the immunogenic or the allergenic effects in the recipient infant, and the safety and efficacy of HA infant formulas can only be determined by clinical trials using scientifically appropriate standards. Some of the advances on these topics are discussed below.

10.2.1 Molecular characterisation of hydrolysates

The molecular properties of proteins change as a result of hydrolysis. Hydrolysis, for example, can lead to increased charge, exposure of hydrophobic groups and revelation of reactive amino acid side-chains (Nielsen, 1997; Caessens *et al.*, 1999). Important indicators of the extent of hydrolysis are the degree of hydrolysis (DH) and molecular weight distribution (MWD). Further separation and analysis of hydrolysis products by chromatographic methods and capillary electrophoresis (CE) provides complementary information on the properties of hydrolysates. The availability of on-line spectroscopic methods provides a whole new additional domain of information, which can aid greatly in the determination of peak identity.

Degree of hydrolysis

The degree of hydrolysis (DH) represents the percentage of peptide bonds released by enzymatic hydrolysis. There are several methods to evaluate this parameter, such as quantification of amino groups using compounds that react specifically with them, yielding chromogenic derivatives that can be detected spectrophotometrically; titration of the released protons after the cleavage of peptide bonds at determined pHs; and determination of soluble nitrogen in trichloroacetic acid or others precipitation agents.

The oldest technique for quantification of α -amino groups uses ninhydrin (Moore and Stein, 1948), which reacts with them to produce a heavy blue product that is detected at 570 nm. This method is very sensitive but present several disadvantages, such as the interference of ammonia and the oxygen sensitivity of the reagent. In addition, the analysis requires a lot of time, due to the heating and cooling phases required for chromophore formation (Turgeon *et al.*, 1991). The utilisation of trinitrobenzenesulphonic acid (TNBS) and orthophthaldialdehyde (OPA), other specific reagents for α -amino groups released by the hydrolysis of a peptide bond, for the analysis of protein hydrolysates was proposed by Adler-Nissen (1979) and Church *et al.* (1983), respectively. Samples are incubated with TNBS for 1 hour at 37°C, and subsequently the absorbance is measured at 420 nm. Some disadvantages of this technique are the duration of the analysis, reagent contamination by picric acid giving high values of blank, the interference of reducing sugars and ammonia, and the lack of reactivity of proline and hydroxyproline. Moreover, the ϵ -amino groups of lysine are also capable of reacting with TNBS, altering the results (Silvestre, 1997). In contrast, the reaction of α -amino groups with o-phthaldialdehyde (OPA) takes place quickly at room temperature (Church *et al.*, 1983) and quantification of coloured compound can be done after 2 min of incubation by measuring the absorbance at 340 nm. However, this method also presents some disadvantages, since cystein reacts only slightly and proline does not react at all with OPA, and the OPA derivatives are only stable during the first 20 min (Panasiuk *et al.*, 1998). The results obtained with OPA and TNBS correlate well, whereas those with ninhydrin give in general much lower DH values.

Another method of evaluation of DH consists of the quantification of the protons released during proteolysis by addition of acids or bases. This method is known as pH-stat. The amount of acid or base added is related to the amount of dissociated amino groups and released protons, since the amino and carboxyl groups are more or less (de)protonated depending on the pH of the solution. This method is generally used for continuous determination of DH, due to its simplicity, rapidity and reproducibility (Silvestre, 1997). It is not a denaturant technique, but it presents the disadvantage that the protonation and deprotonation of the acid/base groups are in equilibrium at pH 5–6, and consequently the method is not applicable at these pHs. In addition, the pH values, are not constant during enzymatic hydrolysis since they depend on the peptide chain length and on the side chain of the terminal amino acid (Diermayr and Dehne, 1990; Camacho *et al.*, 2001). For this reason, the values of DH determined by this method need to be checked by other methods.

The nitrogen content of the soluble fraction in TCA also gives information on the yield of enzymatic hydrolysis and it can be evaluated by the Kjeldhal method (A.O.A.C., 1995), spectrophotometric analysis in the visible region after Biuret reaction (Hung *et al.*, 1984), or in the UV region by quantification of peptides with aromatic groups (Pelissier, 1984).

Molecular weight distribution (MWD)

The estimation of MWD of peptides in hydrolysates may be useful for predicting their antigenicity and functional properties, as well as for showing differences in the effects of different denaturant pre-treatments on the substrate or proteases used. There are different methods to determine the nature and MWD of peptides in a hydrolysate and, the most widely method used is sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) because of its simplicity and rapidity (Haque and Mozaffar, 1992; Parrado *et al.*, 1993; Leaver and Thomson, 1993; Schmidt and van Markwijk, 1993; Perea *et al.*, 1993; Guo *et al.*, 1995; Galvao *et al.*, 2001; Calvo and Gómez, 2002). Several authors studied the proteolysis of dairy whey proteins (WP) (Schmidt and Poll, 1991), bovine and ovine serum albumin hydrolysed by different proteases (Fiocchi *et al.*, 1995) using SDS-PAGE. Hayashi *et al.* (1987) successfully employed this method to study the selective proteolysis of β -Lg in WP concentrate when high pressure (HP) in combination with thermolysin was used. Other authors also studied the combined effect of HP and enzymatic treatment on MWD of peptides in hypoallergenic hydrolysates from WP (Peñas *et al.*, 2006a) or purified bovine β -Lg (Maynard *et al.*, 1998; Bonomi *et al.*, 2003). However, in molecular weight range below a few thousand Da the SDS-PAGE method is operating near the limits of resolution, and small peptides are lost during the colouring and washing steps (Silvestre, 1997).

Gel permeation (Adachi *et al.*, 1991; Tchobanov and Iliev, 1993; Chen *et al.*, 1995) and size exclusion chromatography (SE-HPLC) are alternative

techniques to SDS-PAGE (Visser *et al.*, 1992), providing fast quantification of peptides and estimation of their MWD (Irvine, 2003). TSK columns have been used for these purposes (Mahmoud *et al.*, 1992; Nakamura *et al.*, 1993; González-Tello *et al.*, 1994; Guadix *et al.*, 2000).

Peptide and amino acid composition

Evaluation of peptide and amino acid contents of hydrolysates can be done directly with or without a preliminary step for the separation of nitrogen components. Quantification of amino acids by the Kjeldahl procedure and by using an amino acid analyser are the oldest methods (Mont and Jost, 1978; Cogan *et al.*, 1981; Adachi *et al.*, 1991; Zhang *et al.*, 1992; Parrado *et al.*, 1993). Silvestre *et al.* (2003) determined amino acids and peptides contents from casein hydrolysates using a cuprimetric assay, described by Lati *et al.* (1992). This method seems to provide better results than the classical α -amino nitrogen assay for differentiating between homogeneous hydrolysates and mixtures consisting of amino acids and poorly hydrolysed proteins. Besides, this technique showed no interference by lipids and carbohydrates and could be used for analysing commercial preparations that generally contain these compounds (Silvestre, 1997).

The ability to separate and recover peptides in nanomole amounts makes reversed-phase high performance liquid chromatography (RP-HPLC), based on the difference in hydrophobicity between amino acids, a particularly convenient means of separating peptides for hydrolysates characterisation. For small peptides (< 15 residues), the elution time from the column is determined by the hydrophobicity of the amino acid side chains in the peptides (Meek, 1980; Guo *et al.*, 1986; Hearn *et al.*, 1988), while the peptide length also influences for larger peptides (Pearson *et al.*, 1984; Mant *et al.*, 1988; Chabanet and Yvon, 1992). In the literature, the application of RP-HPLC has been reported for separation of peptides in hydrolysates from β -casein (Maynard *et al.*, 1998; Knudsen *et al.*, 2002; Bonomi *et al.*, 2003; Izquierdo *et al.*, 2005) or whey proteins and different proteases (Perea *et al.*, 1993; Peñas *et al.*, 2006a, b); tryptic and chymotryptic hydrolysates from α s1-casein and β -casein, as well as κ -casein hydrolysed by rennet (Yvon *et al.*, 1989); separation of A and B casein-macropptides obtained by hydrolysis of κ -casein by rennet (Imbert and Nicolas, 1993); detection of low contents of milk protein hydrolysates in soybean protein preparations (Dziuba *et al.*, 2004). Another use of RP-HPLC relates to the separation and characterisation of bioactive peptides obtained by enzymatic hydrolysis. Thus, the formation of opioid peptides from proteolysis of bovine caseins and β -casein has been evaluated by Pihlanto-Leppäla *et al.* (1994) and Bouhallab *et al.* (1993), respectively. Recently, Gibbs *et al.* (2004) separated bioactive peptides from soy-fermented foods and enzymatic hydrolysates from soybean.

Other techniques for separating peptides from protein hydrolysates are capillary-HPLC using micro columns having a diameter below 1 mm (Davis and Lee, 1992), ion-exchange chromatography (Dizdaroglu, 1985), ligand

exchange chromatography (Salmona *et al.*, 1982; Aubry *et al.*, 1992), fast protein liquid chromatography (FPLC) (Leaver and Thomson, 1993; Harwalkar *et al.*, 1993), size-exclusion chromatography (SE-HPLC) (Cordle *et al.*, 1991; Mahmoud *et al.*, 1992; Guadix *et al.*, 2000; de Freitas *et al.*, 1993; Boza *et al.*, 1994, 1995; Sato *et al.*, 1996; Lin *et al.*, 1997; Madsen and Qvist, 1997; Otte *et al.*, 1997; Iametti *et al.*, 2002) and gel permeation chromatography in high performance liquid mode.

FPLC has been used by Gallagher *et al.*, (1994) in the characterisation and MWD of bitter peptides from casein hydrolysates. In addition, Picot *et al.*, (2004) and Kappel *et al.* (2006) used FPLC system, respectively, for characterising fish protein and alfalfa white protein hydrolysates, in this last case hydrolysates obtained by continuous proteolysis of an industrial concentrate. Different soft gels have been reported as the support in SE-HPLC, such as Sephadex G-25 (Amiot and Brisson, 1980; Zhang *et al.*, 1992), Sephadex G-10 (Pellerin *et al.*, 1985; Landry *et al.*, 1988) and Bio-gel P-2 (Iliev and Tchobanov, 1992). In addition, different silica gels chemically bonded with hydrophilic compounds are commercially available (Silvestre, 1997) and are used in the SE-HPLC separation of peptides from protein hydrolysates (Vijayalakshmi *et al.*, 1986; Lemieux *et al.*, 1991; Visser *et al.*, 1992). The different methods mentioned above show limitations for the separation of small peptides with molecular weight lower than 1000 Da (Lemieux and Amiot, 1989, 1990; Lemieux *et al.*, 1991), and SE-HPLC shows secondary interactions with the stationary phase (Kopaciewicz and Regnier, 1982; Golovchenko *et al.*, 1992). The development of efficient methods to separate these peptides is important, especially in the production of hydrolysates to be used in the formulation of enteral diets and infant formulas, where essentially di- and tripeptide are required.

The availability of on-line spectroscopic methods can aid greatly in the characterisation of hydrolysates. In addition to retention time information, which in a complex peptide map from enzymatic hydrolysates is of only limited use, mass spectra or ultraviolet (UV) spectra can be employed to gain further insight into the chemical makeup of a chromatographic peak (Sievert, 1996). The introduction of electrospray ionisation and related liquid chromatography-mass spectrophotometry (LC-MS) technology (Léonil *et al.*, 2000), gas chromatography coupled with mass spectrometry (Ford *et al.*, 1986; Aubry *et al.*, 1992) or mass spectrometry by fast atom bombardment (Pucci *et al.*, 1992) makes the use of molecular weight information for the identification of peptide fragments possible. However, these techniques require expensive equipment and an experienced operator, and at this point cannot be considered suitable for routine use. HPLC with diode array detection (HPLC-DAD), is a much simpler and less expensive technique that can also aid in the identification of peptides. However, comparison of the spectral properties of an unknown peak to those of well characterised standards is needed to arrive at the most likely identification.

10.2.2 Predictive testing of hydrolysates allergenicity

In addition to physicochemical characterisation of hydrolysates described above, currently several methods may be applied to generate some relevant information with respect to the antigenicity and allergenicity of hydrolysates. Allergen testing should have a definite place in hazard analysis of critical control plans (HACCP) of hypoallergenic hydrolysates. There is clearly a need for analytical methods, which are rapid, robust, reliable, and cost-effective.

In vitro immunochemical assays such as enzyme-linked immunosorbent assay (ELISA), radioallergosorbent (RAST) and enzyme allergosorbent (EAST) tests, as well as protein (Western) blotting method, are very useful to characterise the presence of reactive epitopes in the hydrolysates or in the study of possible immunological cross reactivity and, thus to determine suitability of hypoallergenic products. In addition, these analyses can be used to determine the possible sensitisation of patients (diagnostic procedures) when sera collected from them are used as antibodies. On the other hand, several well validated assays are available for determining the antigenicity of proteins/peptides. These assays are based on parenteral application of the test sample to laboratory animals. Guinea pigs and rats are the most regularly used test species.

In vitro immunochemical methods

Immunochemical methods involving specific serum IgE from sensitised subjects are suitable for the characterisation of food allergens. However, these serological tests are not very suitable for reliable allergen determination in food, since the specificity of IgE from sensitised individuals differs considerably and the amount of serum is usually limited. Besides, cross-reactivities to more than one allergenic food may be present in human serum IgE. In order to overcome the disadvantages associated with the use of human sera, immunoassays relying on antisera raised by repeated immunisation of suitable animals such as rabbits, mice, goats, sheep, and more recently chickens have been developed. These antibodies detect antigens used for immunisation rather than allergens.

ELISA is the method most frequently used by food industries and agencies for food inspection and allergen control in foods (Poms and Anklam, 2004). This method may be used for determining IgG and IgE binding epitopes potentially present in protein hydrolysates, using animal and human patient sera, respectively. Two approaches are available for the semi-quantification of milk antigens: sandwich and competitive ELISAs. The assays combine the specificity of antibodies with the sensitivity of simple enzyme assays, by using antibodies or antigens coupled to an easily assayed enzyme that possesses a high turnover number. ELISAs can provide a useful measurement of antigen or antibody concentration.

The sandwich ELISA requires two antibodies that bind to epitopes that do not overlap on the antigen. This can be accomplished with either two monoclonal antibodies that recognise discrete sites or one batch of affinity-

purified polyclonal antibodies. To utilise this assay, one antibody (the 'capture' antibody) is purified and bound to a solid phase attached to a solid phase support on a plate well. Antigen is then added and allowed to complex with the bound antibody. Unbound products are then removed with a wash, and a labelled second antibody (the 'detection' antibody) is allowed to bind to the antigen. The assay is then quantitated by measuring the amount of labelled second antibody bound to the matrix, through the use of a colorimetric substrate. The competitive ELISA assay is preferred for the detection of small proteins. Although there are several different configurations for this assay, briefly, the animal anti-sera are incubated with the diluted test sample before being added to the solid phase (wells of microtiter plates) which contains the immobilised antigen. The measured absorbance is inversely proportional to the concentration of antigen in the sample.

The detection of β -Lg epitopes by ELISA has been reported by several authors. Otani *et al.* (1987) investigated the immunological properties of quimotryptic digests of cow milk caseins, by direct ELISA using rabbit's sera, and observed reduced their immunoreactivity. Rosendal and Barkholt, (2000) studied the β -Lg content of twelve commercial hypoallergenic milk formulas by using a direct ELISA and purified rabbit anti- β -Lg antibodies. Iametti *et al.*, (2002) assessed the immunochemical properties of the products of β -Lg hydrolysis by competitive ELISA by using monoclonal or polyclonal rabbit anti- β -Lg antibodies. Cave and Guilford (2004) reported reduced residual antigenicity of chicken-protein hydrolysates, when canine serum IgG produced by dogs sensitised to the intact protein was used. Peñas *et al.*, (2006a) observed reduced residual immunochemical reactivity in WP hydrolysed under high pressure by trypsin, chymotrypsin and pepsin, when a direct ELISA test and a pool of seven sera from allergic children to bovine milk was used. The same group (Peñas *et al.*, 2006e) also studied the combined effect of high pressure (HP) and enzymatic treatments on the residual antigenicity of hydrolysates from bovine whey proteins (WP) by competitive ELISA test using polyclonal rat anti- β -Lg antibodies. In this case four food grade protease preparations (alcalase, neutrase, corolase 7089 and corolase PN-L) were used.

An alternative to conventional ELISA may be flow systems, which have been applied to perform immunoassays (IAs) for some analytes, such as an enzyme linked immunoaffinity chromatography (ELIAC). The flow systems allow greater control of reaction times, of addition of reactives, and also of the kinetics, compared with classic IAs (de Frutos *et al.*, 1996). Puerta *et al.* (2006) detected β -Lg at the pM level in a formula based only on caseins, and at the nM to μ M level in those based on hydrolysed whey proteins, applying the ELIAC method.

RAST and EAST methods allow the detection of allergen-specific antibodies in human sera, after their incubation with optimal amounts of the allergen insolubilised onto a solid phase. The response is measured as counts per minute bound to the allergosorbent or optical density in RAST or EAST,

respectively. The signals are directly or inversely proportional to the amount of specific IgE antibody in the original serum, depending on the use of direct (Fig. 10.1) or inhibition tests (Fig. 10.2), respectively (Hamilton and Adkinson, 2003). Although the original description of the RAST procedure was based on the use of dextran-derived materials (Wide *et al.*, 1967), nowadays other solid phases are widely used such as cellulose discs (Ceska *et al.*, 1972), polystyrene tubes (Zeiss *et al.*, 1973; Poulsen *et al.*, 1989), microtitre plates (Olivieri *et al.*, 1993), magnetic microparticles (Kleine-Tebbe *et al.*, 1992) or aluminium hydroxide gel (Poulsen and Weeke, 1985). Fremont *et al.* (1996) investigated the presence of α -lactalbumin allergen from cow's milk as a contaminant of an infant product guaranteed free of cow's milk proteins, detecting 1 μ g of this protein in 1 g of the baby product using the RAST test.

Immunoblotting technique combines the resolution of gel electrophoresis with the specificity of antibody detection. Native polyacrylamide gel electrophoresis (PAGE) can be used to separate individual proteins, by size,

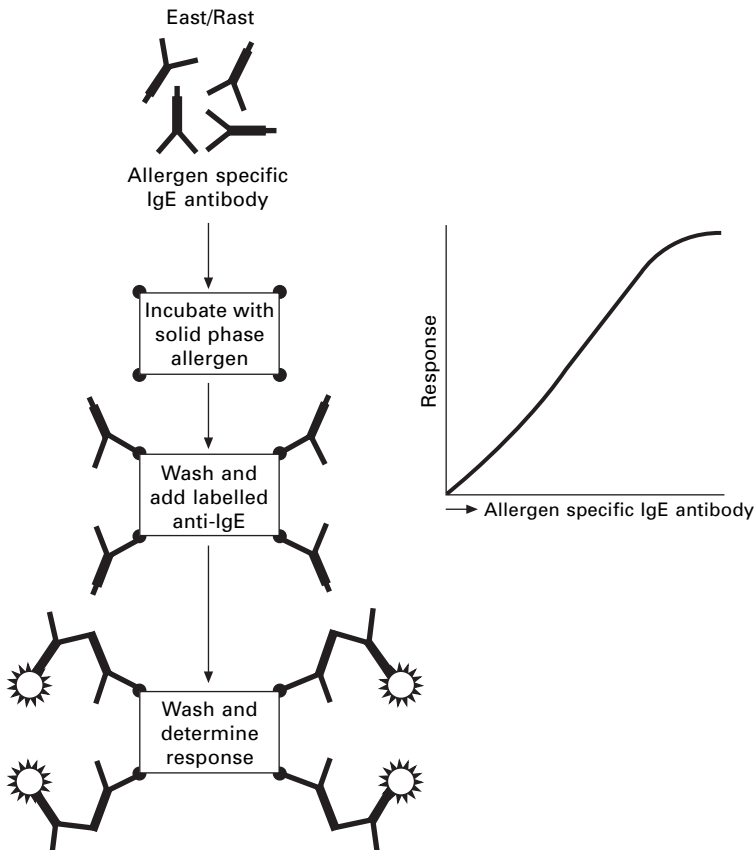


Fig. 10.1 Scheme of the steps involved in the allergosorbent direct tests (RAST/EAST). (Hamilton and Adkinson, 2003).

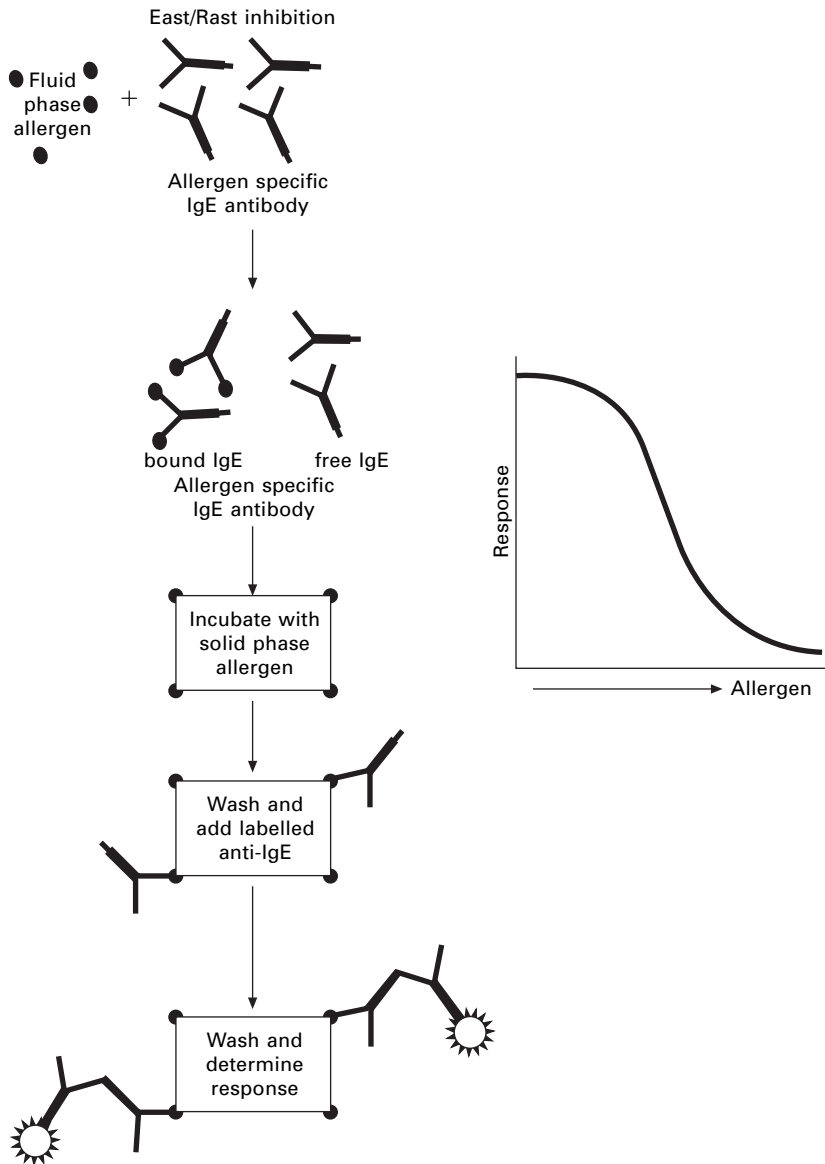


Fig. 10.2 Scheme of the steps involved in the allergosorbent (EAST/RAST) inhibition assays. (Hamilton and Adkinson, 2003).

charge and shape, and it can also be used under denaturing conditions, in the presence of a molar excess of sodium dodecyl sulfate (SDS-PAGE) and/or under reducing conditions using beta mercaptoethanol or dithiothreitol (DTT). Transfer of the proteins fractionated by PAGE to a solid support membrane can be accomplished by either capillary blotting or by electroblotting, this last the most efficient and widely used method. In this procedure, a sandwich

of gel and solid support membrane is compressed in a cassette and immersed in buffer between two parallel electrodes. The efficiency with which a particular antigen will be transferred to the blot is dependent on the protein-binding capacity of the membrane used, the transfer method and conditions employed as well as the nature of the antigen itself. A blotting membrane that binds all proteins at the same degree is not available because of the heterogeneous nature and size of proteins/peptides. The choice of blotting membrane is a key step in the procedure. Nitrocellulose, the most widely used, binds proteins via hydrophobic interaction regardless of the charge of the proteins, giving reliable and reproducible results. However, this kind of membrane presents certain disadvantages, such as its fragility and poor retention of low molecular weight proteins (Lin and Kasamatsu, 1983; Harper, *et al.*, 1990). Nowadays, polyvinylidene difluoride (PVDF) and polypropylene membranes are employed because they present greater mechanical strength than nitrocellulose. Positively charged nylon membranes that bind negatively charged proteins are also used, however they require careful blocking and, only certain kinds of stains may be used because of the positive charge of the membrane (Gershoni and Palade, 1982).

Several authors have used the immunoblotting method for detecting residual antigenicity of hypoallergenic infant formulas and protein hydrolysates from different origins. Restani *et al.*, (1995) evaluated the reactivity of circulating IgE (from sera of children with CMA) with the residual intact proteins and with the peptides present in commercial hydrolysed formulas. Docena *et al.*, (2002) investigated the presence of residual antigenic and allergenic components in different milk substitutes employed in the prevention and treatment of CMA. According to the authors, residual cow's milk proteins in pHF and eHF from different mammalian milks, as well as cross-reactive proteins in different mammalian milks can be detected by immunoblotting using a cow's milk specific antiserum and casein specific monoclonal antibodies. Restani *et al.*, (2000) studied the potential presence of bovine proteins in human milk, as a possible cause of allergic symptoms in breast-fed children, by using monoclonal antibodies specific for β -Lg and caseins. The authors conclude that the presence of β -Lg in human milk is due to cross-reactivity between bovine milk and human proteins. Beretta *et al.* (2001) identified the BSA antigenic determinants after limited enzymatic hydrolysis of the protein by using commercial murine IgG antibody and sera from children sensitised to BSA. Sequential and conformational epitopes of BSA in homogenised and freeze-dried derivatives of beef were detected by using this technique and sera from children allergic to beef (Restani *et al.*, 2004). The residual immunoreactivity of WP hydrolysates obtained by combination of enzymatic hydrolysis by five food grade proteases and high pressure treatment was assessed by immunoblotting using PVDF membrane and anti β -lactoglobulin monoclonal antibodies, and the sera from pediatric patients allergic to cow's milk proteins by Peñas *et al.* (2006d). Moreover, anti-cow's milk protein polyclonal antibodies were also used to evaluate the

presence of residual trace amounts of casein in the hydrolysates. By using sera from children allergic to soybean proteins, the same group (Peñas *et al.*, 2006b) could detect and identify residual antigenic components in soybean whey hydrolysates.

In vivo animal models

Several tests based on oral/parenteral application of the test proteins to laboratory animals, can be applied to qualitatively and quantitatively determine specific immune responses and immune-mediated effects as a measure for the antigenicity and allergenicity of the test proteins. In general, the parenteral antigenicity assays demonstrate highest sensitivity. The two phases of IgE dependent allergic reaction, inducing and provoking phases, are commonly examined by using *in vivo* animal models. The immune system of the host is sensitised by the allergen with the production of specific IgE anti-allergen antibodies which are then fixed by mast cells in target organs; the second step or provoking phase is mediated by the allergen binding to these IgE and stimulating mediator (histamine) release from mast cells. Historically, guinea pigs (Devey *et al.*, 1976; Coombs *et al.*, 1978; Anderson *et al.*, 1979; McLaughlan *et al.*, 1981a, b; Baird *et al.*, 1984; Pahud *et al.*, 1985, 1988) and rats (Fritsché and Bonzon, 1990) have been used to study immunoreactivity of food proteins. Guinea pigs may be sensitised by the oral via without adjuvants, making them good models close to the human situation, although their use to study CMA is limited because of difficulties associated with passive cutaneous anaphylaxis testing.

In contrast, the rat has been reported to be a suitable model for the investigation of food allergy in general and, dairy products in particular. Intraperitoneal sensitisation of rats generates IgG and IgE antibodies to a range of milk proteins that are of similar specificity to those produced by humans (Atkinson and Miller, 1994). The parenteral rat model provides also a good indication of the IgE inducing capacity of standard and hypoallergenic infant formulas. Fritsché and Bonzon (1990) reported that pHF and eHF induced, respectively, 100 and 10000 times fewer IgE antibodies than a standard milk formula, when this *in vivo* test was used. In the rat model, the specific protease (RMCP II) is released into blood after intestinal mast cell triggering (Fritsché 2003), a protease that can be determined by ELISA in serum and, which is an indicator of the IgE mediated allergic provoking capacity of infant formulas at the intestinal level. However, the oral murine models do not seem suitable models because of their innate tendency to develop oral tolerance to ingested antigens.

10.2.3 Assessment of allergenicity of hydrolysates

Skin sensitisation tests in human

The skin prick test (SPT) provides a useful and rapid method to screen patients with suspected IgE mediate food allergies in general (Bock *et al.*,

1978; Sampson, 1999), and allergy to cow's milk in particular. The basis of the method is that by introducing a small volume of allergen in the skin, either intradermally or via a small puncture, the skin mast cells sensitised with specific IgE are activated via allergen cross-linking of this IgE. The activation of mast cells results in release of mediators, primarily histamine, which induces a reaction of the skin. The biological response is measured as the area of the wheal or the flare (Poulsen *et al.*, 1993, 1994), calculated either with planimetry, which is time-consuming and less precise, or by means of a computerised scanning method (Petersen *et al.*, 1994).

SPT responses may be standardised by dividing the original wheal area of a prick by that obtained for the histamine control (Bolhaar *et al.*, 2005). Positive SPT responses suggest the possible association between the food tested and the reactivity of the patient to that specific food. However, false positives results may occur because of skin irritation caused by bleeding. Besides, the degree of skin response does not correlate always with the severity of the clinical reaction on the ingestion of the food (Ives and Hourihane, 2002) and, the positive predictive accuracy of the test is only of 50–60% (Bock *et al.*, 1977). Moreover, children less than two years of age may have smaller wheals, probably because of lack of antigen-specific IgE and skin reactivity (Menardo *et al.*, 1985). For these reasons the interpretation of results should be made with caution (Norgaard and Bindslev-Jensen, 1992; Bock *et al.*, 1978; Sampson and Albergo 1984; Sampson, 1988; The British Nutrition Foundation, 2002; Sicherer and Sampson, 2006).

The use of skin patch test is for research studies rather than clinical, since allergy diagnosis by patch tests are less frequently positive than by SPT (Rasanen *et al.*, 1992). However, combining patch tests with other assays such as SPT or detection of specific IgE in serum increases the detection rate of cases with delayed type of cow's milk hypersensitivity (Saarinen *et al.*, 2001). Niggemann (2001) has shown an increase of the positive predictive value to 100% when the patch test and a positive SPT were used. The main question surrounding the use of skin sensitisation tests in human is that of their ethical acceptance. This has limited the use of such studies in many countries with some resistance by authorities to accept data generated by them and no internationally agreed test protocols are available, although several approaches are supported by published data (Botham *et al.*, 1991; Bolhaar *et al.*, 2005).

Double-blind placebo controlled food challenges (DBPCFC)

The ultimate determination of the biological activity of food allergens is the effect on sensitised food allergic patient. This is normally carried out by DBPCFC, that it is considered the gold standard in diagnosis of food allergy (Benlounes *et al.*, 1996; Bindslev-Jensen *et al.*, 2004; Sicherer and Teuber, 2004), and is recommended by the European Academy for Allergy and Clinical Immunology (1993) as the only conclusive evidence of a food allergy, where a patient suspected of food allergy is challenged and observed in an allergy

clinic. The patients receive the suspected food (active) on one occasion and a placebo on another occasion, in order to investigate the spontaneous variation of the disease. Thus, the challenge procedure becomes placebo-controlled. To further reduce the psychological factor, the food is given to the patient in a double-blind fashion and, neither the patient nor the health care personnel know on which day the patient receives the active and the placebo. The experiment begins with exclusion of the suspect foods for 7 to 14 days before the challenge. Then, an equal number of randomly alternating food allergen and placebo challenges, starting with 125 to 500 mg of lyophilised food, are administered to the patient, doubling the dose every 15 to 60 minutes. Clinical reactivity can be ruled out once 10 g of lyophilised food is tolerated (Hamilton and Adkinson, 2003). To avoid possible false-negative results, negative DBPCFCs must be confirmed by open feedings (or single-blind challenges). The average false-positive rate has been reported to be 0.7% and the false-negative rate of 3.2% (Sampson, 1998).

If the formula being tested is not derived from cow's milk proteins, the formula must also be evaluated in children with documented allergy to the protein from which the formula was derived (American Academy of Pediatrics, 2000). It is also recommended that after a successful double-blind oral challenge, an open challenge using an objective scoring system to document allergic symptoms during a period of seven days is performed (Kleinman *et al.*, 1991), in order to detect late-onset reactions to the formula (Hill *et al.*, 1995). For both practical and ethical reasons, the patients can not be routinely challenged to allergenic preparations, and the main reason for conducting controlled food challenges in patients is to verify or rule out suspicion of food allergy for their benefit.

10.3 New methods to produce hypoallergenic hydrolysates

The use of protein hydrolysates by the food industry is increasing because they have properties that make them attractive as a source of essential amino acids in human nutrition (Peñas *et al.*, 2004). Nowadays, protein hydrolysates are included in specific formulations, such as geriatric products, high-energy supplements, weight control and therapeutic diets (Frokjaer, 1994; Schmidt *et al.*, 1994), as well as in infant formulas for children with CMA, due to the reduced antigenicity of the hydrolysates in comparison to intact protein (Clemente, 2000). The hydrolysates are physiologically better than elementary diets in which the nitrogen components consist exclusively of a mixture of free amino acids. Absorption of amino acids from short chain peptides is more efficient than the equivalent amount of free amino acids (Siemensma *et al.*, 1993). On the other hand, peptides are less hypertonic than free amino acid mixtures, favouring the absorption of other dietary components (Adibi, 1989; Parrado *et al.*, 1991).

Enzymatic proteolysis and heat treatment are normally used to reduce the content of β -Lg and other intact proteins and therefore reduce the antigenicity of milk proteins in the production of pHP or eHF from cow's milk. These techniques are also used to prepare hypoallergenic formulas based on protein hydrolysates from other sources (Pahud *et al.*, 1985; Nakamura *et al.*, 1993; Siemensma *et al.*, 1993; Wahn, 1995). Heat treatment and enzymatic hydrolysis seem to affect to conformational and sequential epitopes, respectively, depending on the temperature used. At temperatures below 60–65°C, only reversible modifications of pure β -Lg at neutral pH occur, and the hydrophobic protein cores are unfolded and solvent exposed (Cairolì *et al.*, 1994, Iametti, 1996, 1997; Roefs and de Kruif, 1994; Bonomi *et al.*, 2003), thus making the inner parts of protein accessible to enzymes. At higher temperatures, dissociation of the β -Lg dimer into monomers occurs, and soluble and relatively small aggregates are formed in heated solutions (Cairolì *et al.*, 1994, Iametti *et al.*, 1997; Bonomi *et al.*, 2003), that may hide sites of attack from the action of proteases, some of which may be relevant to allergenic properties of the protein. Finally, ultrafiltration is commonly used as post-hydrolysis process, for removing residual high-molecular weight peptides and proteins and the enzymes used in the digestion, contributing to the reduction of the antigen content of hypoallergenic formulas (Gorthler *et al.*, 1995).

The residual antigenicity of infant formulas will depend on the hydrolysis grade obtained, the specificity of the enzyme and the filtration technique used. However, residual allergenicity has been reported in several of these commercial preparations (Oldaeus *et al.*, 1991; Restani *et al.*, 1995; Van Beresteijn *et al.*, 1995; Rosendal and Barkholt, 2000; Caffarelli *et al.*, 2002; Calvo and Gómez, 2002), which could be due to inaccessibility of some epitopes to proteases even in the denatured protein. Therefore, in recent years there has been growing interest in the development of a new range of hypoallergenic (HA) hydrolysates with limited hydrolysis. High pressure (HP) and microwave irradiation (MWI) may be useful tools in their development. Excess hydrolysis may cause some disadvantages such as low palatability, bitterness, off-flavour and loss of nutritional value of hydrolysates. However, relatively few data are available on the reduction of protein allergenicity by combination of these physical treatments and enzymes.

10.3.1 High pressure and microwave treatments

High pressure

High pressure treatment (HP) may be an alternative to thermal processing in the production of HA hydrolysates, since this physical treatment induces enhanced hydrolysis and conformational changes in proteins, as shown by several scientific papers. Hayashi *et al.*, (1987) studied the selective digestion of β -Lg in milk whey by thermolysin under HP. Besides, acceleration under HP of the proteolysis of β -Lg by thermolysin (Okamoto *et al.*, 1991), pepsin

(Dufour *et al.*, 1995; Stapelfeldt *et al.*, 1996) and trypsin (Maynard *et al.*, 1998; Stapelfeldt *et al.*, 1996; Van Willige and Fitzgerald, 1995) was confirmed. Our group studied the effect of HP, applied during or after enzymatic digestion, on the proteolysis of bovine whey (WP) by digestive enzymes (Peñas *et al.*, 2006a). The extent of proteolysis by chymotrypsin and trypsin hydrolysis was significantly enhanced ($p \leq 0.05$) when the digestion took place at at 100 and 200 MPa, respectively. However, there were no significant differences between control samples and those hydrolysed by both enzymes after HP treatment. In addition, HP treatment enhanced proteolysis by pepsin, and the highest degree of hydrolysis by this enzyme was obtained at 300 MPa. In this case, though, there were no significant differences when high pressure was applied prior to or during enzymatic digestion.

The effect of HP on the enzymatic hydrolysis of soybean whey proteins by digestive enzymes (Peñas *et al.*, 2004) and by food grade proteases (Peñas *et al.*, 2006b) has also been assessed. Our findings confirmed the enhancement of the digestion of soybean whey proteins by pepsin, trypsin and chymotripsin when HP was applied, and better results were obtained at 100 MPa. Increased hydrolysis of these vegetable proteins by three different food grade proteases, such as alcalase, neutrase, and corolase PN-L, was also observed at 200 and 300 MPa, while the enzymes showed similar proteolytic activity at atmospheric pressure and at 100 MPa.

On the other hand, the dissociation of oligomeric proteins into its subunits under moderately HPs (< 150 MPa) (Cheftel, 1991; Lullien-Pellerin and Balny, 2002; Tauscher, 1995) and the unfolding of the protein monomers under pressures up to 200 MPa (Iametti *et al.*, 1997) have been reported. These phenomena seem to lead to the transient exposure of the antigenic epitopes to the proteases which were not accessible in the native protein (Bonomi *et al.*, 2003), reducing consequently the protein antigenicity.

Some of the earliest studies in this field have already addressed the possibility of combining pressure and enzyme hydrolysis to reduce the allergen content of dairy whey (Okamoto *et al.*, 1991). According to the authors, the binding affinity of five kinds of anti- β -Lg monoclonal antibodies with different epitopes was decreased by thermolysin hydrolysis of bovine WP concentrate, and it was almost completely removed by treating at 200 MPa at 30°C for 3h. However, positive response on anti- α -lactoalbumin antibody was found in all pressurised hydrolysates, results related by the authors to the resistance of this protein to the pressure. Nakamura *et al.* (1993) observed an important reduction in antigenicity related with the presence of β -Lg, in WP concentrate pressurised at 400 or 600 MPa prior to their enzymatic digestion with papain and proleather®, compared to those samples unpressurised and hydrolysed at atmospheric pressure.

The nature, properties and antigenicity of the products obtained from bovine β -Lg and ovalbumin (OVA) at short incubation periods by trypsin and chymotrypsin using different pressure/temperature combinations have been studied (Bonomi *et al.*, 2000, 2003). Increased hydrolysis of both proteins

by either enzyme was observed at 600 MPa, while the proteins were resistant to activity of both enzymes at atmospheric pressure. A synergistic effect of temperature increase from 37 to 44 °C in reduction of immunoreactivity was observed only for trypsin (Fig. 10.3). Hajós *et al.* (2004) studied the effect of high pressure (600 MPa, 20 min) on immunoreactivity of sausage batter proteins and observed a significant reduction of antigenicity due to conformational modification of the antigenic epitopes.

An important decrease of immunoreactivity was also found by our group in bovine WP hydrolysates by combining 200 or 300 MPa and trypsin or pepsin, respectively, in comparison to untreated bovine milk (Fig. 10.4) and enzymatic treatment alone by using an ELISA test and a pool of seven sera from patients allergic to bovine milk (Peñas *et al.*, 2006a). Our results showed, however, no decrease of immunoreactivity in the hydrolysates obtained by chymotrypsin under pressure, results consistent with the residual α -lactalbumin in them. The discrepancy between our results and those reported by Bonomi *et al.* (2003) for β -Lg could be related to the substrate used in each study, and potential interference by other proteins and non-protein components of bovine whey used by us.

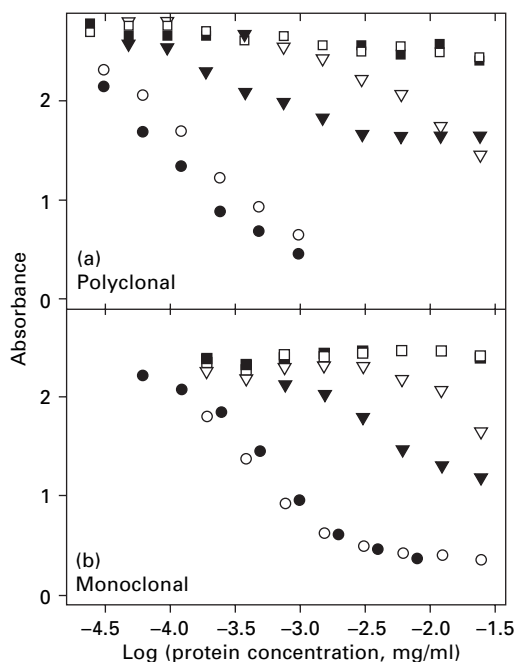


Fig. 10.3 Residual immunochemical reactivity of β -Lg following combined pressure/enzyme treatment. ELISA assay using (a) rabbit antiserum IV or (b) monoclonal antibody M 5G6. Hydrolysis with either $\nabla\nabla$ trypsin or $\blacksquare\blacksquare$ chymotrypsin was performed at 37°C (solid symbols) or 44°C (open symbols). Controls were: $\bullet$ untreated β -Lg and $\circ$ pressure treated β -Lg with no enzymes added. (Bonomi *et al.*, 2003).

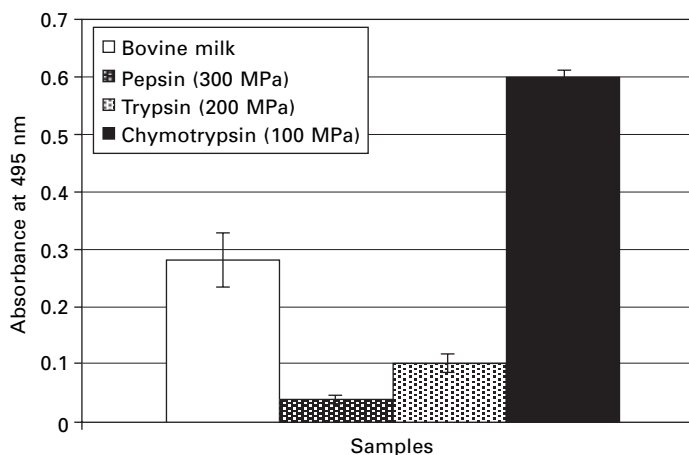


Fig. 10.4 Residual immunochemical reactivity determined by an ELISA assay (expressed as absorbance units at 495 nm) of bovine whey hydrolysates obtained by combination of high pressure and digestive enzymes, using a pool of seven sera from allergic children to bovine milk.

Peñas *et al.* (2006d) also evaluated the effect of the combination of pressure treatment and enzymatic hydrolysis with food grade proteases (alcalase, neutrase, corolase 7089, corolase PN-L or papain) on the antigenicity of bovine WP hydrolysates, evaluated by immunoblotting using anti β -Lg monoclonal antibodies, anti cow's milk protein polyclonal antibodies and sera from paediatric patients allergic to cow's milk proteins. Positive reactions on the band corresponding to β -Lg were detected for corolase PN-L and corolase 7089 hydrolysates, except for those obtained under 300 MPa by the last protease, when anti- β -lactoglobulin monoclonal antibody was used (Fig. 10.5d and c). On the contrary, no residual immunochemical reactivity associated with presence of β -lactoglobulin was observed in the hydrolysates obtained with alcalase and neutrase under HP and in all hydrolysis products obtained by papain (Fig. 10.5a, b, and e). Neutrase hydrolysates showed, however, antigenic reactions associated with polymeric forms of β -Lg. The polyclonal antibody permitted to exclude the presence of a significant quantity of caseins in alcalase, corolase 7089 and papain hydrolysates obtained at atmospheric pressure and by HP applied prior to or during the enzymatic digestion (Fig. 10.6a, c and e). In contrast, a positive reaction related to caseins was found in the hydrolysis products obtained by neutrase and HP (Fig. 10.6b), and also by corolase PN-L hydrolysates obtained from dairy WP pressurised before enzymatic digestion (Fig. 10.6d). These results were confirmed with those obtained with human sera.

The digests by corolase PN-L and neutrase, that show different RP-HPLC peptide profile and maximum proteolysis grade, were also assessed by inhibition ELISA test using polyclonal rat anti- β -Lg antibodies (Peñas *et al.*, 2006e). Figure 10.7 shows the inhibition ELISA to β -Lg by the hydrolysates obtained

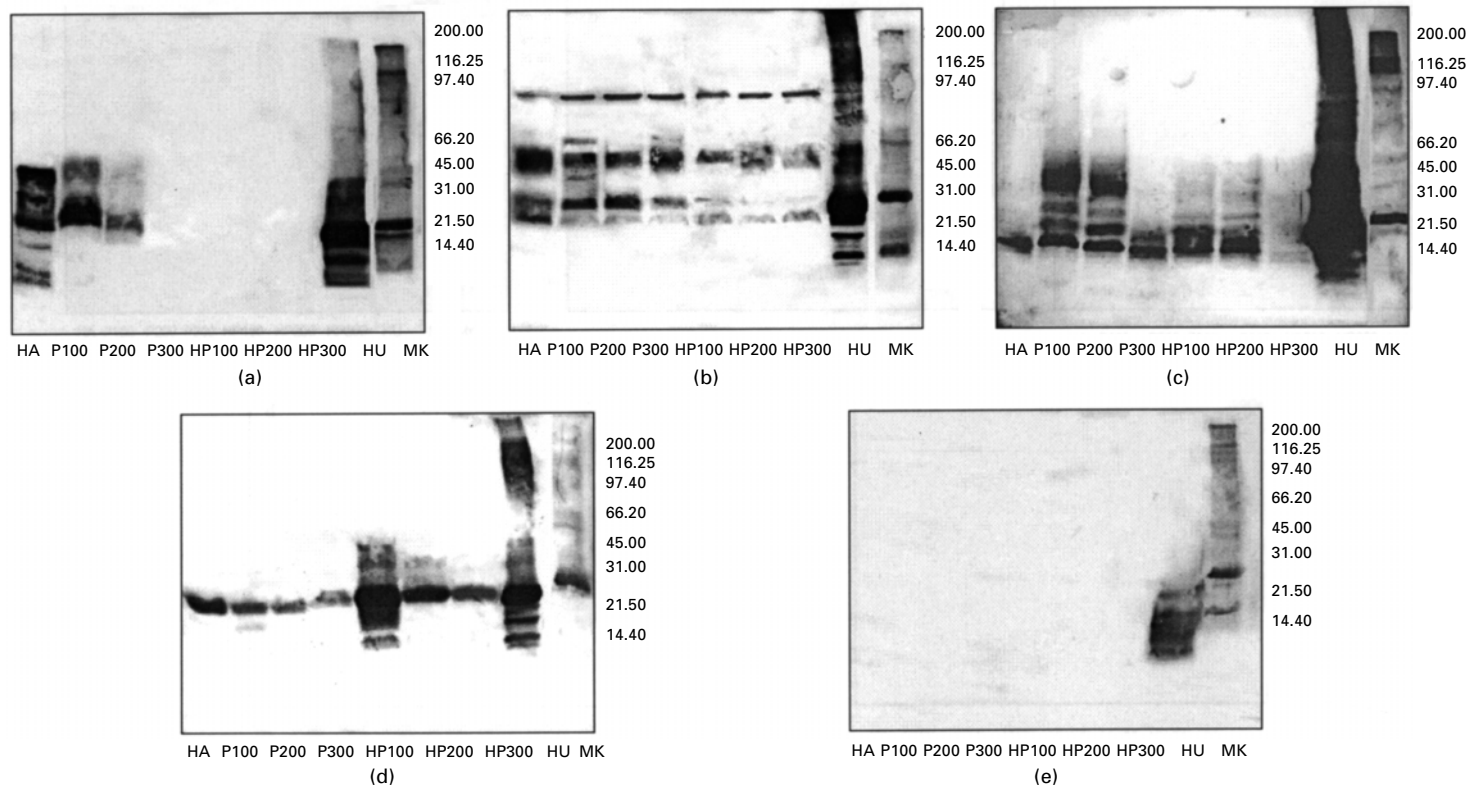


Fig. 10.5 Immunoblotting of polypeptides obtained by proteolysis of bovine whey with alcalase (a) neutrase (b) corolase 7089 (c) corolase PN-L (d) and papain (e) incubated with monoclonal anti- β -lactoglobulin antibodies. MK = Molecular weight standard solution, HU = unhydrolysed bovine whey, HA = hydrolysis at atmospheric pressure (0.1 MPa), P100 = hydrolysis after pressurisation at 100 MPa, hydrolysis after pressurisation at 200 MPa, P300 = hydrolysis after pressurisation at 300 MPa, HP100 = hydrolysis at 100 MPa, HP200 = hydrolysis at 200 MPa, HP300 = hydrolysis at 300 MPa.

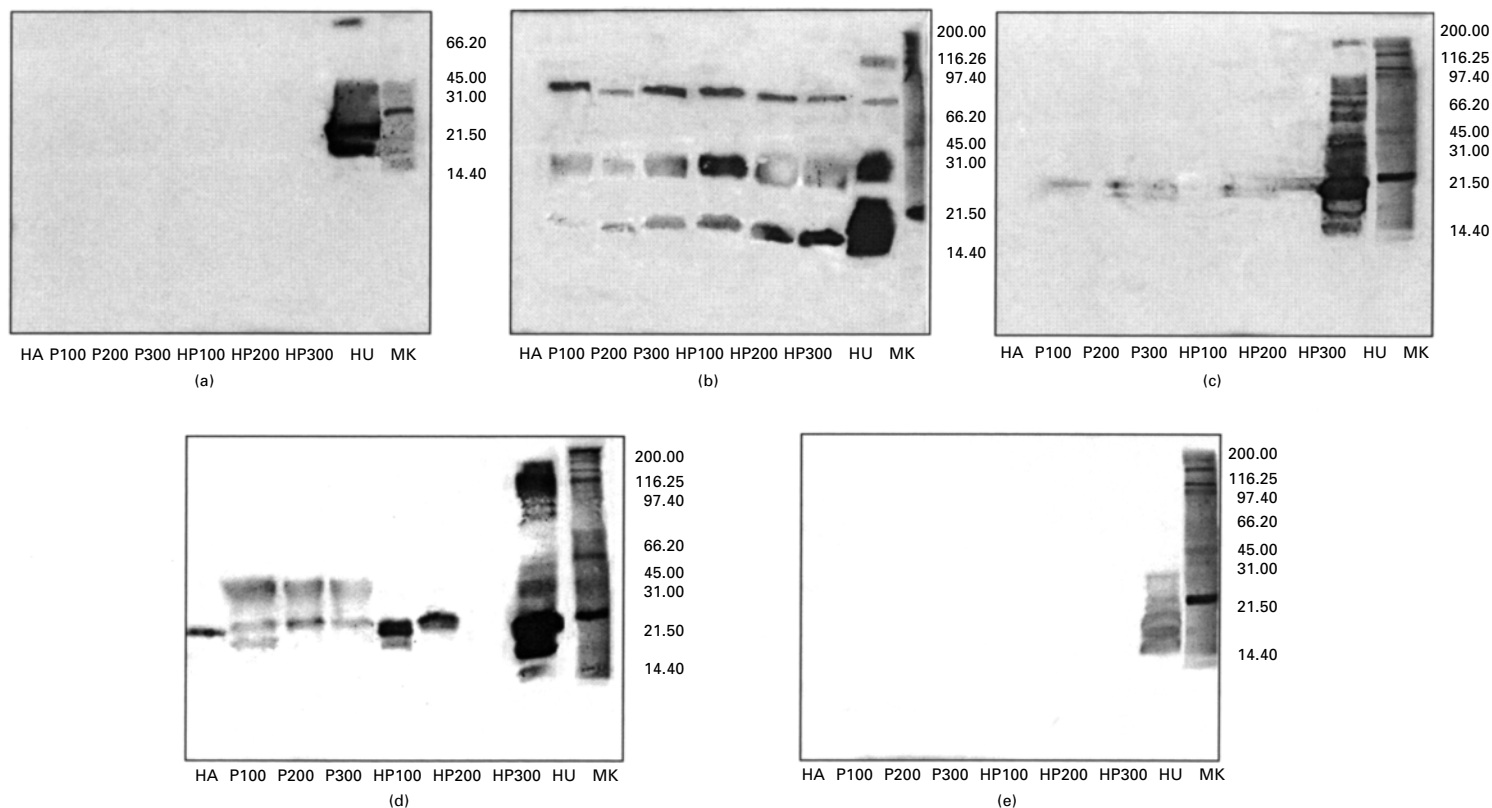


Fig. 10.6 Immunoblotting of polypeptides obtained by proteolysis of bovine whey with alcalase (a) neutrase (b) corolase 7089 (c) corolase PN-L (d) and papain (e) incubated with polyclonal anti cow's milk protein antibodies. MK = Molecular weight standard solution, HU = unhydrolysed bovine whey, HA = hydrolysis at atmospheric pressure (0.1 MPa), P100 = hydrolysis after pressurisation at 100 MPa, hydrolysis after pressurisation at 200 MPa, P300 = hydrolysis after pressurisation at 300 MPa, HP100 = hydrolysis at 100 MPa, HP200 = hydrolysis at 200 MPa, HP300 = hydrolysis at 300 MPa.

by combined treatments HP/corolase PN-L or neutrase. Corolase PN-L hydrolysates obtained at 300 MPa showed the lowest antigenicity, followed by those obtained at 100 MPa (Fig. 10.7a). Neutrase hydrolysates obtained at 100 and 300 MPa presented a small reduction of the immunochemical reactivity as compared with control hydrolysis products (Fig. 10.7a). No significant differences between 100 and 300 MPa were found. Our results suggest that the reduction of antigenicity of hydrolysates by the combined enzymatic/HP treatments may be a compromise between the specificity of enzymes used to eliminate sequential epitopes (Van Beresteijn *et al.*, 1995) and structural unfolding and denaturation of WP proteins induced by HP

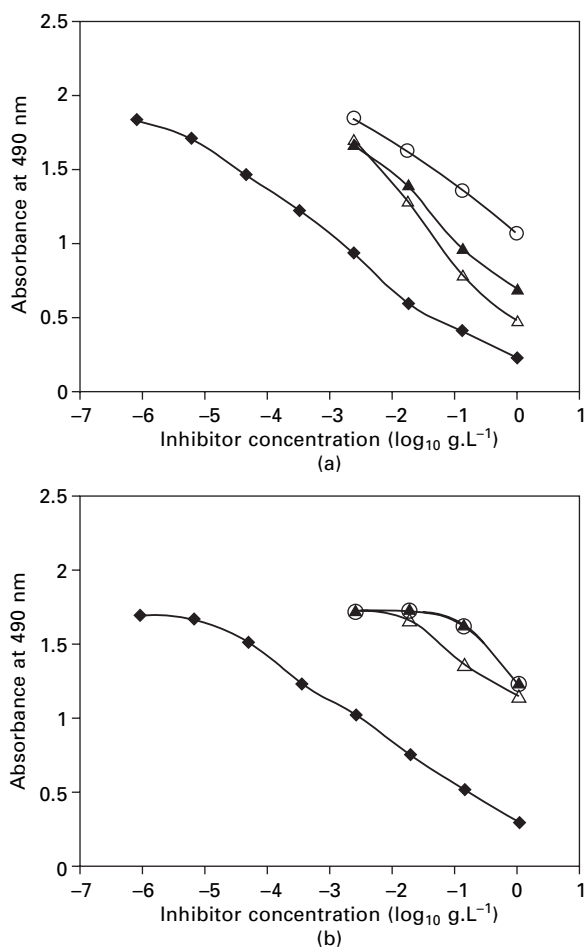


Fig. 10.7 Inhibition of IgG binding to β -lactoglobulin in hydrolysates from bovine whey (WP) proteins by combining enzymatic and high pressure (HP) treatments. (a) corolase PN-L, (b) neutrase. ◆ β -lactoglobulin standard; △ control; ▲ Hydrolysis of WP under 100 MPa; ○ Hydrolysis of WP under 300 MPa.

Table 10.1 Concentration of Gly m 1 allergen ($\mu\text{g/ml}$) in soy-whey untreated (0.1 MPa) and treated by HP at 100, 200 and 300 MPa, determined by ELISA and monoclonal anti-Gly m 1 antibody

Soybean whey	
	Gly m 1 ($\mu\text{g/ml}$)
0.1 MPa	14.17
100 MPa	12.53
200 MPa	10.97
300 MPa	13.01

Table 10.2 Concentration of Gly m 1 allergen ($\mu\text{g/ml}$) in soy-whey hydrolysed by alcalase, neutrase, corolase 7089 and corolase PNL at atmospheric pressure (0.1 MPa) or under high pressure at 100, 200 and 300 MPa, determined by ELISA and monoclonal anti-Gly m 1 antibody

	Alcalase	Neutrase	Corolase 7089	Corolase PN-L
	Gly m 1 ($\mu\text{g/ml}$)			
0.1 MPa	8.53	9.48	8.08	8.01
100 MPa	8.89	8.29	20.17	19.53
200 MPa	7.63	14.42	10.63	10.16
300 MPa	8.52	10.82	10.69	6.54

(Dumay *et al.*, 1994), which could favour the exposure of conformational antigenic epitopes to proteases.

The combined HP and enzymatic treatment also affect the antigenicity of soybean, as assessed by Peñas *et al.* (2006b) by measuring the reduction of the main allergen (Gly m1) by inhalation of the soybean. This allergen is a globular glycoprotein, stabilised by disulphide bonds (Gonzalez *et al.*, 2000), rich in cysteine, and located in the hull of the soybean seed. Ours findings show the reduction of this allergen after pressurisation, reaching the lowest value at 200 MPa (Table 10.1), antigenicity measured by ELISA and anti-gly m1 monoclonal antibody. The modification of secondary, tertiary and quaternary structures of proteins under high pressure (Balny and Mason, 1993; Silva and Weber, 1993), seemed to be responsible of the removal of the conformational antigenic epitopes of this allergen. The combination of enzymatic hydrolysis and HP treatment reduced the antigenicity of soy WP (Table 10.2) and the highest reduction was obtained at 300 MPa for corolase PN-L, followed by 200 MPa for alcalase hydrolysates and 100 MPa for neutrase hydrolysates. For corolase 7089 hydrolysates, however, the lowest antigenicity related with Gly m 1 was obtained at atmospheric pressure (0.1 MPa).

Peñas *et al.* (2006c) also studied the immunoreactivity of soybean hydrolysates by immunoblotting using sera from children allergic to soybean,

when alcalase, neutrase and corolase PN-L in combination with HP were used. Our results show absence of residual immunoreactivity in alcalase and neutrase hydrolysis products obtained both at atmospheric pressure and under high pressure (Fig. 10.8a and b). On the contrary, residual immunoreactivity was found in the hydrolysates obtained by corolase PN-L at atmospheric pressure and at 100 and 200 MPa (Fig. 10.8c; HA, H100, and H200). The reactivity was associated with a band having molecular weight of about 70 kDa, which could be correlated to a digestion product and its immunoreactivity was not detectable in the undigested soybean WP sample. In contrast, a synergic effect between the pressure treatment and the proteolysis was appreciable at 300 MPa (Fig. 10.8c, H300), where no residual antigenicity was detected. In this case, the application of HP improves the exposure of some epitopes to proteases, epitopes that were hidden in the intact protein, and for this reason, the antigenicity associated with the band of 70 kDa was not detected at 300 MPa.

Microwave

Another physical treatment that it is known to affect the hydrolysis and structure of proteins is microwave irradiation (MWI). Microwaves are electromagnetic waves and the heating of proteins by microwaves is accomplished by absorption of microwave energy, rotation of the bipolar water molecules and oscillatory migration of the ionic components of the proteins (Ohlsson and Bengtsson, 2001). Numerous studies have dealt with the application of MWI as an alternative method to conventional heat (CH) treatments of foods. Enzymatic processes under this non-conventional energy source are accelerated as described by several authors. Pramanik *et al.* (2002) reported the effectiveness of this treatment for protein mapping of cytochrome c, ubiquitin, lysozyme, myoglobin and interferon α -2b by trypsin or lysine C. The potential advantages of MWI treatment have been reported also for acceleration of protein hydrolysis in the preparation of samples for amino acid analysis (Chen *et al.*, 1987; Chiou and Wang, 1989; Marconi *et al.*, 1995); for milk pasteurisation (López-Fandiño *et al.*, 1996) without adverse effects on flavour during cold storage (Valero *et al.*, 2000), and for preparation of samples for atomic absorption analysis (de la Fuente and Juarez, 1995). However, relatively few data are available on the application of MWI to enzymatic reactions of dairy proteins (Izquierdo *et al.*, 2005; Izquierdo *et al.*, 2006), and the effects of this non-conventional heat on protein allergenicity remain largely unexplored.

On the other hand, several authors reported the unfolding and denaturation of β -Lg (Bohr and Bohr, 2000a, b) and bovine serum albumin (BSA) in solution (de Pomerai *et al.*, 2003) under MWI. Conformational changes of the β -Lg at the moment the proteases are present would favour the exposure of some residues specifically recognised by proteases as well as favouring the initial proteolysis, as has been reported for high pressure treated β -Lg (Dumay *et al.*, 1994; Bonomi *et al.*, 2003), bovine WP (Peñas *et al.*, 2006a, c)

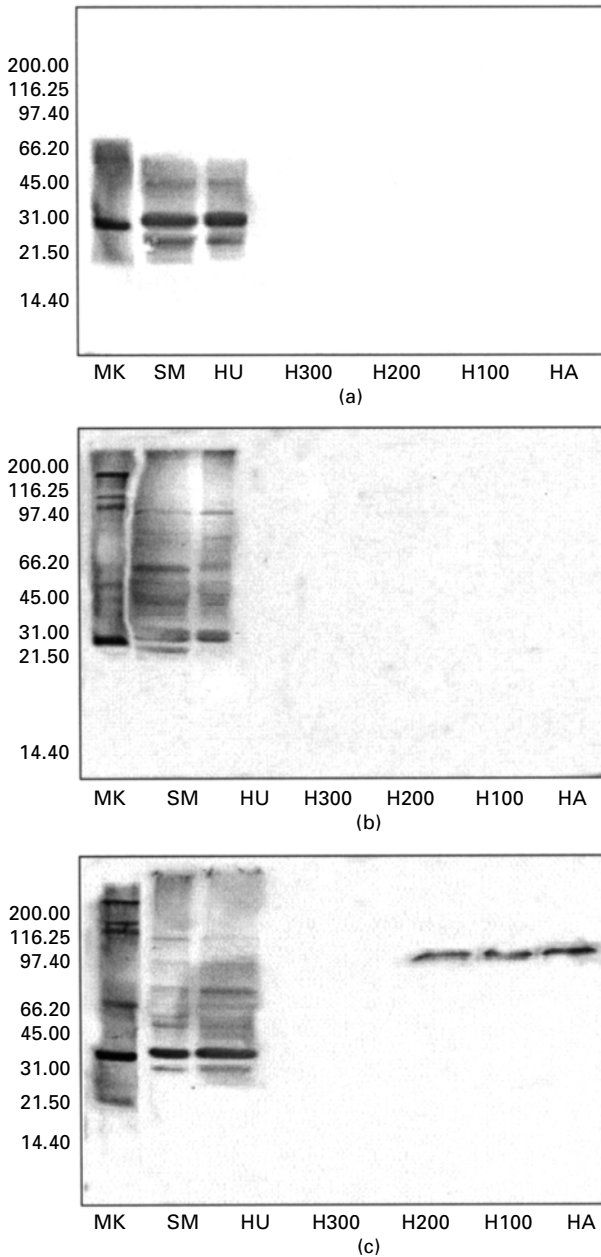


Fig. 10.8 Immunoblotting of polypeptides obtained by hydrolysis of soybean whey with alcalase (a) neutrase (b) and corolase PN-L (c) incubated with the serum of allergic children. MK = Molecular weight standard solution, SM = soybean milk, HU = unhydrolysed soybean whey, H300 = hydrolysis at 300 MPa, H200 = hydrolysis at 200 MPa, H100 = hydrolysis at 100 MPa, HA = hydrolysis at atmospheric pressure (0.1 MPa).

and soybean (Peñas *et al.*, 2006b, c). The potential effect of the treatment on enzymes has been reported by Porcelli *et al.* (1997) who found that 10.4 GHz caused conformational changes in two enzymes (S-adenosylhomocysteine and 5'-methylthioadenosine phosphorylase), detected by fluorescence spectroscopy and circular dichroism spectroscopy. These authors suggest that the MWI treatment induce structural rearrangements in the enzymes/proteins which are not related to temperature.

Our group investigated comparatively the influence of pressurisation and MWI on β -Lg AB hydrolysis by pronase and α -chymotrypsin (Izquierdo *et al.*, 2005) and, whether MWI treatment of β -Lg during its digestion would affect the kinetic parameters of the enzymes, as well as the nature of the end products (Izquierdo *et al.*, 2006). Pronase hydrolysis under HP and MWI (40 °C for 10 min) resulted in significant ($P \leq 0.001$) increasing release of [-NH₂] groups in comparison with CH digestion; there were no significant differences between the proteolysis by pronase under 200 and 300 MPa or MWI irradiation. Besides, significant amounts of A and B genetic variants of β -Lg were not found in these digests. On the other hand, the levels of α [-NH₂] groups released in the pronase digestion under MW in 1 and 4 min were similar to those released after 7 and 10 min of CH digestion, respectively. The advantage of MW irradiation when compared to CH is less evident for α -chymotrypsin than as described above for pronase, since 30% undigested β -Lg B and significant lower hydrolysis was found in the digestions performed for 10 min under MWI, in comparison to the most effective performed under 100 and 200 MPa, that show insignificant amount of A and B genetic variants of the protein. These findings suggest that the HP seems to favour the hydrolysis of B genetic variant by both enzymes, while the MW effect is enzyme dependent.

The application of MWI during the enzymatic digestion of β -Lg affects the kinetic parameters of the enzymes, as well as the nature of the end products (Izquierdo *et al.*, 2006). The Michaelis-Menten constant (K_m) for pronase in the hydrolysis performed under MWI (0.25 ± 0.02 mM) was significantly ($p < 0.05$) lower than under CH (1.01 ± 0.14 mM). A similar trend was obtained for α -chymotrypsin, exhibiting K_m values of 0.18 ± 0.03 mM (under MWI) and 0.44 ± 0.07 mM (under CH) (Table 10.3). Higher V_{max} and K_{cat} were found for pronase when the reaction took place under MWI, with significant differences ($p < 0.05$) in comparison with CH digestion. However, the two kinetic parameters for α -chymotrypsin were not significantly altered by MWI. Significantly ($p < 0.05$) higher catalytic effectiveness (K_{cat}/K_m^{-1}) values were also found in the pronase and α -chymotrypsin digestions performed under MWI (7793 and 2073 min⁻¹ mM⁻¹, respectively) in comparison to the values in the respective CH digestions (1802 and 941 min⁻¹ mM⁻¹, respectively). These results suggest highest substrate-enzyme affinity under MWI, since this catalytic parameter is commonly used to compare substrate specificity as reported by Price and Stevens (1989). This fact could be explained by either increased susceptibility of the substrate or

Table 10.3 Kinetic parameters¹ for hydrolysis of β -lactoglobulin digestion by different enzymes under conventional heating and microwave irradiation

	Pronase				Chymotrypsin			
	Heating		Microwave		Heating		Microwave	
	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
K_m ($\mu\text{moles mL}^{-1}$)	1.0 ^a	0.14	0.25 ^b	0.02	0.44 ^c	0.07	0.18 ^d	0.03
$V_{\max}$ ($\mu\text{moles mL}^{-1}\text{min}^{-1}$)	60 ^a	0.2	64 ^b	0.1	16 ^c	1.7	15 ^c	1.0
K_{cat} (min^{-1})	1820 ^a	6.7	1948 ^b	2.4	414 ^c	41.2	373 ^c	24.7
$K_{\text{cat}} K_m^{-1}$ ($\text{min}^{-1} \text{mM}^{-1}$)	1802 ^a	6.6	7793 ^b	9.7	941 ^c	93.7	2073 ^d	137.5

¹Values are the mean and standard deviation of duplicate analysis of sextuple samples. The statistical analysis was made for pronase and α -chymotrypsin, independently.

^{a,b}means in the same rows (pronase) without a common superscript differ ($p < 0.05$),

^{c,d}means in the same rows (α -chymotrypsin) without a common superscript differ ($p < 0.05$).

increased activity of the proteases under MWI and, further research will be necessary with MWI-insensitive substrates and/or synthetic substrates to distinguish the two processes. Our findings also show that pepsin presents very low activity on the β -Lg at pH 4.0 regardless of the heating procedure used. This fact could be a result of the resistance of β -Lg to peptic digestion, as reported by Dufour *et al.*, (1995) and Stapelfeldt *et al.*, (1996). In addition, Peñas *et al.*, (2006a) reported greater peptic hydrolysis of β -Lg under high pressure of about 300 MPa than at atmospheric pressure, where the activity of the protease was very low. The resistance of β -Lg to pepsin action may reflect its stable conformation at pH 4.0, which has been explained by the strong stabilising action of the two disulfide bonds present in its tertiary structure (Papiz *et al.*, 1986; Kella and Kinsella, 1988; Iametti *et al.*, 1995).

The electrophoregrams of enzymatic hydrolysates support the kinetic data, showing that the highest pronase and chymotrypsin proteolysis occurred under MWI (Fig. 10.9), with production of smaller peptides, although the specificity of both enzymes remained unchanged upon this physical treatment applied. It seems that the MWI effects were not thermal, since CH reactions were also performed at 40°C. Its main effect seems to be related to the rate at which primary hydrolysis products were further hydrolysed to end products, the nature of them being the same. Our findings are consistent in qualitative terms with those reported by several researchers regarding enhancement of enzymatic hydrolysis of substrates under MWI. Pramanik *et al.*, (2002)

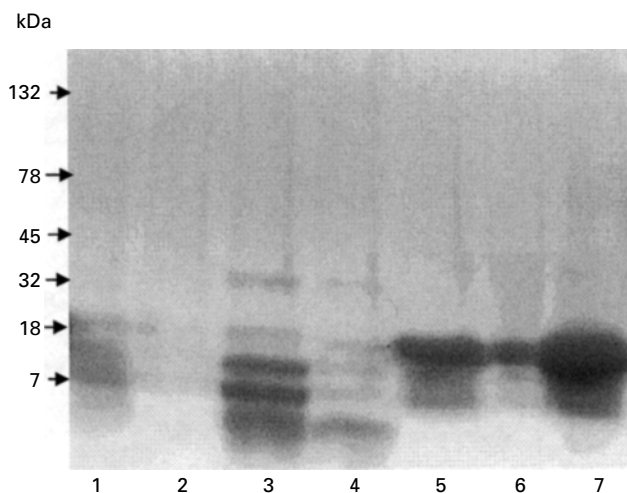


Fig. 10.9 SDS-PAGE pattern of enzymatic hydrolysates from β -lactoglobulin (0.273 mM) under conventional heating (CH) or microwave irradiation (MWI). The enzymatic reactions were performed at 40 °C for 3 min in 50 mM phosphate buffer (pH 8.0) for pronase and α -chymotrypsin, and in 50 mM citrate buffer (pH 4.0) for pepsin. Lanes: 1, pronase, CH; 2, pronase, MWI; 3, α -chymotrypsin, CH; 4, chymotrypsin, MWI; 5, pepsin, CH; 6, pepsin, MWI; 7, β -lactoglobulin in phosphate buffer (50 mM, pH 8.0)

described that the MWI-assisted digestion by endoproteases trypsin or lysine C of several biologically active proteins, led to smaller peptides in minutes, in contrast to the hours required for the peptide fragmentation by conventional methods. Roy *et al.* (2003) described that the MWI pretreatment of the chitin was successful for their efficient hydrolysis by chitinase and pectinase. According to the authors, greater accessibility of the susceptible bonds in the MWI treated chitin occurs as demonstrated by SEM and X-ray diffraction data. MWI induced enhancement of enzymatic proteolysis of β -Lg, one of the main allergen of cow's milk, may allow the development of hypoallergenic hydrolysates. However, relatively few data are available on the effects of this non conventional heat on protein allergenicity and, further studies are needed. This type of work is now in progress in our laboratory and the preliminary results are promising.

10.4 Future trends

Through research and innovation, new approaches for improving the composition of hypoallergenic dietary products for infant nutrition may be found, since it is desirable that the hydrolysates used as proteic ingredients present the minimum degree of hydrolysis compatible with absence of antigenicity. Extensive enzymatic digestion of proteins leads to a loss of nutritive value, low palatability, bitterness and off-flavours. Besides, high molecular weight peptides have an essential role in emulsion stabilisation of infant formulas, improving other functional properties such as foam-forming ability. In this way, emerging technologies such as HP and MWI may be useful tools for obtaining protein hydrolysates with low antigenicity and acceptable sensorial/functional properties, although studies are needed to confirm their safety for allergic patients.

On the other hand, such formulas should also achieve in infants all the functional effects that are observed in breast-fed infants. In this way, scientific efforts are focused on the supplementation of infant formulas with probiotic bacteria and prebiotic components, which beneficially affect the host by improving its intestinal microbial balance and play an important role in the control of allergic inflammation at an early age. Further research, however, is needed to know which species of probiotic bacteria and new non-digestible oligosaccharides show high specificity to prevent allergic diseases.

10.5 Sources of further information and advice

Review articles on food allergy, prevention and diagnosis, in addition to those mentioned in references, include the following:

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11

Plant sterols and stanols as functional ingredients in dairy products

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11.1 Introduction

Plant sterols were first described as cholesterol-lowering agents over 50 years ago. (Lesesne, 1955) but have only been used in food products since 1995 when Raisio introduced stanol-containing spreads into Finland. Dairy products containing plant sterols and stanols have become widely available in the last 2–3 years with EU approval of most of the products listed below having occurred in 2004. The whole European market for plant sterols was 7,990 tons in 2005 with a value of \$185M. Although there are 25 active competitors in the field, 79% of the market is controlled by the top three companies.

11.2 Range of dairy products fortified with sterols and stanols

Raisio of Finland produces esterified stanol (derived from both soy and tall oil sterols) which has been added into milk, butter milk, yoghurt, drinking yoghurt, single shot drink (2 g stanol esters in 70 ml) and cheese spread by various dairy companies. These products are all sold under the Benecol brand with distribution of at least one of these products in 16 countries. Benecol is marketed in the USA and the EU excluding the Scandinavian countries by McNeil. Raisio is the only company currently producing stanol esters.

Unilever produces a semi skimmed milk drink containing plant sterols derived from soy and other vegetable oils as well as yoghurt and a mini drink

(2 g plant sterols in 100 ml) as Flora Pro.Active brands and Lipton Take Control. Suppliers of vegetable oil sterol esters include Cargill (Corowise), Cognis (Vegapure) and Archer Daniels Midland (CardioAid). Multibene produces free and esterified sterols for a wide variety of dairy (e.g. yoplait yoghurt sold by Glanbia) and non dairy products including ketchup and spicy sauces. Other dairy products include a Lifeline Food Company low fat cheese containing Corowise (phytosterol esters launched in 2003). Danone sells sterol containing yoghurt under the Danacol brand. Triple crown AB sell free sterols from vegetable oils and tall oil.

Teriaka from Finland sells sterols (Diminicol) in a special formulation of free sterols or stanols partly dissolved in fat and in semicrystalline form and is approved in Europe the USA and Mexico. Adumin is a small Israeli company marketing encapsulated free sterols in cooking oil.

The Canadian company Forbes Meditech produces pine-derived free sterols and stanols (tall oil) under the brand Reducol which is sold by a variety of dairy companies (e.g. in the UK by Fayrefield Foods sold by Tesco; in Finland the Pirkka brand sold by Kesko). The largest supplier of tall oil in the USA is Arboris LLC. Cargill also supplies wood-derived sterols. There is quite a strong demand in Europe for GM-free derived sterols and ensuring the GM identity of soy is at times difficult. Wood-derived sterols are more expensive than vegetable sterols but the commercial products are generally sold for the same price (or even cheaper). Prime Pharma derives its tall oil sterols from Derives Resiniques et Terpeniques (DRT) which is the largest European supplier of tall oil. Other suppliers of tall oil sterols include Triple Crown (Prolocol).

Table 11.1 shows sterols suppliers, the source and formulation of the products and the available dairy products and their manufacturers and distributors.

11.3 The role of plant sterols and stanols in functional dairy products in reduction of cholesterol

Plant sterols and stanols appear to be as effective in dairy products as in spreads with lowering of LDL cholesterol of 5–10%. No significant changes in HDL cholesterol or triglyceride occur with either food carrier.

Volpe (2001) was the first to show an effect of low fat yoghurt containing 1 g/day of soybean derived sterols (whether free or esterified was not specified) in 30 men and women with elevated LDL cholesterol. LDL cholesterol was lowered by 6.2% after four weeks compared with placebo. Eleven of the volunteers continued for another four weeks on 2 g/day of sterols and achieved an LDL cholesterol-lowering of 15.6% but there was no placebo period to compare it with. Given that during the placebo period in the first part of the study LDL cholesterol fell by 4.9% it would appear that 2 g/day of sterol in yoghurt lowers LDL cholesterol by about 10%.

Table 11.1 Sterol sources and products

Supplier	Type/name	Dairy product	Other Food Brands/distributors
Raisio	vegetable, tall oil stanol ester (Benecol, Scandinavia)	Valio produces and markets milk, butter milk, yoghurt, and single shot drink containing Benecol	Benecol-McNeil (Europe, USA)
Unilever	vegetable sterol ester	milk, yoghurt	Flora Pro.activ (Europe, Australia) Take control (Lipton, USA)
Forbes Meditech	tall oil free sterol (Reducol)	12% fat cheese (Heartfelt+) yoghurt Tesco milks, yoghurts, probiotic drinks	Fayrefield Foods (UK) Pirkka yoghurts (Finland)
Archer Daniels Midland	vegetable oil sterols/esters (Cardioaid)	Cardioaid milk, yoghurts (Europe)	
Cargill	vegetable sterol esters (Corowise)	low fat cheese (Lifetime, USA)	Lifeline Food Co
Cognis	vegetable sterol esters (Vegapure)	Heart Choice yoghurts, milks (Europe, S. America, Japan, Korea)	
Teriaka	free sterols (Diminichol)	milk drinks, yoghurt (Europe, Korea)	
Central Soya	soy vegetable sterols		
Derives Resiniques et Terpeniques 1 ⁰ Prime Pharma 2 ⁰	tall oil free Beta sitosterol	yoghurt	? Danacol from Danone (oil source not clear)
Multibene	sterols	yoghurt	Yoplait essence (Glanbia)
Triple Crown AB	soy or tall oil-free (Prolocol)		

Mensink (2002) tested 1 g/day of esterified plant stanols in a low fat yoghurt (0.7% fat) in 60 subjects in a four week placebo controlled randomized study. LDL cholesterol fell by 13.7% compared with placebo and this effect occurred within 1 week. Beta carotene/LDL cholesterol ratio fell by 14.4% while total tocopherol/LDL cholesterol level increased. Decreases in absolute beta-carotene concentrations were found in all apoB-containing lipoproteins. LDL-cholesterol standardised phytofluene levels decreased by 21.4+/-25.7% ($P < 0.001$), while other plasma carotenoid (lutein/zeaxanthin, beta-cryptoxanthin, lycopene and alpha-carotene) levels did not change significantly.

Non esterified plant sterols (2 g/day) were tested in low fat yoghurt, low fat hard cheeses and low fat fresh soft cheeses and lowered LDL cholesterol by 9.8% compared with sterol-free products in a six-week parallel study containing 164 subjects. LDL cholesterol adjusted carotenoid levels did not change (Korpela, 2006).

A 40% butter fat based spread containing 2.4 g/day of esterified soybean sterols lowered median LDL cholesterol by 18.7% in a small study of 15 subjects. Carotenoids and tocopherols did not change (Nestel, 2001). In a dose response study Thomsen, (2004) tested a low fat (1.2%) vegetable oil filled milk in 71 subjects in a three way crossover comparing placebo with 1.2 and 1.6 g/day of free plant sterols. The placebo adjusted LDL cholesterol-lowering was 7.1 and 9.6% respectively but there was no significant difference between the two doses. Again carotenoids and tocopherols did not change after LDL cholesterol adjustment. Noakes (2005) fed 40 subjects either a sterol-enriched yoghurt (1.7 g/day) or a stanol-enriched yoghurt (1.8 g/day) or a placebo yoghurt and demonstrated an 5–6% lowering of LDL cholesterol after three weeks. In another paper from the same group 2 g/d sterol ester in low fat milk was equally efficacious at lowering LDL cholesterol as the same amount of sterol in a spread (Noakes, 2005) with 8–10% lowering of LDL cholesterol. Adding the two food sources together did not increase the degree of cholesterol-lowering.

Doornbos (2006) in a large study of 185 people showed that LDL cholesterol was lowered by 9.3–9.5% when a single shot yoghurt drink containing 2 g plant sterols was taken with a meal compared with 5.1–6.9% when taken without a meal. Varying the fat content of the drink from 2.2 to 3.3% had no effect.

Korpela (2006) carried out a parallel, double-blind study in three locations in Finland involving 64 mildly or moderately hypercholesterolaemic subjects who were randomly divided into two groups: a plant sterol group and a control group. The subjects consumed the products for six weeks after a three-week run-in period with a targeted plant sterol intake of 2 g/day in the sterol group. Yoghurt, low-fat hard cheese and low-fat fresh cheese enriched with a plant sterol mixture reduced serum LDL cholesterol by 10.4%. The HDL/LDL cholesterol ratio increased by 16.1% in the sterol group and by 4.3% in the control group ($P = 0.0001$). None of the fat-soluble vitamin

levels decreased significantly when changes in serum total cholesterol were taken into account.

Jauhiainen (2006) conducted a randomized double-blind parallel-group in 67 mildly hypercholesterolaemic volunteers (24 men, 43 women). During the five-week intervention, the subjects in the stanol group consumed a hard cheese enriched with 2 g of plant stanols per day, and the subjects in the control group, a control cheese with no plant stanols. In the stanol ester group, as compared to the control group, LDL: cholesterol decreased by 10.3% ($P < 0.001$). There were no significant changes in high-density lipoprotein cholesterol (HDL), triglycerides or apolipoprotein B concentrations between the groups. The lack of change in the latter suggests LDL particles have got smaller and contain less cholesterol but have not been reduced in number. Apo B has not often been measured in plant sterol and stanol interventions.

Pouteau (2003) showed that with 1.8 g of free plant sterols completely dissolved in low fat milk cholesterol absorption was reduced from 70.1 $\pm$ 4.2% with control to 41.1 $\pm$ 4.0% with milks containing plant sterol ($P < 0.001$).

11.3.1 The relative effectiveness of plant sterols in different foods

As noted above, dairy products whether high or low in fat are as effective as non dairy spreads in lowering LDL cholesterol but there have been few head to head comparisons in the same individuals except the study by Noakes (2005). The only head to head comparison of the relative effectiveness of different foods as sterol carriers was performed by Clifton (2004). Fifty-eight people ate 1.6 g/day of esterified sterols in either milk, yoghurt, bread or breakfast cereal for three weeks each. Serum total and LDL cholesterol levels were significantly lowered by consumption of phytosterol-enriched foods: milk (8.7 and 15.9%) and yoghurt (5.6 and 8.6%). Serum LDL cholesterol levels fell significantly by 6.5% with bread and 5.4% with cereal. They were both significantly less efficacious than sterol-enriched milk ($P < 0.001$). Plasma sitosterol increased by 17–23% and campesterol by 48–52% with phytosterol-enriched milk and bread. Lipid-adjusted beta-carotene was lowered by 5–10% by sterols in bread and milk, respectively. Thus although bread did not lower LDL cholesterol to the same degree as milk the plant sterols appeared to be available for absorption and to interfere with beta carotene absorption. Whether cholesterol absorption was inhibited equally is not known.

Richelle (2004) compared the effects of free and esterified sterols in low fat milk on cholesterol absorption. Inhibition of cholesterol absorption was equal (about 60%) but the esterified sterol interfered with beta carotene (50% inhibition) and tocopherol absorption (20% inhibition) significantly more than free sterol.

11.4 Future trends

As free sterols are cheaper than esterified sterols and hydrogenated esterified stanols are more expensive again, an increasing number of products will make use of free sterols. These products will need to ensure that the sterol is completely solubilized and may require in vitro tests with cell lines to show that they do not interfere with cholesterol absorption. There are negative studies in the literature and these almost certainly relate to the insolubility of the sterol preparation used. Permission has been given recently both in the USA and Europe for a wide variety of sterol formulations and a wide variety of products, so market availability will increase. Capsule and tablet formulations of free sterols are widely available and their use will provide a significant competition to functional foods.

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Part III

Product development

12

Regulation of functional dairy products

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12.1 Introduction

The regulatory principles relevant to functional foods in general also apply to functional dairy products. Also in the case of probiotic products the laws and regulations on the use of starters, or intentionally added live microbial cultures that are used in the food manufacturing processes, should also be taken into account. These practices vary considerably in different countries but, in general, the regulatory status of human probiotics is rather unclear, while microbiological feed additives are subject to a detailed regulation in the EU.

12.1.1 Definition of functional foods

Functional foods do not exist as a legal term either in the US or EU. The concerted action ‘Functional food Science in Europe’ (FUFOSE) produced the following often cited definition (Bellisle *et al.*, 1998):

A food can be regarded as ‘functional’ if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional benefits, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease. Functional foods must remain foods and they must demonstrate their effects in amounts that can normally be expected to be consumed in the diet: they are not pills or capsules, but part of normal food pattern.

A functional food can be a natural food, a food to which a component has been added, or a food from which a component has been removed by technological or biotechnological means. It can also be a food where the

nature of one or more components has been modified, or any combination of these possibilities. A functional food might be functional for all members of a population, or for particular groups of population, which might be defined, for example, by age or by genetic constitution.

In Japan the Ministry of Health and Welfare (now Ministry of Health, Labour and Welfare, MHLW) introduced in 1991 the concept of 'Foods for Specific Health Uses' (FOSHU) or 'Foods that are designed to be consumed as a constituent part of a regular diet, and to help promote and maintain health by regulating bodily functions, and protect against a range of conditions and diseases, including heart disease, hypertension, diabetes and osteoporosis.' (Chadwick *et al.*, 2003; Ohama *et al.*, 2006).

Despite the somewhat different wording in the various definitions, the general principles of functional foods being foods intended to be consumed in normal quantities and exerting a beneficial action not related to purely nutritional effects, are common and also apply to functional dairy products.

12.1.2 The central regulatory issues

The regulations on functional foods should specifically focus on questions of safety, efficacy and the claims associated with the proposed beneficial effects. While the general principle that all foods should be safe applies naturally to functional foods, the special safety aspects associated with the specific functional component or modification may need to be addressed. The beneficial effects should be unequivocally demonstrated, and they should be communicated to the consumers in a way that the claims do not blur the distinction between foods and pharmaceuticals – a distinction that is universally considered essential by law makers. In the following chapters examples of the implementation of these general principles within different legal and regulatory traditions are outlined.

12.2 The present regulatory framework

12.2.1 The US

As stated above, in the US functional foods are not recognized as a special legal entity, but are covered by the federal Food, Drug and Cosmetic Act. Other relevant legislation includes the Nutrition, Labeling and Education Act (1990) and the Dietary Supplement, Health and Education Act (1994), which basically define what kinds of claims can be attached to food products (Chadwick *et al.*, 2003). The Food and Drug Administration (FDA) has a key role in assessing the evidence behind health claims, although other bodies of the US Government or the National Academy of Sciences can also provide authoritative statements on health effects associated with foods.

12.2.2 Japan

Japan is apparently a unique country, where there is a formalized procedure for the approval of functional foods (Ohama *et al.*, 2006). The FOSHU system of 1991 (see Section 12.1.1) was revised in 2001 by MHLW by the introduction of a new category: 'Foods with Health Claims' (FHC), which is divided into two subcategories: namely 'Foods with Nutrient Function Claims' (FNFC) and FOSHU. Until 1991 only conventional foods were eligible for FOSHU status, but subsequently food supplements (in form of pills and capsules) have also been included in the system (it should be noted that these are not functional foods according to the European FUFOS definition). In 2005 FOSHU category was also divided into three subsystems: Individual approval FOSHU, Standardized FOSHU and Qualified FOSHU.

To obtain FOSHU status the applicant must identify the main functional ingredient(s), elucidate the action mechanism (for standardized FOSHU), and clarify, how the product will contribute to the improvement of diet. The safety of the product can be demonstrated in various ways, on the basis of history of safe use or with toxicological studies. The efficacy of the product must be demonstrated by clinical trials. The standardized FOSHU foods require statistical significance of $P\text{-value} < 0.05$ against the control in the clinical trial for the effect(s) to be demonstrated. In qualified FOSHU, the mechanism of action of the functional component can be unknown, and the $P\text{-value}$ for statistical significance can be < 0.1 . If the mechanism of action is known, non-randomized controlled clinical trials are also acceptable for Qualified FOSHU status.

12.2.3 The EU

In the EU there is no special legislation on functional foods distinct from the General Food Law Regulation 178/2002 EC. This regulation defines the general principles and requirements of food law, including food safety, and established the European Food Safety Authority (EFSA) (Coppens *et al.*, 2006). Regarding the safety and efficacy aspects of functional foods (and feeds) the specific EU regulations and directives on novel foods, genetically modified foods and feeds, and on feed additives are, of relevance, however.

The Novel Food Regulation

The Novel Food Regulation (258/97 EC) defines foods or food ingredients that were not consumed to a significant extent in the EU before 1997 as novel, and subject to a safety assessment, before they can be introduced into market (Chadwick *et al.*, 2003). Genetically modified or GM foods have subsequently been removed from the sphere of the Novel Food Regulation and subjected to specific regulations on GM foods and feeds (see page 268).

According to the regulation, novel food must not pose a danger or mislead the consumer or differ nutritionally to such an extent from the conventional counterpart that it is going to replace, that a nutritional disadvantage could

occur. These aspects are taken into account in the assessment of a novel food intended for the EU markets.

The assessment procedure starts in the Member State, where an applicant first wants to introduce the novel food product. The application is directed to the Competent Authority of that country, which performs the initial assessment of the product within 90 days. This assessment together with the details of the application is passed to Commission and to other Member States. The Member States have 60 days to question, comment or object. The disagreements are discussed in a Standing Committee on Food Chain and Animal Health (SCFCAH), in which the Member States are represented and which makes decision according to a qualified majority vote. Also a scientific opinion of the European Food Safety Authority (EFSA) is routinely required.

In case SCFCAH cannot reach a decision, the application is directed to Council of Ministers. If the Council of Ministers fails to act or does not obtain a qualified majority on the matter within three months, the final decision is shifted to the Commission.

Although a functional food is not necessarily a novel food, several functional products (mainly products, including dairy products, containing cholesterol-lowering phytosterols or -stanols) have been authorized according to the procedures outlined in the Novel Food Regulation (http://ec.europa.eu/comm/food/food/biotechnology/novelfood/authorisations_en.htm).

Regulation on genetically modified foods and feeds

Regulation 1829/2003 EC on genetically modified food and feed defines the procedures for the authorization of genetically modified (GM) foods or feeds in the EU. Again the applicant submits the application to the Competent Authority of the Member State in which the product is first intended to enter the market. Within 14 days the Competent Authority sends the application and the supporting data to EFSA, who will also inform the Commission and other Member States. Within six months EFSA is supposed to formulate an opinion and submits it to the applicant, Commission and to the Member States. The final authorization occurs according to the Committee procedure outlined in the previous subsection.

So far, no GM functional foods have been submitted for assessment, although the possibility of this, though taking into account present consumer opinion regarding GM foods in the EU, of course exists.

Regulations on feed additives

While there are no specific EU-level regulations on functional foods, the situation is rather different with microbiological feed additives, which in most cases could also be classified as animal probiotics. Because many of the micro-organism species and even strains that are used in animal nutrition occur also in both conventional and functional food products, the regulations on feeds could also have some relevance to functional foods, particularly in the event of eventual harmonization of legislation on micro-organisms in the

food chain. These aspects have recently been reviewed and discussed by Wessels *et al.* (2004), von Wright (2005) and Anádon *et al.* (2006).

The relevant regulation defining the authorization procedure of feed additives is 1831/2003 EC on additives for use in animal nutrition. The applicant submits the application to Commission, who will inform the Member States and forward the material to EFSA. EFSA will also receive relevant information defined in Article 7 of the regulation directly from the applicant at the time of application. EFSA will give its opinion within six months of receipt of a valid application and mediate it to the Commission, Member States and applicant. The Commission shall formulate a draft decision within three months of the receipt of the opinion. This draft decision is subject to a Committee procedure similar to the one used with novel foods or GM foods.

The specific requirements for approval of a microbiological feed additive are defined in the Opinions of the former Scientific Committee on Animal Nutrition (SCAN). The Scan Opinion of 2001 'Guidelines for the assessment of additives in feedingstuffs, part II: Enzymes and Microorganisms' (http://europa.eu.int/comm/food/fs/sc/scan/outcome_en.html) is still basically valid, although new guidelines are currently being formulated.

According to the guidelines, the efficacy of the additive must be demonstrated in at least three field trials assessing appropriate zootechnical parameters. The safety requirements for micro-organisms are rather stringent, requiring:

- Tolerance study in the target species using, if possible, at least a tenfold overdose of the additive and spanning from one month (young, rapidly growing animals) up to three months according to target animal category.
- Studies on operator safety including eye and skin irritation and possibly also skin sensitization. In general, all microbiological additives are treated as potential respiratory sensitizers and protective measures are recommended.
- Genotoxicity studies (a bacterial reverse mutation test and an *in vitro* mammalian clastogenicity assay) and a 90-day rodent feeding study. These studies are for consumer protection in order to exclude the possibility of unknown or undetected harmful microbial metabolites in the feed, which could form a consumer risk, if accumulated in animal products.

In addition to these general requirements SCAN has published separate opinions addressing special problems associated with *Bacillus*-species used as animal probiotics, and with the risk of transmissible antibiotic resistance genes in micro-organisms used as feed additives.

The SCAN opinion 'The safety of use of *Bacillus*-species in animal nutrition, 2000' (http://europa.eu.int/comm/food/fs/sc/scan/outcome_en.html) deals with the risk of toxigenic *Bacillus* species entering the food chain. The *Bacillus* species intended as feed additives should be checked for the production of enterotoxins or the emetic toxin. The procedure involves accurate taxonomic

characterization of the strain, PCR-tests for the known enterotoxin genes and cytotoxicity assays for the final exclusion of enterotoxin or emetic toxin.

The SCAN opinions regarding the presence of transmissible antibiotic resistant genes: 'The criteria for assessing micro-organisms resistant to antibiotics' of 2001 and 2002 (http://europa.eu.int/comm/food/fs/sc/scan/outcome_en.html) have subsequently been updated by an EFSA document 'Opinion of the FEEDAP Panel on the updating of the criteria used in the assessment of bacteria for resistance to antibiotics of human or veterinary importance' (http://www.efsa.eu.int/science/feedap/feedap_opinions/993_en.html). In these opinions certain breakpoints for MIC values for 13 clinically important antibiotics are presented (Table 12.1). The genetic background of resistances exceeding these breakpoints has to be clarified and confirmed to be intrinsic, physiological or mutational but not transmissible, before authorization. This requirement has apparently resulted in similar concerns on antibiotic resistances in starters and also probiotics used in human foods. At present there is a European 6th framework research programme Assessment and Critical Evaluation of Antibiotic Resistance Transferability in Food Chain (ACE-ART) which elucidates these questions.

12.3 The regulatory status of health claims

The claims associated with foods have received considerable attention from legislators. One of the central principles has been that a clear distinction between food and medicines should be maintained. This principle is rather forcefully formulated in Directive 2000/13/EC which categorically forbids claims to 'attribute to any foodstuff the property of preventing, treating or curing human diseases or refer to such properties'. This principle makes the marketing of functional foods rather difficult, because it prevents conveying to the general public even scientifically valid information on the health-promoting effects of functional foods or food ingredients.

The claims that are generally allowed to a variable degree are nutrition claims and functional claims. A functional claim typically consists of two parts, one stating the presence or concentration of a certain ingredient in the product and the other its physiological role. Particularly relevant to functional foods are enhanced function claims relating to a special beneficial effect of a food component beyond that of nutrition, and reduction of disease risk claims. The latter ones imply that a food component reduces a major risk factor in the development of human disease.

12.3.1 Health claims allowed in the US

As stated in Section 12.2.1. the relevant laws in the US are the Nutrition, Labeling and Education Act and the Dietary Supplement, Health and Education Act. The allowed health claims or health-related statements include nutrient

Table 12.1 Microbiological breakpoints categorizing bacteria as resistant (mgL⁻¹). Strains with MICs higher than the breakpoints below are considered resistant

	<i>Lactobacillus</i> obligate homo- fermentative	<i>Lactobacillus</i> hetero- fermentative*	<i>Lactobacillus</i> <i>plantarum</i>	<i>Enterococcus</i>	<i>Pediococcus</i>	<i>Leuconostoc</i>	<i>Lactococcus</i> <i>lactis</i>	<i>Streptococcus</i> <i>thermophilus</i>	<i>Bacillus</i> spp	other Gram+
ampicillin	4	4	4	8	4	4	4	4	n.r.	2
vancomycin	4	n.r.	n.r.	8	n.r.	n.r.	4	4	4	4
gentamicin**	8	8	64	512	4	4	8	8	4	4
kanamycin**	16	16	64	1024	4	8	8	8	8	8
streptomycin**	16	16	64	1024	4	8	16	16	8	8
neomycin**	16	16	32	1024	8	8	8	8	8	8
erythromycin	4	4	4	4	4	4	4	4	4	4
clindamycin	4	4	4	4	4	4	4	4	4	4
quinupristin + dalfopristin	4	4	4	4	4	4	4	4	4	4
tetracycline	8	8	32	16	4	4	4	4	8	4
chloramphenicol	4	4	8	8	4	4	8	8	8	4
trimethoprim**	8	8	8	8	8	8	n.r.	n.r.	8	8
linezolid	4	4	4	4	4	4	4	4	4	4

n.r. not required.

* including *L. salivarius*

** possible interference of the growth medium

content claims, structure and function claims (functional claims), dietary guidance claims, qualified health claims conveying a relationship between components in the diet and risk of disease (as approved by FDA and supported by the weight of credible scientific evidence available) and health claims confirming a relationship between components in the diet and risk of disease or health condition (as approved by FDA and supported by significant scientific agreement) (Burdock *et al.*, 2006). The significant scientific agreement (SSA) is a standard mainly defined by FDA. So far 12 SSAs on the relationship between a dietary component and disease or health-related condition exist ([http:// www.cfsan.fda.gov/~dms/flg-6c.html](http://www.cfsan.fda.gov/~dms/flg-6c.html)), namely:

1. calcium and osteoporosis
2. dietary lipids and cancer
3. sodium and hypertension
4. dietary saturated fat and cholesterol and risk of coronary heart disease
5. fibre-containing grain products, fruits and vegetables and cancer
6. fruits, vegetables and grain products that contain fibre, particularly soluble fibre, and risk of coronary heart disease
7. fruits and vegetables and cancer
8. folate and neural tube defects
9. dietary non-cariogenic carbohydrate sweeteners and dental caries
10. soluble fibre from certain foods and risk of coronary heart disease
11. soy protein and risk of coronary heart disease
12. plant sterol/stanol esters and risk of coronary heart disease

The qualified health claims were introduced, because the burden of evidence to obtain the SSA level is considered rather difficult to reach. With qualified health claims the relationship between a food component and a disease does not need to be as firmly established as to reach SSA. Consequently, the FDA statements that can be used as claims are also conservatively and cautiously worded. As an example the FDA formulated claim for green tea and cancer:

Based on FDA's review of the strength of the total body of publicly available scientific evidence for a claim about green tea and reduced risk of breast cancer, FDA ranks this evidence as the lowest level for a qualified health claim. For reasons given above, FDA concludes that it is highly unlikely that green tea reduces the risk of breast cancer.

12.3.2 Health claims in Japan

In Japan specified health claims are allowed for FOSHU foods. For example, functional components such as oligosaccharides, lactobacilli and bifidobacteria, Psyllium husk, indigestible dextrin, wheat bran, low molecular weight sodium alginate and partially hydrolyzed guar gum can be associated with claims such as: 'Maintaining good gastrointestinal conditions'. Also disease risk reduction claims are allowed for FOSHU products rich in calcium or folic acid.

Nutrient function claims are permitted for FNFC foods containing allowed vitamins and minerals. The labels should clearly indicate the recommended dosage and warning that the excessive use of the product does not heal the illness or improve health (Ohama *et al.*, 2006).

12.3.3 The developing system in the EU

In the EU, a proposal for a regulation on nutrition and health claims has been in preparation (Coppens *et al.*, 2006), and was passed on 20 December 2006. The regulation (1924/2006/EC) entering in force on 1 July 2007 establishes the principle of premarketing authorization, especially with claims on the reduction of risk and on claims related with the development and health of children. In these cases the applications submitted via the National Competent Authorities are subjected to evaluation by EFSA and approved using the Standing Committee procedure.

12.4 The special case of probiotics

Probiotics have been defined as ‘mono- or mixed cultures of live micro-organisms which, when applied to man or animals, affects beneficially the host by improving the properties of indigenous microflora’ (Huis in’t Veld and Havenaar, 1991). According to a more recent definition by Joint FAO/WHO working group report on drafting guidelines for the evaluation of probiotics in food: ‘Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host.’ (http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf).

As viable micro-organisms intentionally entering the food chain they resemble many traditional food starters that have been used in a variety of foods since ancient times. The use of starters is an age old practice, and no particular attention has been paid to their safety aspects in human foods. Consequently, they have received relatively little attention of the legislators. Consequently, there is little experience of experimental safety assessment of a food micro-organism, probiotic or starter. As pointed out on pages 268–70 the situation is drastically different with micro-organisms intended as feed supplements in the EU, in which case detailed safety studies are required. Even with animal probiotics, however, many of the formally required safety tests (such as genotoxicity tests) are not often required in practice due to methodological difficulties.

Eventual regulations on probiotics consequently also have implications for the status of conventional starters and vice versa, in case a harmonization of legal practices concerning micro-organisms intentionally added to the food chain is attempted. In the following sections the present and emerging situations concerning the regulatory status of starters, probiotics and other food micro-organisms are briefly reviewed.

12.4.1 The regulatory status of starters

The existing starter cultures are generally classified either as food ingredients, processing aids or ingredients (Feord, 2002). In Europe currently only in Denmark and France is there a formal notification or approval systems for new strains intended for food use (von Wright, 2005). The French guidelines are based on a decision tree approach also recommending toxicological studies (including animal studies) if there is a need to guarantee an absence of risk.

12.4.2 The GRAS system

While the regulatory framework for food micro-organisms is not very defined in the EU, in the US a micro-organism used in food could be classified either as an additive, in which case it must be approved by FDA, or it could be considered as generally recognized as safe (GRAS). A product can have a GRAS status either having a history of safe use in food dating before 2 January 1958, or it has been recognized as safe by qualified experts under the conditions of the intended use. The responsibility to provide expert opinions on the safety of a substance or a micro-organism rests solely with the applicant; FDA only either accepting or rejecting them (Wessels *et al.*, 2004, von Wright 2005). The GRAS status also usually only applies to a special use of a micro-organism (i.e., *Bifidobacterium lactis* Bb12 and *Streptococcus thermophilus* in infant formula).

12.4.3 The QPS-system and the emerging European legislation

When SCAN guidelines for the evaluation of micro-organisms used as feed additives were formulated, no exemption was made for the species and strains that have a history of human use. It was soon recognized that this led to an anomalous situation where a strain is subjected to a much more stringent safety evaluation, if it is intended as feed additive, than would be the case if it were used as a food starter or human probiotic. The large number of species and strains already used in food production further makes their safety evaluation strain by strain a practical impossibility. Consequently a position paper 'Safety assessment and regulatory aspects of micro-organisms in feed and food applications' was published by SCAN in 2002 (http://europa.eu.int/comm/food/fs/sc/Scan/outcome_en.html). In this paper it was suggested that specific micro-organisms with a history of safe use or otherwise established safety, could be compiled in order to allow for a generic approval of micro-organisms for food and feed use. The list would be based on 'Qualified Presumption of Safety (QPS) – presumption being defined as a belief or assumption based on reasonable evidence and qualified to allow certain restrictions to apply'. The basic idea is that for a micro-organism having a QPS status the safety assessment should be focused only on those aspects that are relevant for the organism (such as on the possible presence of antibiotic resistance markers) rather than on a global safety assessment of the strain.

The QPS concept was further elaborated in 2003 by involving members from several EU scientific committees (SCAN, Scientific Committee on Food, Scientific Committee on Plants) who produced a working document 'On a generic approach to the safety assessment of micro-organisms used in feed/food and in feed/food production' (http://ec.europa.eu/comm/food/fs/sc/scf/out178_en.pdf). This document was subject to public consultation.

After the establishment of EFSA a scientific colloquium on the topic was organized in Brussels at the end of 2004. The QPS concept was generally seen as a good way of introducing the useful elements of the GRAS system without compromising the safety aspects. Consequently, EFSA has established a working group to provide a proposal for a list of micro-organisms that could be considered for QPS. This work is currently in progress.

12.5 Conclusions and future trends

The regulatory aspects relevant to functional dairy products are to a large extent similar to those of functional foods in general. Functional foods, with the exception of Japan, are not officially recognized in food law as a separate entity, and consequently regulations on the crucial aspects, safety and efficacy are mostly lacking. It can be assumed that this situation will change, and formal criteria for the authorization of functional food products both at national and international level will be established.

The question of allowed health claims is naturally important regarding both the product development and marketing of functional foods. Again, apparently Japan currently has the most coherent policy regarding the health claims that can be accepted. In the US and particularly in the EU the regulations on health claims are still under development. While the distinction between foods and drugs should be kept clear, a more relaxed policy towards enhanced function claims and reduction of disease risk claims would encourage the further development of functional foods.

The specific questions on the probiotic dairy products and the safety evaluation of probiotic strains are connected to the development of regulations on the general use of micro-organisms in foods, whether conventional starters or probiotics. The GRAS system and the eventual European QPS approach would allow the use of traditional safe starters and probiotics without an extensive safety assessment focusing the limited resources to where they are needed, namely situations, where completely novel micro-organism are intentionally introduced into the food chain.

12.6 Sources of further information and advice

Further information on the regulatory developments can best be obtained from the EFSA and FDA web pages (<http://efsa.eu.int>, and <http://www.fda.gov>,

respectively) and direct links to various EU regulations and directives can be found at <http://europa.eu.int/eur-lex/>. Partial lists of GRAS micro-organisms and their uses in foods can be presented at <http://www.cfsan.fda.gov/~dms/opa-micr.html>. The ACE-ART project, which has relevance to the question of the presence of transmissible antibiotic resistance markers in starters or probiotics, and might also have regulatory implications, is presented at <http://www.aceart.net/asp/>.

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Using biomarkers and other indicators of efficacy to demonstrate the health benefits of functional dairy products

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13.1 Introduction: importance of biomarkers in demonstrating health benefits

It is becoming generally accepted that several human disorders in the Western world can be linked, at least in part, to a suboptimal diet. Among them we can observe obesity, diabetes, allergy, cardiovascular disease and cancer. Hence, the dairy industry has considerable market opportunities to produce and develop new and better foodstuffs that without doubt could be profitable both for the industry and for society. However, the industry can only achieve this goal if new strategies are developed to understand the basic underlying molecular mechanisms accounting for these problems. Interest in the role of nutrition in health, not just to stay alive but also to stay healthy and improve the quality of life, has grown tremendously during the past ten years. Traditionally, maintaining health has been more a question of treating diseases through medicines. In the future, the possibility to diminish the risk of disease by eating the right food is an interesting opportunity for the food industry as well as for the health authorities. To make real progress in this area, there will be a need for close collaboration between medical research, clinical knowledge and the food industry. A colleague in the dairy industry (Ulla Svensson, Arla Foods, Stockholm) recently made the following comment:

The dairy industry has a great interest to develop healthy foods where the health effects have been documented. Thus, there is a need for good methods to make this possible. Today, we have a shortage of knowledge on health effects and a shortage of methods to study these. We need to know more about what health effects different foods or food components have and the underlying mechanisms and more importantly, we need

good markers for effects on health/homeostasis. The new technique, 'genomics', offers tremendous possibilities here. By using techniques such as this, we will get the possibility and the tools to carry out tailor-made human intervention studies on health effects leading to new dairy foods on the market with well documented health effects.

Thus, the concept of using functional dairy products to maintain/enhance health is of considerable interest and holds great promise. However, at present, the major problem is being able to document and scientifically demonstrate such effects on health. According to the WHO: 'Health is a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity' (WHO, 1946). In my opinion, it is unlikely that the vast majority of so-called healthy individuals in our society are really healthy according to this strict definition. Indeed, it would be difficult to find individuals with complete well-being considering that many individuals who generally consider themselves healthy often have minor ailments and many individuals without illness/disease symptoms will consider themselves not healthy. Thus, rather than attempting to compartmentalize people into one of the two groups, healthy or not healthy, it may be more valuable/descriptive to depict health as a continuum (Fig. 13.1). A more practical (less restrictive) definition for health might be a state with no recognizable disease (normal functions), no symptoms that require treatment (according to well-defined guidelines) and no nutritional deficiencies. When one takes this approach, it then becomes more evident that the broad spectrum of healthy individuals could indeed benefit from the use of functional dairy products.

13.2 Range of biomarkers used to investigate health benefits

Critical to the above discussion is the availability of biomarkers to enable effects of food components on 'health status/maintenance' or 'reduction of disease risk' to be measured/documented. It can be mentioned, at this stage, that most of the biomarkers presently available address 'reduction of disease risk' rather than health maintenance/improvement. This is not surprising as many of these markers result from an understanding of the biological changes associated with disease progression. However, this is something that must change in the future as the dairy industry and the general population become more interested in functional food products that address normal health maintenance and health improvement. Indeed, the tools and knowledge are available and work is underway to develop such biomarkers of health (see Section 13.4).

Key targets for intervention with functional dairy products include gut and oral microbiota, gastrointestinal functions, immunological and defense systems, mucosal systems and integrative functions (e.g., quality of life).

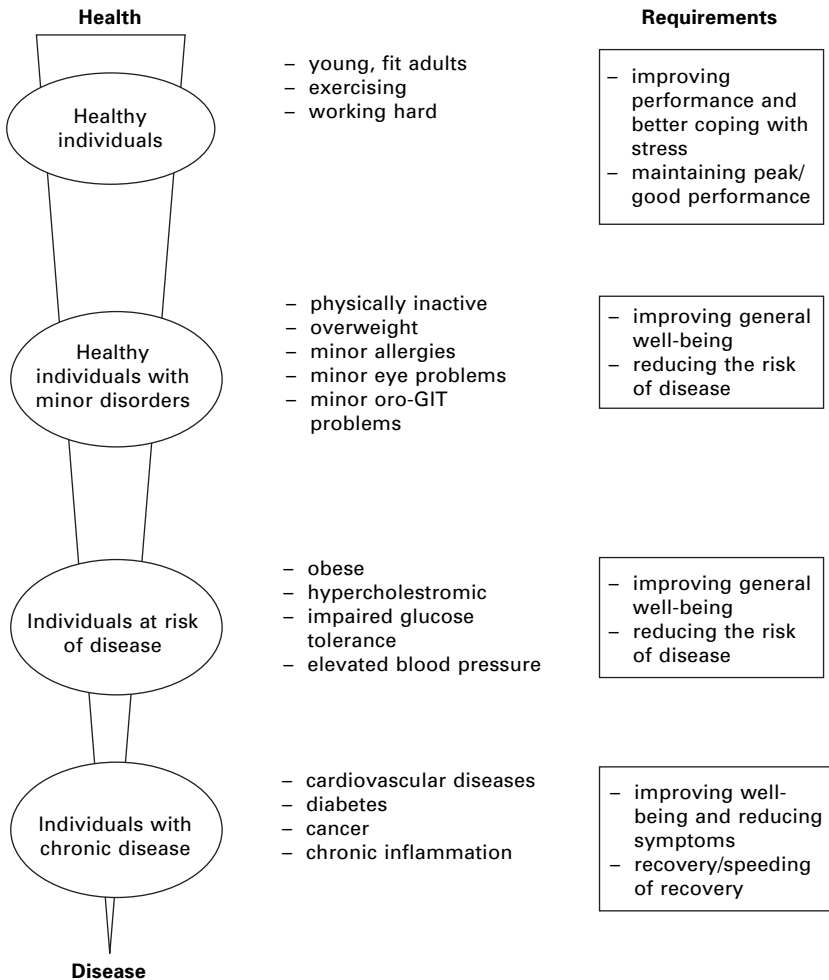


Fig. 13.1 Consumer groups with varying health status and requirements regarding functional foods.

Some promising or developing targets include bone and cardiovascular health and mental state/performance. Thus, numerous biomarkers related mostly to disease have been exhaustively listed in previous publications (Crews *et al.*, 2001; Saris *et al.*, 2002). The reader is particularly referred to two recent supplements of the *European Journal of Nutrition* (Asp *et al.*, 2003, 2004). In these supplements, biomarkers presently available for assessing the effects of food components on cardiovascular disease; bone health and osteoporosis; physical performance and fitness; body weight regulation, insulin sensitivity and diabetes risk; diet-related cancers; mental state and performance; gut health and immunity are thoroughly described and discussed.

In general terms, three levels of generic markers for health are available that can be exploited to assess changes in health status over time with specific dietary interventions. These include: (i) health status and quality of life measures, (ii) physiological/biological markers, and (iii) psychosocial markers/factors.

There are presently available a number of validated tools for measuring health status (Nottingham Health Profile, Hunt *et al.*, 1985), activities of daily living [Physical Self Perception Profile (Fox and Corbin, 1989; Fox, 1990)], and quality of life (QOL) [MOS General Health Status Questionnaire 36-SF (Ware and Sherbourne, 1992)]. Quality of life incorporates the individual's perceptions of health and functional status and is reflective of the perception of his or her health. Its measurement incorporates psychosocial (e.g., daily function, recreation, sexual function, etc.) (see below) as well as physiological (e.g., blood pressure, weight) factors. It is important to point out that the validation of the measure rests with the individual. Several standardized instruments to measure QOL are available including single-item global measures or multi-item health measures; generic measures or condition specific measures. Other clinical measures might also be useful although they do not directly measure individual's perception of own health. These might include daily functioning measures, health care utilization, absenteeism from work or school, prevention of disease. The specific measure should be chosen according to the study population, design, and the specific question to be answered.

There are several different categories for physiological/ biological markers that include:

- integrative, such as infection incidence, skin test, growth rate
- specific system function, such as natural killer cell function, gut transit
- response to a challenge test, such as vaccination or hydrogen breath test
- mechanism, such as DNA repair
- regulatory system, such as insulin secretion
- metabolites, such as short chain fatty acid (SCFA) production.

In order to assess effects of dietary intervention on psychosocial well-being, validated tools such as the Symptom Checklist-90 (Derogatis *et al.*, 1973) or the Mental Health Index SF-36 (Strand *et al.*, 2003)] should be used. The individual subjective sense of well-being is influenced not only by presence and severity of a disease or physical symptoms, but also by the individual's physical, emotional, and psychosocial status. Therefore, clinical research, particularly in healthy people, must use sensitive and responsive measures to assess these subjective domains of health.

13.3 Using biomarkers to demonstrate health benefits

It is possible to conduct human intervention studies looking at the effects of functional dairy products on healthy people. It must be remembered, however,

that ‘healthy people’ is not a homogeneous group (Fig. 13.1). When conducting studies on healthy people, the target population must be considered and specified and appropriate biomarkers must be carefully chosen to measure and demonstrate the effect that is of interest.

Healthy target groups that could potentially benefit from functional dairy product dietary intervention include:

- infants (e.g., in families with atopic disease; prevention of diarrhea)
- toddlers (e.g., prevention of upper respiratory tract infections)
- teenaged girls (bone mass)
- pregnant women (atopic disease, bone mass)
- adults with acute symptoms (e.g., with functional gastrointestinal symptoms)
- elderly (e.g., with impaired immune functions)
- athletes (e.g., restoration of natural killer cell functions)
- travelers (e.g., prevention of diarrhea)

When planning functional dairy product dietary intervention studies, the researcher should choose relevant physiological/biological markers belonging to the above-listed categories. However, within a single study, markers representing one/some categories can be selected – not all categories have to be represented. Guidelines for the proper use of markers are available (Howlett and Shortt, 2004). Particular attention should be paid to the following:

- Markers should be validated methodologically (precision and accuracy, specificity and sensitivity, reproducibility and repeatability) and biologically (they should reflect closely the process leading to the claimed health effect and respond appropriately with changing events).
- Within a study, the marker should change in a biologically relevant way and be statistically significant for the target group.

13.4 Future trends

There is a growing awareness that the intestinal microbiota is not only playing a role in the etiology of the above-mentioned diseases (Introduction) but is also playing an hitherto unexpected role in the maintenance of ‘normal health’. As the microbiota mediates the effects of many of our present health-promoting foods, a greater understanding of the role of these microbes in health and disease will allow the development of the next generation of these foodstuffs. The identification of *Helicobacter pylori* and stomach ulcers and the eradication of this disease as a major hospital problem illustrate the importance of further knowledge of microbe-host interactions. The growing notion that many of the major health problems of chronic inflammation in part have microbial components also underlines the need to understand how the commensal microbes regulate and control homeostasis at the global level.

Thus, accumulating evidence strongly indicates that local and systemic effects of food components are mediated, to a large extent, by crosstalk between microbes and the alimentary tract. Therefore, a major goal of future research must be to better understand the mechanisms underlying gut homeostasis and metabolic function, as well as the influence of the normal microbiota of the intestine. Work must focus on the post-embryonic maturation process, i.e. the microbe mediated colonization of the host that takes place immediately after birth. During this early period in life many of the basic homeostasis mechanisms are set, e.g. the number of fat cells within the body. There is also substantial support for a bidirectional communication between the gut and the brain, possibly via the Hypothalamic-Pituitary-Adrenal (HPA) axis. A deeper understanding of this communication would pave the way for the development of a whole new and exciting range of health-promoting food products.

A strong goal of future research must be to identify microbe regulated biomarkers that can be used to monitor and measure/sustain 'health effects'. These biomarkers will be critical for the development and design of novel functional dairy products and will supply the dairy industry with methods to assess health-promoting effects of their products.

The human colon contains close to 10^{12} bacteria per gram of contents, comprising at least 400 different species. Although the signals provided by bacteria are still poorly characterized, the consequences of microbiota ablation on the GI tract is becoming increasingly apparent through the use of germ-free (GF) mouse models. Using GF mice is essential for these experiments; the noise of the complete microbiota in conventional animals renders them unsuitable for detailed molecular analyses of host-microbe interactions. This extraordinarily complex microbiota (~1 kg biomass of bacteria in man) is gradually established from birth and likely plays an important role in the health of the individual. First and foremost, the normal microbiota influences the function of the intestine, such as breakdown and uptake of foodstuffs, but it also protects the host from colonization with pathogenic bacteria and increases the threshold for activation of the immune system. In addition, conventionally raised animals require 30% less caloric intake to maintain their body weight compared to their GF counterparts, indicating that microbes aid their host in extracting maximum nutritional value from the diet. Thus, GF animals have proven to be a powerful experimental system for studying the molecular cross-talk between mammals and bacteria. DNA microarray analyses have shown that colonization of GF mice with *B. thetaiotaomicron* affects expression of host genes that regulate postnatal maturation, nutrient uptake, metabolism, processing of xenobiotics and angiogenesis (Hooper *et al.*, 2002).

In the future, it will be important to carry out a descriptive identification of genes or gene products in appropriate models that are affected by intestinal colonization with bacteria, locally and systemically. From this information, we will be able to characterize a subset of genes that in the model are

regulated by gut microbes. These genes will constitute biomarkers for normal gene regulation influenced by microbes. It would then be interesting to investigate the possibility of stabilizing a healthy gene expression profile and homeostasis in the colon with functional dairy products. It will also be interesting to follow up the initial description of normal gene expression in the colon, by examining the effects of external stress on the colon. The possible protective effects of functional dairy products could then be tested. These comparative analyses may identify new markers of health effects, and might also explain why and how microbes influence our health. A longer-term goal should be, by carrying out SNP analyses on these health markers in the general population, to identify subgroups who display high versus low reactivity with respect to the markers. Those displaying low marker reactivity could then be the target for intervention trials with functional dairy products in the future.

13.5 Sources of further information and advice

There is already considerable material in the literature dealing with the use of 'biomarkers' for assessing the effects in human trials of dietary interventions with health promoting foods. Thus, for additional information, the reader is referred to some recent reviews (Crews *et al.*, 2001; Saris *et al.*, 2002; Asp *et al.*, 2003, 2004).

13.6 Acknowledgements

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14

Experimental models to investigate the effect of functional dairy products: the case of colon carcinogenesis

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14.1 Introduction

Epidemiological and experimental evidence suggests that functional dietary foods containing probiotics such as lactobacilli or bifidobacteria may be associated with beneficial effects on health such as immunostimulation, alleviation of symptoms of lactose intolerance, decreased incidence of bacterial or virus-induced diarrhea, inflammatory bowel diseases, and, importantly, anticarcinogenesis activity (DiRienzo, 2000). Experimental studies have suggested that probiotic consumption may protect against various types of cancers, and colon cancer in particular (Roberfroid, 2000).

Colorectal cancer is the second most common type of cancer in Europe (Boyle and Ferlay, 2005). The risk of this disease is strongly modified by food and nutrition which may act either by increasing or decreasing the risk. Diets high in vegetables and a regular physical activity decrease the risk, while alcohol, diets high in red meat, sugar and fat are considered possible risk factors (World Cancer Research Fund and American Institute for Cancer Research, 1997). Among potentially protective foods, growing attention has been dedicated to probiotics, since some studies suggest that their consumption decreases experimentally-induced colon cancer in animals (Rowland *et al.*, 1998, Koo and Rao, 1991, Wollowski *et al.*, 2001). Probiotics are defined as viable microbial food ingredients supposed to be beneficial through their effect in the intestinal tract (Roberfroid, 2000). Probiotics consist of either yeast or bacteria, especially lactic acid bacteria and therefore probiotics are mostly consumed as fermented dairy products such as yogurt or freeze-dried cultures (Roberfroid 2000).

In this chapter I will illustrate experimental models that can be used to

study the effect of putative protective foods (whether probiotic dairy foods or not) on colon carcinogenesis. To better understand these models, I will also briefly describe what is known about the pathogenesis of colon cancer. Finally I will review some of the studies in which probiotics or dairy foods have been tested for their anticarcinogenic activity in the colon.

14.2 Pathogenesis of colon cancer

Colon carcinogenesis is a multistage process in which normal cells are initiated to form preneoplastic lesions which may then evolve into macroscopic adenomas and cancers (Chang, 1984, Morson, *et al.*, 2003). The genetic alterations underlying the pathogenesis of colorectal carcinogenesis have been extensively studied (reviewed in Vogelstein and Kinzler, 2004) and it is now believed that neoplastic progression occurs as a stepwise process in which each step is caused by a genetic change (either in an oncogene or in a tumor suppressor gene) so that the development of the different histological stages is accompanied by an accumulation mutational burden (Morson *et al.*, 2003). The study of hereditary bowel cancer syndromes such as familial adenomatous polyposis (FAP) or hereditary non polyposis colon cancer (HNPCC) have led to the discovery of important cancer genes which have also been found mutated in sporadic colorectal cancers. For instance, *APC* gene mutations are inherited in patients with FAP (a disease with a high predisposition to form colorectal polyps and cancer in the colon) and are also frequent in sporadic colorectal cancers (Morson *et al.*, 2003). APC protein interacts with β -catenin, forming, together with other proteins (e.g., GSK-3 β), a macromolecular complex which phosphorylates β -catenin and induces its degradation (Polakis 1999). Mutations in both *CTNBI* (coding for β -catenin) or *APC* genes render β -catenin resistant to degradation, thus causing its accumulation in the cell and activation of the Wnt-signaling pathway (Polakis 1999). Other genes mutated in colon cancer are K-ras, TP53, TGF β RII, DCC. Non-mutational inactivation by mechanisms such as CpG island hypermethylation in the promoter region of genes such as CDKN2A (p 16) and DNA repair genes such as MGMT (O6-methylguanine-DNA methyltransferase) and hMLH1 are also important (Esteller *et al.*, 2000).

14.3 Preneoplastic lesions in colon carcinogenesis

Preneoplastic lesions are considered an obligatory step in the development of cancer, and many efforts have been dedicated to the identification and characterization of preneoplastic lesions in experimental animals and humans. In fact, when preneoplastic lesions are easily identifiable, they can be used as biomarkers in experimental studies of cancer prevention.

In 1987, Bird described foci of aberrant crypts (aberrant crypt foci: ACF), identifiable in whole mount preparations of unsectioned colons in rodents treated with specific colon carcinogens as early as few weeks after carcinogen treatment (Bird, 1987). ACF are visible using a simple methylene blue staining of the colonic mucosa and can be observed under a light microscope at low magnification (Fig. 14.1). ACF appear as crypt aggregates, morphologically altered (larger, intensely stained and with a higher peri-cryptic area (Bird, 1987). ACF have also been identified in apparently normal colonic mucosa of patients with colorectal neoplastic diseases (carcinomas, adenomas, familial adenomatous polyposis) and also in patients with benign colon diseases (Roncucci *et al.*, 1991, Pretlow *et al.*, 1991, Shpitz *et al.*, 1998). Histological analysis showed that some ACF possess typical cytological and histological features of dysplastic lesions, others are hyperplastic lesions, others are characterized only by an increase of the cryptic lumen diameter (Di Gregorio *et al.*, 1997, Roncucci *et al.*, 1993, Pretlow *et al.*, 1994, Siu *et al.*, 1997). Genetic alterations consisting in increased expression of oncogenes, tumor suppressor gene mutations and microsatellite instability, have also been reported in ACF (Augenlicht *et al.*, 1996, Heinen *et al.*, 1996). Based on these data it has been concluded that ACF represent one of the first steps of the colon carcinogenesis process. However, some studies have demonstrated that only a small portion of ACF, probably constituted by the most dysplastic ones, will become tumors (Jen *et al.*, 1994, Papanikolau *et al.*, 2000, Paulsen *et al.*, 2001).

Other premalignant lesions such as β -catenin accumulated crypts (BCAC) have been described in carcinogen-treated rats (Yamada *et al.*, 2000). These lesions are defective in β -catenin, a transcriptional activator frequently mutated in colorectal carcinogenesis. BCAC identification is based on

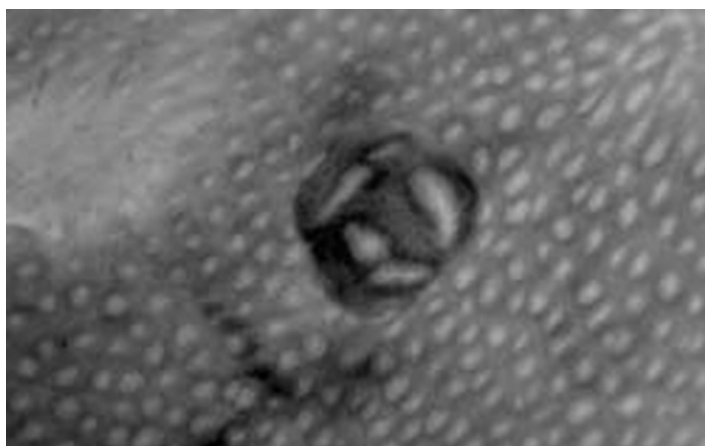


Fig. 14.1 Topographical identification of an ACF formed by five aberrant crypts identified in a rat colon stained with methylene blue (original magnification: 100x).

immunohistochemical techniques, which, unlike the ACF determination, do not permit an easy evaluation of the entire unsectioned mucosal surface. Therefore, BCAC cannot be easily used as biomarkers of carcinogenesis. Similarly, although dysplastic ACF described by other authors (Papanikolaou *et al.*, 2000, Paulsen *et al.*, 2001) may represent preneoplastic lesions, their identification and quantification in the unsectioned colon is problematic, since there is no practical way to look at the entire colon histologically.

Recently, we identified new lesions in the colon of rats treated with azoxymethane (AOM), formed by crypts characterized by the absence or scant production of mucus (mucin-depleted foci, MDF) (Caderni *et al.*, 2003). MDF are easy to quantify in the entire unsectioned colon (Fig. 14.2) and show clear features of dysplasia in histological sections (Caderni *et al.*, 2003). The number of MDF/colon increases in rats treated with promoters of colon carcinogenesis, such as cholic acid, while it is decreased by chemopreventive agents (Femia *et al.*, 2004). MDF are induced dose-dependently by the specific colon carcinogen 1,2-dimethylhydrazine (DMH) and progressively increase in size after carcinogen administration (Femia *et al.*, 2005). Recently, we showed that MDF carry alterations in the Wnt signaling pathway and mutations in the β -catenin gene, two characteristic phenomena in colon tumorigenesis (Femia *et al.*, 2005). Therefore, MDF are suspected preneoplastic lesions.

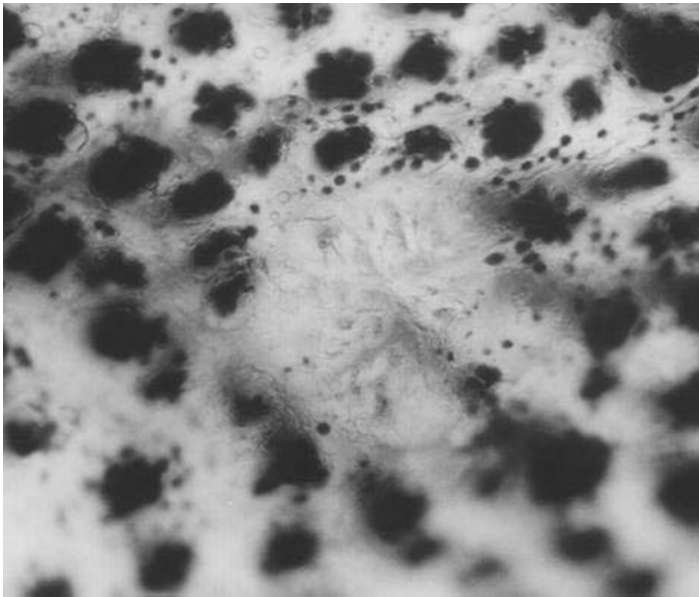


Fig. 14.2 Topographical identification of a MDF in a rat colon stained with HID-AB (original magnification 100x).

14.4 Experimental models to study the effects of nutrients on colon carcinogenesis

14.4.1 Carcinogenesis induced by chemicals

Among the various experimental models used to study colon carcinogenesis, those using azoxymethane (AOM) or 1,2-dimethylhydrazine (DMH) to induce colonic cancer in rodents are very important, since these two carcinogens induce tumors through the sequential formation of histopathological lesions similar to those observed in spontaneous carcinogenesis in humans (Chang, 1984). Accordingly, these methods have been widely used to study the biology of the various phases of colon cancer but also to study the correlation between diet and cancer, by comparing cancer incidence in DMH/AOM initiated rodents fed with different dietary regimens (Fig. 14.3). The DMH/AOM model is also very popular for study of the effect on colon carcinogenesis of putative chemopreventive chemicals such as non steroidal anti-inflammatory drugs (Corpet and Taché, 2002).

Other carcinogens more related to food, such as heterocyclic amines have also been used to induce intestinal tumors in rodents (Ochiai *et al.*, 2003). However, AOM or DMH are less expensive than these carcinogens (e.g., 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine: PhIP) and can more

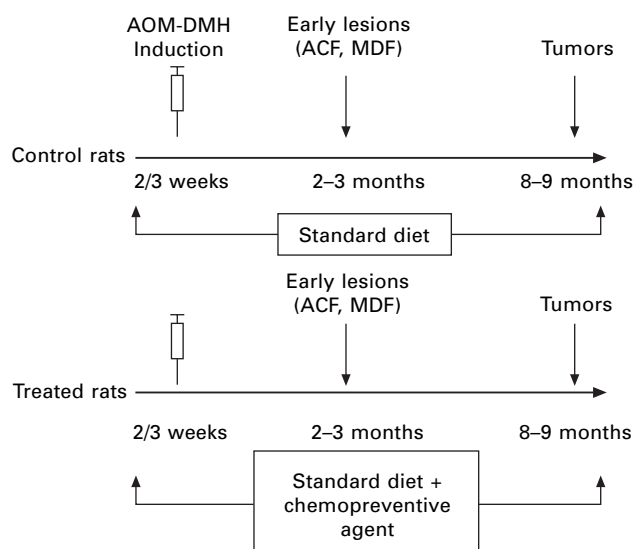


Fig. 14.3 Scheme of an experimental model to study the effects of nutrients on colon carcinogenesis. In both treated and control rats, colon carcinogenesis is induced with injections of azoxymethane (AOM) or 1,2-dimethylhydrazine (DMH). The putative chemopreventive agent, for instance a dietary compound, can be given through the experiment mixed in the diet. Animals are sacrificed after 2–3 months to observe early preneoplastic lesions (ACF or MDF) or later, to observe macroscopic tumors. The effect of the putative chemopreventive agent is evaluated comparing the frequency of early lesions or tumors in the controls and treated rats.

conveniently be administered via subcutaneous or intraperitoneal injections. On the contrary, PhIP has to be administered mixed in the diet for a long period of time (Ochiai *et al.*, 2003).

Rats are the animals used most frequently in the AOM/DMH models, since cancer can be easily induced with just two injections of the carcinogen in these animals, whereas multiple injections are required in mice. However, mice have a smaller body weight than rats so smaller amounts of the chemicals are required. Various dose regimens can be used. We used two subcutaneous injections, one week apart, of DMH, 150 mg/kg each (total dose 300 mg/kg), or, alternatively, two subcutaneous injections, one week apart, of AOM, 15 mg/kg each (total dose 30 mg/kg) in rats. With these dosages and schedule we obtained a good yield of preneoplastic lesions (ACF and MDF) and tumors (Femia *et al.*, 2005). Tumor induction with doses either higher or lower than those we use has been reported (for the website <http://www.inra.fr/reseau-nacre/sci-memb/corpet/indexan.html> for a review).

Rats treated with two injections of AOM or DMH (total dose 30 mg/kg or 300 mg/kg, respectively) develop tumors about eight months later. Both carcinogens induce the majority of cancers in the colon (about one/two tumors per rat with the dosages reported above), but tumors in the small intestine and in the inner ear are also induced, though at a lower frequency. Animals showing rectal bleeding are sacrificed before time.

Typically, groups of 20–30 animals are treated. Treatment with the putative dietary regimen can be administered during the various phases of carcinogenesis, before induction with the carcinogen, during induction or after, during the promotion-progression phase of carcinogenesis.

At sacrifice using CO₂ asphyxiation, all organs are macroscopically examined for the presence of tumors or other pathological lesions. Tissues showing a deviation from normal morphology are fixed in 10% buffered formalin and embedded in paraffin blocks. If possible (due to the dimension of the tumor), part of the lesion is also kept frozen at –80°C for further analysis. Paraffin blocks are then sectioned and stained with hematoxylin-eosin to confirm the presence and type of tumors by histopathological examination, which is performed by a pathologist unaware of the codes of the specimens. Before being fixed in formalin, suspected macroscopic lesions are measured with a caliper and their dimensions calculated by multiplying the two main diameters of each lesion. Cancer histological types are evaluated on the basis of the histotype, grading and pattern of growth (Morson *et al.*, 2003). Adenomas are classified on the basis of their microscopic architecture as tubular, tubulovillous and villous according to Morson *et al.* (2003). To circumvent the necessity of waiting a long time to observe the growth of tumors, purported preneoplastic lesions such as ACF have also been used as endpoint related to carcinogenesis. As reported above, ACF have characteristics of preneoplastic lesions, they are easily quantified and are visible as early as one month from carcinogen induction. Moreover, with a standard dose of carcinogen (f.i. 30 mg/kg of AOM) each rat develops about one hundred

ACF, so that the number of animals/group needed to see a significant effect is relatively smaller with ACF experiments than with long-term carcinogenesis studies (12–15 animals compared with 20–30 animals/group). For these reasons and also because of the simplicity of their quantification, ACF determination has been widely used as a short-term assay to predict carcinogenesis outcome (Corpet and Taché, 2002, see also the website: <http://www.inra.fr/reseau-nacre/sci-memb/corpet/indexan.html>). According to PubMed records of the National Library of Medicine, almost 500 papers have been published on ACF as colorectal cancer biomarkers.

ACF are clearly visible two months after induction with the carcinogen. At sacrifice, the colon is carefully removed from the abdomen, washed with saline, longitudinally opened and pinned flat on a polystyrene board or on two filter papers. After fixation in buffered formalin (at least 4 h), the colon can be cut into three segments: proximal (closer to the cecum and characterized by 'herring-bone' folding of the mucosa), mid and distal (obtained by cutting the remaining colon into two equally long segments). ACF are determined according to Bird (1987) using methylene blue (MB) staining. ACF appear as crypt aggregates, morphologically altered (larger, intensely stained and with a higher peri-cryptic area) (Bird, 1987).

Determination of ACF is performed by counting the number of lesions present in the entire colon and also the number of aberrant crypts (AC) forming each ACF (multiplicity of ACF). Another ACF parameter that has been calculated to predict carcinogenesis outcome is the number of 'large' ACF, which have been defined with different criteria (Corpet and Taché, 2002, Corpet, *et al.*, 1990, Pretlow, *et al.*, 1992). For instance, Pretlow and colleagues (1992) defined 'large' ACF as lesions with a multiplicity equal to or higher than four crypts/ACF. Corpet *et al.* defined large ACF (1990) as ACF being of such a multiplicity that at least one large ACF/rat is present in the control group. It is obvious that using different definitions, different results can be obtained.

The number of total ACF, the multiplicity of ACF and 'large' ACF are all considered parameters correlated to cancer outcome. In the last few years some observers have questioned the use of ACF as a parameter correlated with carcinogenesis. For instance, compounds such as genistein, selected as possible chemopreventive agents based on ACF results, have later been shown to increase tumor development (Magnusson *et al.*, 1993, Zheng *et al.*, 1999, Papanikolaou *et al.*, 2000). Different studies have tried to explain the discrepancy between ACF and cancer (Jen *et al.*, 1994, Papanikolaou *et al.*, 2000, Paulsen *et al.*, 2001), suggesting that only a small portion of ACF, probably constituted by the most dysplastic ones, will become tumors.

Other preneoplastic lesions such as MDF, which are easily detectable in the unsectioned colon of rats 10–15 weeks after induction with the carcinogen, have been proposed as endpoint related to colon carcinogenesis. Accordingly, in AOM or DMH-induced rats the number of MDF increases with promoters of carcinogenesis such as cholic acid, high-fat or heme-rich diets (Femia *et*

al., 2004, Pierre *et al.*, 2004) whereas it decreases with chemopreventive agents such as polyethylene glycole, or synbiotics (Femia *et al.*, 2005, Caderni *et al.*, 2003).

MDF determination can be performed in colons which have been processed for the determination of ACF with methylene blue (MB). MB-stained colons can be kept in formalin and then processed with the high-iron diamine Alcian blue staining (HID-AB). Briefly, the entire colon is rinsed in distilled water for 5 min and transferred into a Petri dish containing a freshly prepared solution (referred to as diamine solution), obtained by dissolving simultaneously 120 mg of N-N'-dimethyl-m-phenylene diamine and 20 mg of N-N'-dimethyl-p-phenylene diamine in 50 ml of distilled water and then adding 1.4 ml of 60% ferric chloride. The Petri dish is covered with aluminum foil to protect it from the light and the colon allowed to be stained with the diamine solution for 18 h at room temperature. The colon is then rinsed three times in distilled water and stained for 30 min with 1% Alcian Blue in 3% acetic acid. The colon is then rinsed three times with 80% ethanol followed by distilled water and then observed under the microscope (mucosa side up) for the determination of MDF. To score the colon we use Optiphot-2, NIKON. With HID-AB staining, normal crypts in the distal part of rat colon are stained brown (indicative of a predominant production of sulphomucins) while in the more proximal part of the colon the crypts are blue (indicative of a predominant production of sialomucins). In HID-AB stained colons, ACF appear as brown or blue depending on their multiplicity and dysplasia (Caderni *et al.*, 1995). MDF are identified as focal lesions characterized by the absence or very limited production of mucins (Caderni *et al.*, 2003). Besides this defect in mucin production, MDF can be recognized since they are focal (with a very clear cut distinction between the lesions and the normal adjacent crypts) and are formed by crypts with a lumen which is often, not always, distorted when compared with normal surrounding crypts. Elevation of the lesion above the surface of the colon, and a multiplicity (i.e., the number of crypts forming each focus) of more than three crypts, are also frequent features of MDF. The colon is then coded and the scoring is performed blindly by two observers. For each colon we determine the number of MDF/colon and their multiplicity (i.e., the number of crypts forming each focus).

14.5 Genetic models

Additional experimental models for the study of colorectal carcinogenesis *in vivo* are those using Apc^{min} (multiple intestinal neoplasia) mice, which carry a germline mutation (codon 850) in the murine homologue of the human APC gene (Adenomatous polyposis coli), mutated in familial polyposis (FAP). Although FAP accounts for a small fraction of all colorectal malignancies overall, it is a good investigative model for studying the sporadic disease, as mutations in APC are often also present as the earliest genetic alteration in

sporadic colon carcinomas. Similarly to what is observed in FAP, $Apc^{\min}$ mice spontaneously develop intestinal polyps at an early age, thus providing a good spontaneous tumor model for studying colon carcinogenesis and also for examining approaches to the prevention and treatment of intestinal polyps. Accordingly, $Apc^{\min}$ mice have also been used to determine the chemopreventive activity of compounds such as dietary components (Wasan *et al.*, 1997, Corpet and Pierre, 2003). However, at variance with the tumors induced with DMH/AOM or with sporadic carcinogenesis in humans, tumors in the $Apc^{\min}$ mice develop mostly in the small intestine while colorectal cancers are rare (Corpet and Pierre, 2003). Since the effect of diet on colon carcinogenesis has been explained, at least in part, with variations in the luminal content of the colon, which is quite different from that in the small intestine, one may argue against the use of $Apc^{\min}$ mice to test dietary compounds. Accordingly, in a recent survey on chemoprevention in rodents, it has been concluded that the carcinogen-induced rat model seems better than the $Apc^{\min}$ mouse model for predicting efficacy of dietary chemopreventive agents in humans (Corpet and Pierre, 2005).

14.6 Proliferative activity of the colonic mucosa

One of the mechanisms proposed to explain the effect of different dietary components on colon carcinogenesis is the modification of the proliferative activity of colon mucosa by different nutrients (Risio *et al.*, 1996, Stamp *et al.*, 1993). Diet has been reported to affect colonic proliferation in both humans and rodents (Caderni *et al.*, 1994, Lipkin, 1988). It has been suggested that high proliferation in the colonic mucosa is associated with an increased risk of colorectal cancer (Lipkin, 1988, Einspahr *et al.*, 1997). Clinical studies have demonstrated that subjects at high risk of colon cancer, such as familial adenomatous polyposis, ulcerative colitis or sporadic colon cancer, have a high mucosal proliferative activity as compared to controls (Lipkin, 1988). Besides, in normal subjects colon proliferative activity is confined to the lower part of the crypt and in high-risk subjects an upwards shift of the proliferative compartment from the basal to the upper part of the crypt occurs. Moreover, in experimental animals, substances known to promote or initiate colon carcinogenesis increase mucosal proliferation (Stamp *et al.*, 1993, Newmark *et al.*, 1990, Richards, 1981). In humans, dietary supplementation with putative protective components such as fish oil have been demonstrated to reduce colonic proliferation in healthy subjects (Bartram *et al.*, 1993), or normalize proliferation in the upper compartment of the crypt in subjects at risk (Anti *et al.*, 1992). Therefore, although some question the association with high proliferation-colon cancer risk (Chang *et al.*, 1997), others consider low proliferation as a protective condition (Bostick *et al.*, 1995, Einspahr *et al.*, 1997).

Cell proliferation can be determined with various methodologies in both humans and experimental animals (Einspahr *et al.*, 1997). For instance, histological sections of the colon can be processed with immunohistochemical techniques to assess the presence of antigens in dividing cells such as PCNA or Ki67 (Femia *et al.*, 2002). In this case, three μm sections from paraffin-embedded colons are mounted on electrostatic-treated slides deparaffinized with xylene, hydrated through a graded series of ethanol and finally distilled water. Endogenous peroxidase activity is blocked with H_2O_2 . To unmask antigen protein (e.g., PCNA), tissue sections are immersed in citric acid buffer (pH 6.0) and microwaved for 20 min. The slides are then immersed in phosphate-buffered saline (PBS) containing 0.05% Tween 20. Immunohistochemical staining can be performed with a streptavidin-biotin immunoenzymatic antigen detection system kit. The slides are incubated in a humid chamber with the primary antibody (mouse monoclonal PC-10, Santa Cruz, CA, USA) diluted 1:200 in PBS for 60 min at room temperature. The slides are then rinsed twice in PBS, covered with Biotinylated Goat Anti-Polyvalent as the secondary antibody (LAB Vision Corporation, CA, USA). After incubation with the secondary antibody, slides are finally reacted with 3,3'-diaminobenzidine and weakly counterstained with Harris' hematoxylin. Proliferative activity is evaluated for each rat by counting PCNA-positive nuclei in at least 10 full longitudinal crypt sections (i.e., from the base to the bottom of the crypt) under a light microscope at 400 $\times$ magnification. Only dark brown stained nuclei are considered PCNA-positive. For each crypt we record the number of cells per crypt column (i.e., number of cells from the bottom to the top of the half crypt appearing in the section) and the number and position of the labeled cells (LC) along the crypt, dividing each crypt into three equal parts: lower, mid and upper compartments.

[^3H]-thymidine incorporation *ex vivo*, followed by autoradiography can also be used to assess proliferation. Briefly, biopsies taken from the distal part of the colon (biopsies can also be taken during endoscopy in studies with human subjects) are transferred to a vial containing Eagle's minimum essential medium and ^3H -thymidine (specific activity, 41 Ci/mmol; final concentration, 5 $\mu\text{Ci/ml}$). Oxygen is also injected into the vial. At the end of the incubation, specimens are washed 10 times with saline and then oriented (mucosa side up), set in a plastic frame, and fixed in this orientation with a 2% agar solution at 40°C. After solidification of the agar they are fixed in 10% buffered formalin and embedded in paraffin. A series of sections, 5 μm in thickness and perpendicular to the surface of the mucosa, are dipped in the dark in Nuclear Track Emulsion (NTB-2; Eastman Kodak, Rochester, NY), diluted by 50% in distilled water at 40°C, and kept in the dark at 4°C for 2 weeks. Slides are then developed by standard photographic procedures and stained with hematoxylin-eosin. After processing each biopsy for autoradiography, the number and position of labeled cells along the crypt, and the number of cells scored per crypt are measured. Data on cell proliferation are expressed as labeling index (LI) (number of labeled cells in the crypt

divided by the number of cells scored in the crypt section $\times 100$). Each crypt was also ideally divided into three equal compartments (lower, middle and upper) and the distribution of proliferating cells along the crypt was expressed as the percentage of labeled cells in each compartment over the total labeled cells in the crypt section.

14.7 Apoptosis in colonic mucosa

A low level of apoptosis (programmed cell death) may allow mutated cells to survive and give rise to a mutated clone of cells. Therefore the induction of apoptosis in neoplastic cells is considered a possible mechanism for eliminating cells with a high level of DNA damage (Samaha *et al.*, 1997).

It has been reported that putative chemopreventive substances such as curcumin, phenylethyl-3-methylcaffeate, and 6-phenylhexyl isothiocyanate increase apoptosis in experimentally induced colonic tumors, suggesting increased apoptosis as a mechanism of chemoprevention (Reddy *et al.*, 1997). Apoptosis evaluation can be carried out on paraffin-embedded sections of normal colonic mucosa and tumors stained with hematoxylin-eosin (Reddy *et al.* 1997, Chang *et al.*, 1997) by evaluating the presence of cells in each crypt with the following characteristics of apoptosis: cell shrinkage, loss of normal contact with the adjacent cells of the crypt, chromatin condensation or formation of round or oval nuclear fragments ('apoptotic bodies') (Caderni *et al.*, 2000).

14.8 Composition of the luminal content of the colon

Of the endogenous luminal components which have been suggested to play a role in the induction of colon cancer, bile acids (BA) and short chain fatty acids (SCFA) have been considered of crucial importance for a long time (Narisawa *et al.*, 1974, Reddy *et al.*, 1976, Bugaut and Bentéjac 1993). In fact, BA have a promoting effect on experimental colon cancer models in rodents (Narisawa *et al.*, 1974, Reddy *et al.*, 1976) and have been associated with colon cancer risk in humans (Hill *et al.*, 1975, Hill, 1990), whereas SCFA are supposed to have a beneficial regulatory effect on colon mucosa (Bugaut and Bentéjac 1993). BA have been suggested to alter the process of colon carcinogenesis through different mechanisms, the most widely accepted suggesting that a high level of BA increases colon mucosa cell proliferation. BA are directly toxic for the cell membranes of the intestinal lumen, induce cell death dose-dependently and, consequently, increase cell turnover (Deschner *et al.*, 1981, Bird *et al.*, 1986., Peiffer *et al.*, 1997). However, while most studies correlating high proliferation with fecal BA have been obtained in experimental models, the existing human literature on this topic is far from

conclusive (Stadler *et al.*, 1988, Geltner-Allinger *et al.*, 1991 Dolara *et al.*, 2000).

Contrary to the supposed detrimental effect of BA on the colon, SCFA, derived from bacterial digestion of fiber and complex carbohydrates in the colon, have trophic action on the mucosa and are possibly protective against inflammation and cancer (Bugaut and Bentéjac, 1993, Tonelli *et al.*, 1995).

A potentially beneficial effect of SCFA on the mammalian colon mucosa has long been suggested (Bugaut and Bentéjac, 1993). However, studies relating SCFA and colon proliferation have provided conflicting results. Studies in experimental animals have demonstrated that SCFA have trophic effects on the colon and that butyrate, one of the main SCFA in the colon, stimulates colon proliferation in rats (Sakata, 1987). SCFA also increase cell proliferation in the basal section of the crypt when human colon biopsies are incubated *in vitro* (Scheppach *et al.*, 1992). On the contrary, SCFA have been reported to reduce cell proliferation in pathological conditions such as ulcerative colitis (22). Moreover, two clinical studies have shown that chronic treatment with acarbose, an inhibitor of α -amylase (Holt *et al.*, 1996), or dietary supplementation with amylomaize (Van Munster *et al.*, 1994) are associated with an increase in colonic SCFA and with diminished mucosal proliferation.

14.9 Probiotics and functional dairy products in experimental models of colorectal carcinogenesis

Probiotics alone or in combination with dairy products have been tested for their ability to decrease colon carcinogenesis induced by AOM or DMH.

Rats initiated with AOM and fed with a semipurified diet supplemented with *Bifidobacterium longum*, had a significantly lower incidence and multiplicity of colonic tumors than control rats (Singh *et al.*, 1997). Colonic proliferation and the expression of ras-p21 oncoprotein were also decreased in rats treated with probiotics, suggesting a mechanism for the observed inhibition of colon carcinogenesis (Singh *et al.*, 1997). Although this report suggests a protective effect of probiotics, other studies have shown that this effect is not completely consistent and may depend on the experimental conditions (Goldin *et al.*, 1996). Goldin and coworkers (1996) demonstrated that while *Lactobacillus rhamnosum* (strain GG) reduced colon cancer when given to rats 3 weeks before carcinogen administration, it was not active if given after the ninth week of DMH treatment. Goldin and Gorbach (1980) reported that the incidence of colon cancer induced by DMH in rats treated with *Lactobacillus acidophilus* was lower after 20 weeks of induction, but this protective effect was not observed after 36 weeks, thus suggesting that these bacteria may increase the latency for induction of experimental carcinogenesis.

Other reports have pointed out that the results may depend on the particular strain of probiotic used. Freeze-dried milk fermented by two strains of

Lactobacillus delbrueckii subsp. bulgaricus were tested for the ability to inhibit DMH-induced colon carcinogenesis in BD6 rats (Balansky *et al.*, 1999). Both strains induced pro-differentiation markers and inhibited ear tumors (that are sometimes induced with DMH treatment). However, one of the strains decreased carcinogenesis in both the colon and small intestine, while the other one was inactive in the colon. Different strains of probiotic bacteria were also studied on DMH-induced colon carcinogenesis in Sprague-Dawley rats (McIntosh *et al.*, 1999). Only *L. acidophilus* (strain Delvo Pro LA-1) caused a significant reduction in colon tumors, whereas strains such as *Lactobacillus rhamnosum* (GG), *Bifidobacterium animalis* (CsCC1941) or *Streptococcus thermophilus* were not active (McIntosh *et al.*, 1999). These data together with those of Balansky *et al.* (1999) suggest that different results can be obtained, depending on the probiotic strain tested.

The ability of functional dairy products to decrease colon carcinogenesis has also been tested determining precursor lesions of colon cancer such as aberrant crypt foci (ACF). Lyophilized cultures of *Bifidobacterium longum* used to ferment dairy products were demonstrated by Kulkarni and Reddy (1994) to decrease ACF formation when fed before, during and after initiation with the carcinogen AOM. A similar protective affect of *B. longum* cultures was observed by Challa *et al.*, (1997) who tested this bacterium alone or in combination with lactulose, a keto analogue of lactose, which may serve as a substrate for growth of bifidobacteria. A significant reduction of AOM-induced ACF was observed in rats fed the bacteria singly or in combination with lactulose. Glutathione-S-transferase was also increased by probiotic treatment.

Brady *et al.* (2000), also suggested that lactobacilli and bifidobacteria may inhibit precancerous lesions and tumor development in animal models. However, negative results have also been reported (Abdelali *et al.*, 1995, Gallaher *et al.*, 1996). Abdelali and colleagues (1995) found that milk fermented with a *Bifidobacterium* strain, uninoculated milk and the same lactic acid bacterium administered in the diet were equally active in reducing the incidence of ACF in the colon. Gallaher *et al.*, (1996) using bifidobacteria and lactobacilli, reported a high variability in ACF results as a function of the weight of the rats at the time of DMH administration.

Recently, it has been suggested that a combination of prebiotics and probiotics, the so-called synbiotics, might be more active than the individual components on the colon (Roberfroid, 1998). Accordingly, Rowland and co-workers (1998) showed that concomitant administration of inulin and bifidobacteria to rats resulted in a more potent inhibition of AOM-induced ACF than the administration of the two separately. Similar results, although less marked and consistent, were obtained by Gallaher and Khil (1999) in DMH-induced rats treated with synbiotics.

While these results with synbiotics were obtained using short-term carcinogenesis tests, no data were available on the effect of synbiotics in long-term carcinogenesis experiments. Therefore we studied (Femia *et al.*,

2002) whether probiotics (PRO group: *B. lactis* (Bb12) and *L. rhamnosus* (LGG), each at 5×10^8 CFU/g diet), the prebiotic inulin enriched with oligofructose (PRE group: Raftilose-Synergy1[®], briefly, Synergy1, 10% of the diet), or synbiotics (PREPRO group: a combination of the two) protect rats against AOM-induced colon cancer. Thirty-one weeks after AOM, rats treated with Synergy1 (PRE and PREPRO groups) had a significantly lower number of tumors (adenomas and cancers) than rats without Synergy1. A slight, not significant effect of probiotics in reducing malignant tumors was also observed. Cecal SCFA were higher in the groups treated with Synergy1. Apoptosis was increased in the normal mucosa of the probiotic group, while no variation was observed in the tumors. Therefore in our study prebiotic and synbiotic administration in the diet decrease AOM-induced carcinogenesis in rats, while the effect of probiotics is not strong (Femia *et al.*, 2002).

In conclusion, the scientific literature on the effect of probiotics, synbiotics and dairy foods suggests a possible benefit on experimental colon carcinogenesis. The mechanisms by which these dietary components act are less clear, but some data suggest that they may act through a combination of mechanisms involving an increase in SCFA production, lower proliferative activity and a variation in some enzymes involved in the pathogenesis of colon cancer such as glutathione S transferase. How these variations are causally related to colon carcinogenesis is not yet clear and further studies will be necessary to understand the role of these dietary components on colon carcinogenesis.

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Molecular approaches to assess the activity and functionality of commensal and ingested bifidobacteria in the human intestinal tract

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15.1 Introduction

Bifidobacteria were first isolated and described over a century ago from human infant faeces and were quickly associated with a healthy intestinal tract due to their numerical dominance in breast-fed infants compared to bottle-fed infants (Tissier, 1900; Tissier, 1906). The human gastrointestinal tract is essentially sterile at birth but is rapidly colonized during the first few days of life by microbes acquired from the mother and the surrounding environment. Bacteria capable of aerobic growth, such as enterobacteriaceae, streptococci and staphylococci, initially colonize the gastrointestinal tract and create reducing conditions favourable to the proliferation of anaerobic bacteria, including bifidobacteria, clostridia, ruminococci and bacteroides (Favier *et al.*, 2002; Mackie *et al.*, 1999). Bifidobacteria generally thrive and dominate in the infant intestine due to their selection by the breast milk. Bifidobacteria still remain a relatively dominant group in the intestine of adults (Zoetendal *et al.*, 2006b). Based on initial studies on breast-fed infants that harboured high numbers of bifidobacteria and which resisted intestinal infections better than their formula-fed counterparts, bifidobacteria have been incorporated into probiotic functional foods. Health benefits of bifidobacteria to the host as supported by clinical trials have led to their wide application as probiotic components of health-promoting foods, especially in fermented dairy products in the form of yoghurts and daily shots. Although foods containing probiotic bifidobacteria are widely consumed, there is only fragmentary information about their physiology, ecology, and genetics. Various

species originating from the human intestinal tract including *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium longum* bv. *infantis*, *Bifidobacterium longum* bv. *longum*, and *Bifidobacterium animalis* ssp. *lactis* (see Table 15.1) have been applied during or after the manufacture of fermented milks together with 'traditional' starter cultures to obtain products with satisfactory quality and added health-promoting value (Tamime, 2002). Bifidobacteria are also available as probiotic components in microcapsules following fermentation by 'traditional' starter cultures which enhances survival (Sultana *et al.*, 2000; Kailasapathy, 2006).

Besides being added to functional foods, indigenous commensal bifidobacterial species are also common targets for prebiotic action. These prebiotics are oligosaccharides that are selectively fermented by bifidobacterial species aiming at improving the host's health (Gibson and Roberfroid, 1995; Ouwehand *et al.*, 2005; Crittenden *et al.*, 2005). Thus there is considerable need to understand functional activity of bifidobacteria and their interactions with the host. The conventional way to study the intestinal ecosystem is by focusing on its structure and specifying the number and variety of inhabitants in different sites. Classical culturing methods are used to define the total number of microbes and species-specific numbers of bacteria. Over the last decade it has been established that only a minority of the gastrointestinal tract microbes have been enumerated and isolated in pure cultures owing to insufficient suitable conditions and challenges in cultivating anaerobic microbes (Backhed *et al.*, 2005; Zoetendal *et al.*, 2006d). Similarly, for bifidobacterial species the selective media used today are not sufficient to enumerate and isolate all bifidobacterial species present in gastrointestinal samples (Temmerman *et al.*, 2004; Satokari *et al.*, 2003; Ventura, 2004c). Consequently, molecular approaches are necessary to reveal the complete architecture of the intestinal microbial ecosystem. The majority of culture-independent methods are based on targeting the 16S ribosomal (rRNA) genes (Ben Amor and Vaughan, 2004). A wealth of technologies to study and track the bifidobacteria including fingerprinting techniques such as 16S rRNA PCR-DGGE up to genome-wide targeting DNA microarrays are under development. In addition to revealing the identity and diversity of the intestinal inhabitants, functional genomics approaches are now also providing insight in the activity of the bifidobacteria population within the human intestine. Such a functional analysis of the intestinal ecosystem is developing rapidly and is considered to be essential for the further development of functional foods that impact health.

In this chapter we review molecular and new genomics-based technologies that may be applied in the absence of culturing to gain more insight into the presence and diversity of bifidobacteria in the human gastrointestinal tract, as well as recent advances of these techniques that allow us to study functionality and activity *in situ*. Molecular techniques will provide data that can help explain the physiology of the bifidobacteria and their adaptation to their ecological niche, and health-promoting effects in the host.

Table 15.1 References and habitats of species of the genus *Bifidobacterium*

Species	Type strain*	Source	Reference
Found in human GIT			
<i>B. adolescentis</i>	ATCC 15703T	Infant and adult faeces, appendix, dental caries and vagina	(Reuter, 1963)
<i>B. angulatum</i>		Adult faeces	(Scardovi and Crociani, 1974)
<i>B. bifidum</i>	DSM 20215 JCM 1254 NCIMB 41171	Infant and adult faeces and vagina	(Orla-Jensen, 1924)
<i>B. breve</i>	UCC2003	Infant faeces and vagina	(Reuter, 1963)
<i>B. catenulatum</i>		Infant and adult faeces and vagina	(Scardovi and Crociani, 1974)
<i>B. dentium</i>	Bd1	Human dental caries, oral cavity and adult faeces	(Scardovi and Crociani, 1974)
<i>B. gallicum</i>		Adult faeces	(Lauer, 1990)
<i>B. longum</i> bv. <i>infantis</i>	ATCC 15697	Infant faeces and vagina	(Reuter, 1963; Sakata <i>et al.</i> , 2002)
<i>B. longum</i> bv. <i>longum</i>	DJO10A NCC2705	Infant and adult faeces and vagina Human intestine	(Reuter, 1963; Sakata <i>et al.</i> , 2002) DOE Joint Genome Institute
<i>B. pseudocatenulatum</i>		Infant faeces	(Schell <i>et al.</i> , 2002)
<i>B. scardovii</i>		Infant faeces	(Scardovi <i>et al.</i> , 1979)
<i>B. thermophilum</i>		Blood and urine	(Hoyles <i>et al.</i> , 2002)
		Piglet, chicken and calf faeces and rumen infant	(Mitsuoka, 1969) (von Ah <i>et al.</i> , unpublished)
Found in animal GIT			
<i>B. animalis</i> ssp. <i>animalis</i>		Rat, chicken, rabbit and calf faeces	(Scardovi and Crociani, 1974; Masco <i>et al.</i> , 2004)
<i>B. asteroides</i>		Honey bee	(Scardovi and Trovatelli, 1969)
<i>B. boum</i>		Rumen and piglet faeces	(Scardovi <i>et al.</i> , 1979)
<i>B. choerinum</i>		Piglet faeces	(Scardovi <i>et al.</i> , 1979)
<i>B. coryneforme</i>		Honeybee	(Biavati <i>et al.</i> , 1982)
<i>B. cuniculi</i>		Rabbit faeces	(Scardovi <i>et al.</i> , 1979)
<i>B. gallinarium</i>		Chicken faeces	(Watebe <i>et al.</i> , 1983)

Table 15.1 Continued

Species	Type strain*	Source	Reference
<i>B. indicum</i>		Honey bee	(Scardovi and Trovatelli, 1969)
<i>B. longum</i> bv. <i>suis</i>		Piglet faeces	(Matteuzzi <i>et al.</i> , 1971)
<i>B. magnum</i>		Rabbit faeces	(Scardovi and Zani, 1974)
<i>B. merycicum</i>		Rumen	(Biavati and Mattarelli, 1991)
<i>B. pseudolongum</i> ssp. <i>globosum</i>		Piglet, calf, rat, rabbit, lamb faeces and rumen	(Biavati <i>et al.</i> , 1982)
<i>B. pseudolongum</i> ssp. <i>pseudolongum</i>		Piglet, rat, chicken, calf faeces and rumen	(Scardovi and Trovatelli, 1969; Yaeshima <i>et al.</i> , 1992)
<i>B. psychraerophilum</i>		Pig caecum	(Simpson <i>et al.</i> , 2004)
<i>B. pullorum</i>	ATCC 49618	Chicken faeces	(Trovatelli <i>et al.</i> , 1974)
<i>B. ruminantium</i>		Rumen	(Biavati and Mattarelli, 1991)
<i>B. saeculare</i>		Rabbit faeces	(Biavati and Mattarelli, 1991)
<i>B. simiae</i>		Monkey abdomen	(Falsen <i>et al.</i> , unpublished)
<i>B. thermacidophilum</i> ssp. <i>porcinum</i>		Piglet faeces	(Zhu <i>et al.</i> , 2003; Dong <i>et al.</i> , 2000)
Other origins			
<i>B. animalis</i> ssp. <i>lactis</i>		Fermented milk	(Meile <i>et al.</i> , 1997) (Masco <i>et al.</i> , 2004)
<i>B. minimum</i>		Sewage	(Biavati <i>et al.</i> , 1982)
<i>B. subtile</i>		Sewage	(Biavati <i>et al.</i> , 1982)
<i>B. urinalis</i>		Human urine	(Hoyles <i>et al.</i> , unpublished)

Adapted from (Biavati *et al.*, 2000; Ventura *et al.*, 2004c; Leahy *et al.*, 2005)

* Characterized type strain with 16S rRNA gene sequence available in the database.

15.2 Development of bifidobacteria in the intestine and beneficial effects

Bifidobacteria form part of the normal intestinal microbiota of human infants and adults and are believed to play a beneficial role in maintaining the health of the host. During birth and the first few days of life, bifidobacteria, amongst other intestinal microbes acquired from the mother and the surrounding environment, colonize the infant intestine (Favier *et al.*, 2003). A succession of microbes occurs commencing with aerobes such as enterobacteriaceae, that create reducing conditions favourable to more strict anaerobic bacteria including the bifidobacteria (Franks *et al.*, 1998; Mackie *et al.*, 1999). The changing microbial ecology is coincident with a marked functional and morphological maturation of the infant gut barrier functions and immune system development (Cebra, 1999; Hooper, 2004). The microbiota in breast-fed infants is usually dominated by bifidobacteria within a few days, but following a short delay they also reach high numbers in formula-fed infants (Harmsen *et al.*, 2000a; Favier *et al.*, 2002). Apparently lower numbers of bifidobacteria are found in faeces of pre-term infants (reviewed by Westerbeek *et al.*, 2006). A combination of sequence analysis of 16S rRNA gene clone libraries (Eckburg *et al.*, 2005; Zoetendal *et al.*, 1998; Suau *et al.*, 1999) and the fluorescent in situ hybridization (FISH) approach targeting the 16S rRNA (Harmsen *et al.*, 2002; Lay *et al.*, 2005) has shown that the most abundant bacterial groups in the human intestine belong to, in order of numerical importance, the phyla of the *Firmicutes* (including the large class of *Clostridia* and the lactic acid bacteria), *Bacteroidetes*, *Actinobacteria* (including *Colinsella* and *Bifidobacterium* species) and *Proteobacteria*. Thus, the adult intestinal microbiota comprises a majority of low and high G+C content Gram-positive bacteria.

The association of bifidobacteria with a healthy infant intestinal tract has promoted the addition of bifidobacteria to dairy products as probiotics. Probiotics are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2002). Survival of the probiotic upon gastrointestinal transit, adhesion to the intestinal mucosa, and stability in the product are considered desirable properties for a probiotic, although not necessarily a good indicator for health-improving properties of the probiotic (Tannock, 2005). Ultimately, the efficacy of these probiotic strains in the products for a certain health benefit must be proven in clinical trials. The viability of the ingested probiotic as well as the effect on the autochthonous microbiota has been reported in numerous studies. The therapeutic efficacy of these probiotic strains (and combination species and strains) has been demonstrated in various clinical trials usually involving prevention or alleviation of symptoms of gastrointestinal disorders. The various benefits included alleviation of symptoms of diarrhoea, irritable bowel syndrome, inflammatory bowel disease, and improvement in immunity. Some studies are described in more detail below.

The importance of the microbiota composition may initiate in early infancy when maturation of the gut barrier functions and immune development occurs. A few studies suggest that infants that do not harbour bifidobacterial species or harbour decreased numbers may be more prone to rectal bleeding (Arvola *et al.*, 2006), allergy (Furrie, 2005) and eczema (Mah *et al.*, 2006). Very early administration of *B. breve* to low birth weight infants was shown to be useful in promoting the colonization of bifidobacterial and the formation of a normal intestinal microbiota (Schrezenmeir *et al.*, 2004). In several other studies bifidobacterial supplementation modified the infant gut microbiota in a manner that appeared to alleviate allergic inflammation (Kirjavainen *et al.*, 2002) and reduced the incidence of acute diarrhoea and rotavirus infections (Saavedra *et al.*, 1994). Also combinations of bifidobacterial species with *Streptococcus thermophilus* strains reduced the incidence of rotavirus associated diarrhoea (Saavedra *et al.*, 1994) and antibiotic-associated diarrhoea in children (Correa *et al.*, 2005; Thibault *et al.*, 2004). A mix of *Bifidobacterium* species and *Lactobacillus acidophilus* increased energy intake and promoted weight gain in acutely ill children receiving antibiotics (Schrezenmeir *et al.*, 2004).

The effects of supplementation with probiotic bifidobacteria have also been studied in adults, especially in relation to alleviating symptoms of gastrointestinal disorders. For example, a recent report indicated that oral therapy with a fixed combination of *B. longum* and *Lactobacillus gasseri* shortened the duration and decreased the severity of acute diarrhoea in adults (Margreiter *et al.*, 2006). Other studies showed beneficial effects of bifidobacterial species mixed with lactobacilli and/or *S. thermophilus* strains in the maintenance of antibiotic-induced remission in chronic pouchitis (Gionchetti *et al.*, 2003; Mimura *et al.*, 2004). However, in another acute pouchitis trial, supplements of *L. acidophilus* and *B. lactis* Bb 12 did not lead to an improvement in symptoms (Laake *et al.*, 2003). In collagenous colitis patients, a preliminary probiotic trial involving treatment with *B. animalis* ssp. *lactis* Bb 12 demonstrated amelioration of clinical symptoms (Wildt *et al.*, 2006). In a trial, intake of capsules of live combined *Bifidobacterium*, *Lactobacillus* and *Enterococcus*, significantly decreased relapse and inflammatory activity in the colon of patients with ulcerative colitis (Cui *et al.*, 2004). The probiotic strain *B. infantis* 35624 was able to alleviate inflammation symptoms in patients suffering from irritable bowel syndrome (O'Mahony *et al.*, 2005) suggesting an immune-modulating role for *B. infantis*. Supplements of lactobacilli and bifidobacteria effectively suppressed *Helicobacter pylori* infection in adults (Wang *et al.*, 2004). Different probiotic preparations, including a combination of bifidobacteria and *Lactobacillus* spp. or *Lactobacillus rhamnosus*, *Saccharomyces boulardii*, were found to be superior to the placebo for side effect prevention but had no effect on *H. pylori* eradication (Cremonini *et al.*, 2002). Although recovery of *B. animalis* ssp. *lactis* Bb 12 from faeces was observed in healthy young adults (Christensen *et al.*, 2006), the supplementation had only a slight effect on the immune function (Christensen *et al.*, 2006). Several other feeding

trials reported in healthy individuals to study persistence of the ingested bifidobacteria mainly using faecal samples will be discussed in the section below on 'Tracking bifidobacteria in human intestinal samples'.

Bifidobacteria are not only used as probiotic ingredients but the indigenous bifidobacteria are also targets of prebiotics. A prebiotic is a nondigestible food ingredient that beneficially affects the host by selectively stimulating growth and/or modifying the metabolic activity of one or a limited number of bacterial species in the colon that have the potential to improve host health (Crittenden *et al.*, 2005; Gibson *et al.*, 2006). A formula supplemented with a prebiotic mixture (galacto- and fructooligosaccharides) showed consistently higher faecal SIgA levels which suggested possible stimulation of the mucosal immune response. SIgA antibodies are associated with increased neutralization and clearance of viruses and this effect may be transferred via breast milk to support maturation of humoral immunity (Bakker-Zierikzee *et al.*, 2006). In a previous study the prebiotic supplemented formula resulted in a similar effect on metabolic activity of the microbiota as in breast-fed infants (Bakker-Zierikzee *et al.*, 2005). The increase of bifidobacterial numbers by dietary oligosaccharides (90% galacto-oligosaccharides and 10% fructo-oligosaccharides) reduced the presence of clinically relevant pathogens in preterm formula-fed infant faecal microbiota, indicating protective capacity of prebiotics against enteral infections (Knol *et al.*, 2005).

Despite the variable results obtained in the numerous clinical trials, many scientists argue in favour of bifidobacteria for adjunct therapy. The main reason is the observation that bifidobacteria are the dominant commensal microbiota of most breast-fed babies, and the negative effects seen upon delayed colonization of bifidobacteria in pre-term babies (Westerbeek *et al.*, 2006). The underlying assumption is that at least part of the protective effect of breast-feeding is linked to the presence of bifidobacteria.

15.3 Taxonomy and typing of bifidobacteria colonizing the human intestine

Bifidobacteria were first isolated from the faeces of breast-fed infants by Tissier (Tissier, 1900) and since then it has been established that their predominant habitat is the human gastrointestinal tract. Although the *Bifidobacterium* genus shares phenotypic features typical of lactic acid bacteria, such as acid production, they belong to the *Actinomycetales* branch of the high-G+C Gram-positive bacteria that also includes the corynebacteria, mycobacteria, and streptomycetes (see Fig. 15.1: Phylogenetic tree obtained using the 16S rRNA gene). Today, 37 bifidobacterial species have been characterized in the literature available in the public database, Pubmed. Many species have been isolated from human as well as animal faeces, and sewage, but they have also been isolated from the human oral cavity and the

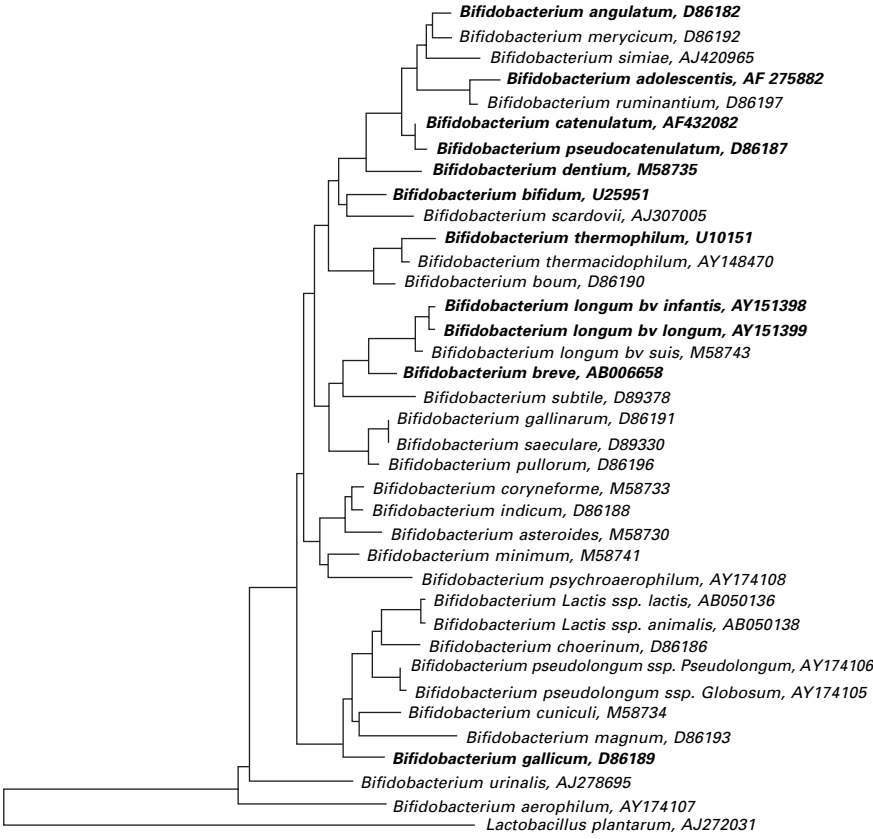


Fig. 15.1 Phylogenetic tree based on 16S rDNA sequences of bifidobacteria rooted with *Lactobacillus plantarum*. The tree was generated from the tree-of-life ARB software package. Accession numbers of the 16S rDNA sequences are indicated. Species isolated from human intestine are indicated in bold.

intestinal tracts of insects such as bees. Species of the genus *Bifidobacterium* and the habitat from which they were isolated are presented in Table 15.1. The *Bifidobacterium* branch forms a coherent phylogenetic unit as their 16S rRNA sequences share over 93% similarity (Miyake *et al.*, 1998). Phylogenetic classification of all 16S rRNA gene sequences of bifidobacterial species originating from the human gastrointestinal tract resulted in a selection of 48 sequences. Twelve of these species were already fully characterized and are part of Table 15.1 (Biavati *et al.*, 2000). The remaining, uncharacterized or uncultured, 36 sequences contain five new unique OTUs (operational taxonomic units), and share less than 97% similarity to the identified 16S rRNA genes of other bifidobacterial species. These five sequences were all acquired from adult faecal samples using culture-independent techniques (Satokari, 2001, Ben-Amor *et al.*, 2005). Thus, the latter study indicates that culture-independent approaches are a valuable tool for the identification of the total community

of microbes in the gastrointestinal tract including not yet identified new bifidobacterial species. As most studies on diversity and identification of microbes are based on 16S rRNA gene sequences, the database of bacterial 16S rRNA genes is expanding daily. Therefore this gene is the general target for bifidobacterial strain and species identification in mixed microbial samples. Nevertheless, characterization of bifidobacterial species remains difficult as the 16S rRNA genes show more than 93–99.5% interspecies similarity (Ventura *et al.*, 2001; Ward and Roy, 2005).

For differentiation between closely related bifidobacterial species other genes including the elongation factor Tu (*tuf*) gene (Ventura *et al.*, 2004c), recombinase A (*recA*) (Ventura and Zink, 2003), chaperone GroEL gene (Ventura *et al.*, 2004b; Masco *et al.*, 2004), ATP synthase subunit B (*atpD*) gene (Masco *et al.*, 2004; Ventura *et al.*, 2004a), pyruvate kinase (Vaugien *et al.*, 2002), and, xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (*xfp*) (Yin *et al.*, 2005) have been introduced as phylogenetic markers besides the 16S rRNA gene. Multiparametric PCR based methods support and complement the 16S rRNA based division of bifidobacterial species and provide a finer taxonomic differentiation between the closely related species such as *B. animalis* ssp. *lactis* (also called *B. lactis*) and *B. animalis* ssp. *animalis*, and *B. longum* bv. *infantis* and *B. longum* bv. *longum*. These and other techniques and definitions on taxonomy are reviewed in more detail in Chapter 17.

The release of the complete genomes of *B. longum* NCC2705 (Nestle Research Centre, Switzerland) and *B. adolescentis* (Gifu University, Life Science Research Centre, Japan/National Center for Biotechnology Information, NIH, USA, Bethesda), two draft assemblies of *B. longum* DJO10A (DOE Joint Genome Institute) and *B. adolescentis* L2-32 project (Washington University Genome Sequencing Center) as well as the initiatives to sequence *B. breve* (University College Cork), *B. dentium* Bd1 (University of Parma, Italy/National University of Ireland, Ireland), and *B. longum* bv. *infantis* ATCC 15697 (DOE Joint Genome Institute/JGI-PGF) are expected to open up a new era of comparative genomics in bifidobacterial biology. The genomes of *B. longum* strains DJO10A and NCC2705 possess a wide range of catabolic pathways which confer a growth advantage where readily fermentable carbohydrates are in short supply, like the competitive ecosystem in the gut depending on the dietary intake of the host. The ability of *Bifidobacterium* species to survive and persist in this competitive environment is due to the use of negative transcriptional regulation as a flexible control mechanism in response to nutrient availability and diversity, as well as predicted genetic features such as exo- and endo-glycosyl hydrolases and high-affinity oligosaccharide transporters. These features likely help to compete for uptake of structurally diverse oligosaccharides released from digestion of plant fibres (Schell *et al.*, 2002; Klijn *et al.*, 2005).

The availability of genome sequences has enabled large scale and high throughput analysis using DNA microarrays. Genome-wide expression profiling

using DNA array technology can be used to determine changes in transcription levels of literally every gene in the genome in response to environmental changes. This will be discussed in the section 'Functional genomics' below. Moreover, the development of microarray technology is being used for the global comparative analysis of gene content between different isolates of a given species without the necessity of sequencing many strains, and may provide information about the degree of relatedness among various strains. This has been performed based on the complete genome sequence for *Lactobacillus plantarum* WCFS1 which showed a high degree of gene content variation among *L. plantarum* strains in genes related to lifestyle adaptation. The *L. plantarum* strains clustered into two clearly distinguishable groups, which coincided with an earlier proposed subdivision of this species based on conventional methods (Molenaar *et al.*, 2005). The comparison of genome sequences provides insight in speciation and evolution. This approach, often termed genotyping, can provide useful information about the degree of relatedness among various species and strains of the genus *Bifidobacterium*.

For the majority of microorganisms for which the complete genome sequence is not yet available, including most bifidobacterial species, clone-based microarrays, on which each spot represents a random genomic fragment, are a valuable alternative to open reading frame-based microarrays. In this approach, a chromosomal DNA library is constructed from the strain of interest, and the inserts of the DNA library, are amplified from the clones by PCR with generic primers and spotted on array-slides, the so-called smartman arrays (Pieterse *et al.*, 2005). Thus, as an alternative to the complete sequencing of genomes, smartman and oligonucleotide microarrays of the sequenced microorganisms can be used to obtain a highly detailed view of the gene content of related organisms, especially of strains of closely related species or of the same species (Molenaar *et al.*, 2005). Large-scale mutation events can often, with a high degree of certainty, be reconstructed from a comparison of related strains. The results obtained with microarray-based genotyping support a model of high evolutionary plasticity of bacterial genomes. It has become clear that horizontal gene transfer is an important mechanism for generating genotypic and phenotypic diversity in bacteria. The phenomenon has been studied in particular in relation to niche adaptations like the emergence of virulence, antibiotic resistance and symbiosis or fitness. A recently constructed clone based mixed-species genomic DNA microarray showed great potential for identification and characterization of bifidobacterial strains and species and appears suitable for biomarker screening (Boesten *et al.*, unpublished).

15.4 Tracking bifidobacteria in human intestinal samples

Bifidobacteria represent one of the most important bacterial groups of the human gastrointestinal tract. Their significant numerical dominance was

demonstrated by culturing from faecal samples on selective medium which indicated levels of between one and ten percent in adults (Harmsen *et al.*, 2000b), and even up to 90% in infants (Harmsen *et al.*, 2000a). However, these conventional plate counts overestimated bifidobacteria counts by 2 to 10% as a result of underestimation of the total microbial counts by culturing (Mitsuoka, 1990). In addition, it is very challenging to obtain pure cultures of the majority of species in the intestinal microbiota due to the largely anaerobic nature of this community and the paucity of suitable enrichment strategies to stimulate intestinal conditions. Less is known about bifidobacterial numbers in other gastrointestinal sites such as the ileum. Studies are limited to ileostomy patients in which the microbiota differs from those of the healthy distal ileum and these indicated that bifidobacterial counts varied between 0.1 and 10% of the total microbiota. The number of bacteria in the terminal ileum of ileostomy subjects has been estimated to be 10^7 – 10^8 per gram compared with 10^5 – 10^6 per gram in the normal ileum (Neut *et al.*, 2002; Finegold *et al.*, 1970).

Molecular techniques that bypass culturing allow a more complete assessment of the bifidobacterial strains present among the complex intestinal microbiota by unravelling the extent of the diversity, abundance and population dynamics of the bifidobacterial population. Molecular tools will greatly expand the capacity to differentiate between bifidobacterial species and evaluate the evolutionary relationship between isolates. Molecular tools can also monitor the functional activity of the bifidobacterial cells and whether they play a role or not at a particular time or at a given site of the intestinal tract. In the following section, various qualitative and quantitative methods are described for this purpose. Figure 15.2 shows the links between the molecular and new genomics-based technologies assessing the diversity and functionality of the intestinal microbiota.

15.4.1 Qualitative fingerprinting techniques

The most widely applied fingerprinting methods are the PCR-denaturing and temperature gradient gel electrophoresis (DGGE and TGGE, respectively) of PCR amplified fragments of 16S rRNA genes (Muyzer and Smalla, 1998). This technique is described in more detail in Chapter 17. When PCR-DGGE of the 16S ribosomal DNA (rDNA) of the gut microbiota is performed, due to the high GC content of bifidobacterial DNA, the bands corresponding to bifidobacterial amplicons usually migrate to the base of the DGGE gel and can be rather conveniently recognized in this manner (Songjinda *et al.*, 2005; Favier *et al.*, 2003) (Fig. 15.3). Following the establishment of the microbiota in infant faecal samples over the first years of life, DGGE profiles showed the dominance of ruminococci and bifidobacterial species based on stability and intensity of the bands over time (Favier *et al.*, 2002).

Owing to the resolution of gels targetting the local community and the properties of the amplicons, different bifidobacterial amplicons do not separate

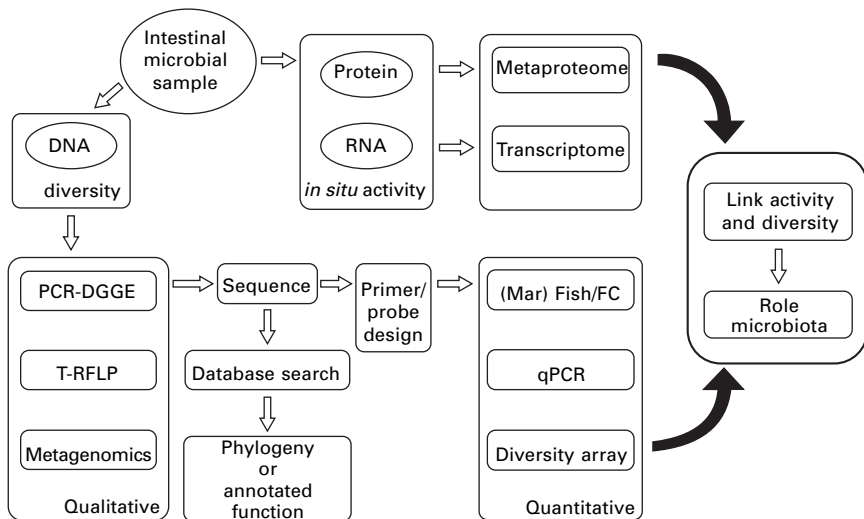


Fig. 15.2 Overview of molecular and new genomics-based technologies to monitor intestinal microbiota at diversity and functionality. Abbreviations: DGGE, Denaturing Gradient Gel Electrophoresis; T-RFLP, Terminal-Restriction Fragment Length Polymorphism; (MAR) FISH/FC, (Microautoradiography) Fluorescent *in situ* Hybridization/ Flow Cytometry; qPCR, Quantitative Real Time PCR.

into distinct bands at the base of the gel. Specific primers have been developed to target the bifidobacterial population within faecal microbiota (Satokari *et al.*, 2001a). These fingerprinting techniques have been invaluable in gaining more insight in the diversity and dynamics of bifidobacteria within individuals. Bifidobacterial-specific PCR-DGGE has shown bifidobacterial species three days after birth and remain constant in time, although the composition of bifidobacterial species was variable (Favier *et al.*, 2003). DGGE profiles of adult faecal bifidobacterial communities were host-specific and stable in time (Satokari *et al.*, 2001a). Comparison of DGGE profiles of specific bifidobacterial strains has suggested their vertical transmission from parents to offspring (Favier *et al.*, 2003), confirming earlier studies based on a culturing approach and diagnostic PCR on colony DNA (Harmsen *et al.*, 2000a). These profiling techniques may also be used to monitor behaviour of probiotics or native bifidobacteria during dietary interventions, for example with prebiotics. PCR-DGGE was used to demonstrate that intake of a probiotic *B. lactis* Bb 12 transiently colonized the adult intestine although the indigenous population remained unaffected (Satokari *et al.*, 2001b). In the same study ingestion of prebiotic galactooligosaccharides showed no effect on the qualitative composition of the indigenous bifidobacterial community (Satokari *et al.*, 2001b). Intake of a potential prebiotic lactulose intensified the bifidobacterial band on DGGEs of healthy adults especially in subjects with lower initial bifidobacterial counts. Quantitative real time PCR confirmed increase of the total levels of bifidobacteria and *B. adolescentis* (Vanhoutte

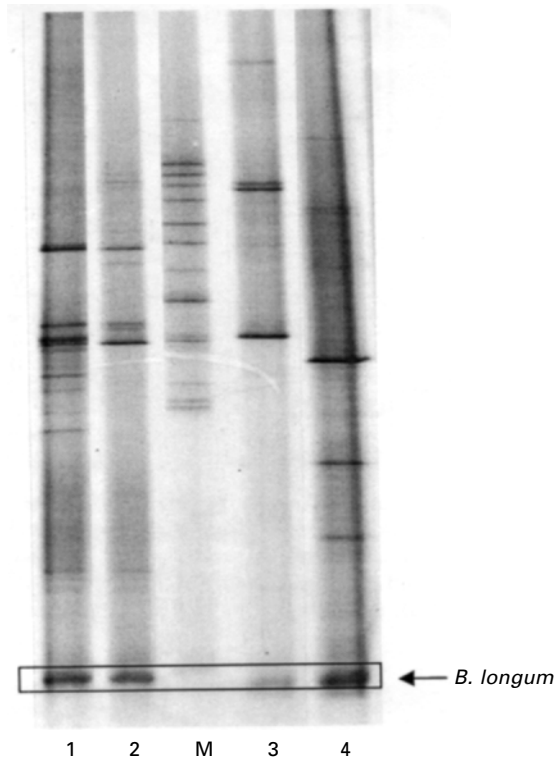


Fig. 15.3 Separation of PCR amplicons obtained from infant faecal microbiota compared to *B. longum* with universal 16S rDNA targeting primers (968f-GC/ 1401r) on 30–60% DGGE gel. 1: infant 1, age 18 weeks; 2: infant 1, age 26 weeks; M: marker; 3: infant 2, age 1 week; 4: infant 2, age 6 weeks; *B. longum* pure culture. Bifidobacterial amplicon in box.

et al., 2006). Comparison of the ascending, transcending, and descending colon wall showed the presence of bifidobacterial species in most sampling sites, but with a very simple profile suggesting few mucosa-associated species (Nielsen *et al.*, 2003).

Qualitative analysis of the bifidobacterial community in faeces has also been studied using terminal restriction fragment length polymorphism (T-RFLP), in detail explained in Chapter 17. Comparison of breast-fed, formula-fed and mixed-fed babies using T-RFLP combined with bifidobacterial species specific PCR revealed no significant differences in the distribution of different bifidobacterial species between the groups (Sakata *et al.*, 2005). Comparison of different locations in the human gastrointestinal tract using T-RFLP revealed bifidobacterial species in the colon and rectum of healthy adults (Hayashi *et al.*, 2005). The inability to generate sequence information from T-RFLP peaks makes the identification of unknown species in a sample difficult. Ideally identification may be artificially determined by performing virtual restriction digests on public database sequences. However this approach

may result in a misidentification as it assumes that only a single species or operational taxonomic unit (OTU) can have a peak of that size in a sample. It is further based upon the assumption that the database sequence is of good quality and accurately identified, which may or may not be the case.

The development of DNA oligonucleotide microarrays offers a fast, high throughput option for detection and estimation of the diversity of microbes in a complex ecosystem (Loy *et al.*, 2005). Typical microarrays contain hundreds of probes, usually based on the 16S rRNA gene, specific for different strains, species or genera of microorganisms that are detected in one single experiment. Both labelled RNA and DNA can be hybridized to the slides with immobilized probes, and following a washing step, be detected by a scanner. A microarray fully covering all species inhabiting the human gastrointestinal known at present is designed and being validated currently with promising reproducible results (Rajilić-Stojanović *et al.*, 2006). More studies are underway to develop and apply this microarray technology to unravel the diversity of the human intestinal microbiota in a wide range of intestinal samples. Palmer *et al.* (Palmer *et al.*, 2006) describe development and validation of diversity arrays for gut microbes. Ultimately, this high throughput technique may be used for quantitative detection of all human gastrointestinal tract microbial inhabitants.

15.4.2 Quantitative detection

Alternative approaches are required in order to obtain culture-independent quantification of the numbers of microbes in human intestinal samples. Within the past decade the most common approach was based on hybridization of oligonucleotide probes to microbial cells in mixed samples (Amann *et al.*, 2001). Just like most fingerprinting analysis the most commonly used target for hybridization, either by dot blot or fluorescent *in situ* hybridization (FISH), is the 16S rRNA gene. FISH is applied to morphologically intact cells and thus provides a quantitative measure of the target microbes. Following fixation, bacterial cells can be hybridized with species-, genus-, or group-specific probes. Prior to hybridization, cells can be either immobilized on glass slides or kept in suspension when analyzed by the flow cytometer (Ben Amor and Vaughan, 2004). The flow cytometer has the possibility to offer a platform for high resolution, high throughput identification and enumeration of microorganisms using fluorescent rRNA-targeted oligonucleotide probes. Species and genus-specific probes have been designed and validated for the bifidobacterial population (see Table 15.2). Several studies using FISH have provided a more realistic estimation of the bifidobacterial numbers in the human intestine, and the current average for the autochthonous bifidobacterial community in human European adults is approximately 4% of the total bacterial count (Mueller *et al.*, 2006). Especially faeces of breast-fed infants were demonstrated to be dominated by bifidobacteria, an estimated 40–90% of the total microbiota, but they also reached high numbers in formula-fed

Table 15.2 Major primers and probes used to study the bifidobacteria in faecal samples and products for PCR/DGGE, FISH and qPCR

Oligo-nucleotide	Sequence (5'–3')	Name	Reference
PCR			
	GGGTGGTAATGCCGGATG	Bif164-f	(Kok <i>et al.</i> , 1996)
	CCACCGTTACACCGGGAA	Bif662-r	(Kok <i>et al.</i> , 1996)
	CGCCCGCCGCGCGCGGGCGGGG	Bif662-GC-r*	(Satokari <i>et al.</i> , 2001)
	CGGGGGCACGGGGG-		
	CCACCGTTACACCGGGAA		
	GATTCTGGCTCAGGATGAACG	Im26-f	(Kaufmann <i>et al.</i> , 1997)
	CGGGTGCTICCCACTTTCATG	Im3-r	(Kaufmann <i>et al.</i> , 1997)
FISH			
	GGGTGGTAATGCCGGATG	Bif164-f	(Langendijk <i>et al.</i> , 1995; Ben Amor <i>et al.</i> , 2002)
	GATAGGACGCGACCCCAT	Bif228	(Marteau <i>et al.</i> , 2001)
qPCR			
DNA binding dye			
<i>Bifidobacterium</i> spp.	GATTCTGGCTCAGGATGAACG	Im26-f	(Kaufmann <i>et al.</i> , 1997; Gueimonde <i>et al.</i> , 2004)
	GATAGGACGCGACCCCAT	Bif228	(Marteau <i>et al.</i> , 2001; Gueimonde <i>et al.</i> , 2004)
<i>Bifidobacterium</i> spp.	GGGTGGTAATGCCGGATG	Bif164-f	(Langendijk <i>et al.</i> , 1995; Bartosch <i>et al.</i> , 2005)
	TAAGCCATGGACTTTCACACC	Bif601R	(Bernhard and Field, 2000; Bartosch <i>et al.</i> , 2005)
<i>Bifidobacterium</i> spp.	CTCCTGGAAACGGGTGG	g-Bifid-F	(Requena <i>et al.</i> , 2002)
	GGTGTTCCTCCCGATATCTACA	g-Bifid-R	(Requena <i>et al.</i> , 2002)
<i>B. adolescentis</i>	CTCCAGTTGGATGCATGTC	BiADog-1a	(Matsuki <i>et al.</i> , 2003)
	TCCAGTTGACCGCATGGT	BiADog-1b	(Matsuki <i>et al.</i> , 2003)
	CGAAGGCTTGCTCCCAGT	BiADO-2	(Matsuki <i>et al.</i> , 2003)
<i>B. angulatum</i>	CAGTCCATCGCATGGTGGT	BiANG-1	(Matsuki <i>et al.</i> , 2003)
	GAAGGCTTGCTCCCCAAC	BiANG-2	(Matsuki <i>et al.</i> , 2003)
<i>B. bifidum</i>	CCACATGATCGCATGTGATTG	BiBIF-1	(Matsuki <i>et al.</i> , 2003)
	CCGAAGGCTTGCTCCCAAA	BiBIF-2	(Matsuki <i>et al.</i> , 2003)

Table 15.2 Continued

Oligo-nucleotide	Sequence (5'–3')	Name	Reference
<i>B. breve</i>	CCGGATGCTCCATCACAC	BiBRE-1	(Matsuki <i>et al.</i> , 2003)
	ACAAAGTGCCTTGCTCCCT	BiBRE-2	(Matsuki <i>et al.</i> , 2003)
<i>B. catenulatum</i> group	CGGATGCTCCGACTCCT	BiCATg-1	(Matsuki <i>et al.</i> , 2003)
	CGAAGGCTTGCTCCCGAT	BiCATg-2	(Matsuki <i>et al.</i> , 2003)
<i>B. longum</i> bv. <i>longum</i>	TTCCAGTTGATCGCATGGTC	BiLON-1	(Matsuki <i>et al.</i> , 2003)
	GGGAAGCCGTATCTCTACGA	BiLON-2	(Matsuki <i>et al.</i> , 2003)
<i>B. longum</i> bv. <i>infantis</i>	TTCCAGTTGATCGCATGGTC	BiINF-1	(Matsuki <i>et al.</i> , 2003)
	GGAAACCCCATCTCTGGGAT	BiINF-2	(Matsuki <i>et al.</i> , 2003)
<i>B. dentium</i>	ATCCCGGGGGTTTCGCCT	BiDEN-1	(Matsuki <i>et al.</i> , 2003)
	GAAGGGCTTGCTCCCGA	BiDEN-2	(Matsuki <i>et al.</i> , 2003)
<i>B. infantis</i> Y 1	GTCAAGTCATGAAAGTGGGTA	Y116SI	(Vitali <i>et al.</i> , 2003)
<i>B. infantis</i> Y 1	GTCAAGTCATGAAAGTGGGTA	Y1ITSr	(Vitali <i>et al.</i> , 2003)
<i>B. breve</i> Y 8	TACAACGGGATGCGACAGC	Y816SI	(Vitali <i>et al.</i> , 2003)
<i>B. breve</i> Y 8	GAACGAGGAATCAAACCCCGTCT	Y8ITSr	(Vitali <i>et al.</i> , 2003)
<i>B. longum</i> Y10	GCAAGGCACTTTGTGTTGAG	Y1016SI	(Vitali <i>et al.</i> , 2003)
<i>B. longum</i> Y10	AAGAACGAGGAATCAAAGGAAACC	Y10ITSr	(Vitali <i>et al.</i> , 2003)
Primer-probe based qPCR			
<i>Bifidobacterium</i> spp.	GGGATGCTGGTGTGGAAGAGA	F_allbif_IS	(Haarman and Knol, 2005)
	TGCTCGCGTCCACTATCCAGT	R_allbif_IS	(Haarman and Knol, 2005)
	TCAAACCACCACGCGCCA	P_allbif_IS	(Haarman and Knol, 2005)
<i>Bifidobacterium</i> spp.	GATTCTGGCTCAGGATGAACGC	F LM26	(Kaufmann <i>et al.</i> , 1997; Gueimonde <i>et al.</i> , 2004)
	CTGATAGGACGCGACCCCAT	R Bif228	(Marteau <i>et al.</i> , 2001; Gueimonde <i>et al.</i> , 2004)
	CATCCGGCATTACCAACCCGTTT CCTC	P Bif164	(Langendijk <i>et al.</i> , 1995; Gueimonde <i>et al.</i> , 2004)
<i>B. adolescentis</i>	ATAGTGACGCGAGCAAGAGA	F_adol_IS	(Haarman and Knol, 2005)
	TTGAAGAGTTTGGCGAAATCG	R_adol_IS	(Haarman and Knol, 2005)
	CTGAAAGAACGTTTCTTTT ^a	P_adol_IS	(Haarman and Knol, 2005)
<i>B. angulatum</i>	TGGTGGTTTGAGAACTGGATAGTG	F_angul_IS	(Haarman and Knol, 2005)
	TCGACGAACAACAATAAACAACAAACA	R_angul_IS	(Haarman and Knol, 2005)
	AAGGCCAAAGCCTC	P_angul_IS	(Haarman and Knol, 2005)

Table 15.2 Continued

Oligo-nucleotide	Sequence (5'–3')	Name	Reference
<i>B. bifidum</i>	GTTGATTTTCGCCGACTCTTC	F_bif_IS	(Haarman and Knol, 2005)
	GCAAGCCTATCGCGCAAA	R_bif_IS	(Haarman and Knol, 2005)
	AACTCCGCTGGCAACA	P_bif_IS	(Haarman and Knol, 2005)
<i>B. breve</i>	GTGGTGGCTTGAGAACTGGATAG	F_breve_IS	(Haarman and Knol, 2005)
	CAAAACGATCGAAACAAACACTAAA	R_breve_IS	(Haarman and Knol, 2005)
	TGATTCCTCGTTCTTGCTGT	P_breve_IS	(Haarman and Knol, 2005)
<i>B. catenulatum</i>	GTGGACGCGAGCAATGC	F_cate_IS	(Haarman and Knol, 2005)
	AATAGAGCCTGGCGAAATCG	R_cate_IS	(Haarman and Knol, 2005)
	AAGCAAACGATGACATCA	P_cate_IS	(Haarman and Knol, 2005)
<i>B. dentium</i>	CCGCCACCCACAGTCT	F_dent_IS	(Haarman and Knol, 2005)
	AGCAAAGGGAAACACCATGTTT	R_dent_IS	(Haarman and Knol, 2005)
	ACGCGTCCAACGGA	P_dent_IS	(Haarman and Knol, 2005)
<i>B. infantis</i>	CGCGAGCAAAACAATGGTT ^a	F_inf_IS	(Haarman and Knol, 2005)
	AACGATCGAAACGAACAATAGAGTT	R_inf_IS	(Haarman and Knol, 2005)
	TTCGAAATCAACAGCAAA	P_inf_IS	(Haarman and Knol, 2005)
<i>B. lactis</i>	CCCTTTCCACGGGTCCC	F	(Gueimonde <i>et al.</i> , 2004)
	AAGGGAAACCGTGTCTCCAC	R	(Gueimonde <i>et al.</i> , 2004)
	AAATTGACGGGGGCCCGACAAGC	P	(Gueimonde <i>et al.</i> , 2004)
<i>B. longum</i>	TGGAAGACGTCGTTGGCTTT	F_long_IS	(Haarman and Knol, 2005)
	ATCGCGCCAGGCCAAAA ^a	R_long_IS	(Haarman and Knol, 2005)
	CGCACCCACCGCA	P_long_IS	(Haarman and Knol, 2005)
<i>B. longum</i>	CAGTTGATCGCATGGTCTT-	F	(Lahtinen <i>et al.</i> , 2005)
	TACCCGTCGAAGCCAC	R	(Lahtinen <i>et al.</i> , 2005)
	TGGGATGGGGTCGCGTCCTATCAG	P	(Lahtinen <i>et al.</i> , 2005)

* Primer with GC clamp for DGGE

F: forward primer, R: reverse primer, P: probe.

infants (Harmsen *et al.*, 2000a). Dot blot hybridization with rRNA-targeted probes was used to show that bifidobacteria constituted approximately 5% of the total community within the adult cecum (Marteau *et al.*, 2001). Interestingly the latter technique of targeting of rRNA from complex microbial ecosystems gives more information about the activity of the microbes rather than FISH which mainly indicated presence. FISH has been employed in many studies successfully to determine especially the bifidobacterial numbers as well as other microbiota groups following dietary interventions with functional foods. For example in one such study, following a week of daily ingestion of 5×10^9 colony forming units (CFU) of the probiotic *B. lactis* Bb 12, the *B. lactis* cells could still be detected in the faeces of five of the nine subjects one week after cessation which indicated at least temporary colonization by the probiotic (Ouwehand *et al.*, 2004). Similarly a feeding trial with the probiotic *B. lactis* LAFTI®B94 indicated survival of the bacterial cells following intestinal transit, detected using a specific probe, and presence of the probiotic in faecal samples for up to a maximum of four weeks after intake, but no permanent colonization (Su *et al.*, 2005).

Quantitative real time PCR (qPCR) assays are currently being developed for detection and quantitation of human intestinal microbiota, which has the advantages of being high throughput as well as having a high dynamic range and high sensitivity. There are different approaches for qPCR. One involves using DNA binding dyes such as SYBR Green I which are easy to design and optimize in the assays, and cost effective (Wittwer *et al.*, 2001). When free in solution, SYBR Green I displays relatively low fluorescence, but when bound to double-stranded DNA its fluorescence increases by over 1000-fold. The more double-stranded DNA that is present, the more binding sites there are for the dye, so fluorescence increases proportionately to DNA concentration. This property of the dye provides the mechanism that supports its application to track the accumulation of PCR product. As the target is amplified, the increasing concentration of double-stranded DNA in the solution can be directly measured by the increase in fluorescence signal. Compared to non-specific chemistries for qPCR such as the latter SYBR Green I dye, a higher level of detection specificity is provided by using an internal probe together with a primer set to detect the qPCR product of interest. In the absence of a specific target sequence in the reaction, the fluorescent probe does not hybridize and therefore the fluorescence remains quenched. When the probe hybridizes to the target sequence of interest, the reporter dye is no longer quenched, and fluorescence will be detected. The level of fluorescence detected is directly related to the amount of amplified target in each PCR cycle. DNA binding dyes allow labelling of probes with different reporter dyes to detect more than one target in a single reaction (multiplex qPCR).

The value of qPCR has been demonstrated in several studies. A study in elderly subjects, using the SYBR green qPCR approach, showed decreased bifidobacterial numbers in patients treated with antibiotics compared to healthy and non-treated patients (Bartosch *et al.*, 2005). The primer probe based

technique has successfully been applied to faecal samples to measure bifidobacterial numbers and can also differentiate between several bifidobacterial species. *Bifidobacterium* genus specific primers targeting infant faecal samples showed decreased numbers in infants born through caesarean section as well as in infants using antibiotics, on the contrary infants with older siblings had slightly higher numbers of bifidobacteria with a sensitivity of $10\text{--}10^6$ cfu/ml (Penders *et al.*, 2006). Another study targeting the *Bifidobacterium* genus showed high specificity and reproducibility and a detection limit of 5×10^4 cfu/ml (Gueimonde *et al.*, 2004). Multiplex qPCR, targeting *B. adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. dentium*, *B. infantis*, and *B. longum* as well as the total number of bifidobacterial species, have been used in a study which compared infants that were breast-fed, formula-fed and a formula supplemented with galacto- and fructo-oligosaccharides. The amount and diversity of bifidobacteria in the infants' faeces receiving the supplemented formula was higher than the standard formula-fed infants and mimicked that of the breast-fed infants. The dynamic range of the study was $10^4\text{--}10^9$ cfu/ml and all results were comparable to FISH (Haarman and Knol, 2005). qPCR targeting the bifidobacterial transaldolase gene instead of the 16S rRNA gene did not always give reproducible results (Requena *et al.*, 2002). Common primer and probe combinations are listed in Table 15.2. It should be taken into account that bacterial quantification by qPCR can be influenced by differences in the number of rRNA operons between the quantified species or groups, sequence heterogeneity between different operons of the same species, and differential amplification of different DNA molecules. Nonetheless, this technique is likely to become more popular in quantifying microbiota numbers owing to its high throughput nature and relatively ease to perform and it is also less expensive compared to the purchase of, for example, a flow cytometer.

15.5 *In situ* activity of bifidobacteria

Molecular identification techniques such as PCR are based on DNA extractions which do not differentiate between live, dead or inactive (dormant or injured) microorganisms within the ecosystem. To study the activity and function of the members of the intestinal microbiota their identity needs to be linked to the role that the different organisms play in the host. Although a percentage of the gut microbes can be studied *in vitro*, physiological characterization cannot be directly linked to their function *in situ*. Laboratory conditions cannot represent the intestinal conditions, and organisms behave differently in pure culture compared to the complex ecosystem that resides inside the gut. There are several approaches being used to study the activity of the microbial gut inhabitants, and their potential for assessing bifidobacteria activity is discussed.

Quantitative hybridization with fluorescent rRNA probes (as in FISH) is a useful indicator of activity as there is a correlation between the growth rate, which is coupled to efficient protein synthesis, and the number of ribosomes. The FISH technique has been used to estimate growth rates of *E. coli* cells colonizing the intestinal tract of mice (Rang *et al.*, 1999). *In situ* activity of pure cultures of the human commensal *L. plantarum* has been measured by correlating the rRNA, as determined by fluorescent intensity, with the cell growth rate (Vries *et al.*, 2004). However, at the very high cell densities which are a typical property of *L. plantarum* at late stages of growth, changes in the cell envelope appeared to prevent effective entry of the fluorescent probe into the cells. This has yet to be ascertained for bifidobacteria. Permeabilization issues may preclude application of this technique to certain microbes in complex environments like the intestine. Furthermore, recent data suggest that cellular ribosome content is not always an indicator of physiological activity. Apparently some bacterial cells might be highly active but possess a low ribosome content (Pernthaler and Amann, 2004), while other bacterial types possess high RNA even after extended starvation periods (Morgenroth *et al.*, 2000).

The metabolic activity of microbes from complex systems like the intestine may be studied using a variety of physiological fluorescent probes together with fluorescent microscopy or flow cytometry. Together with cell sorting using the flow cytometer (FCM) viability of cells can be measured very rapidly in environmental samples (Ben-Amor *et al.*, 2005). Ability to grow in medium is the current standard to recover viable cells, but in many instances suitable medium is not established for gut microbes, and it is further recognized that some cells enter a non-culturable state although still exhibit metabolic activity. The criteria by which viability can be evaluated by the physiological probes include membrane permeability or integrity, enzyme activity, and/or maintenance of a membrane-potential. The most common dye used for assessment of viability is carboxy-fluorescein diacetate, a non-fluorescent precursor that diffuses across the cell membrane, but is retained only in viable cells with intact membranes which convert it into a membrane-impermeant fluorescent dye by non-specific esterases of active cells. Another probe is propidium iodide, a nucleic acid dye, which is excluded by viable cells, but enters cells with damaged membranes and binds to their DNA and RNA. Simultaneous staining of faecal *Bifidobacterium* species with both probes was used to assess their viability during bile salt stress (Ben Amor *et al.*, 2002). Subsequent detection and cell sorting revealed three populations representing viable, injured and dead cells, whereby a significant portion (40%) of the injured cells could be cultured. This approach highlights the importance of multi-parametric FCM as a powerful technique to monitor physiological heterogeneity within populations at the single cell level. In a further study the probes were used to determine activity of human adult faecal microbes (Ben-Amor *et al.*, 2005). Subsequent bifidobacterial-specific PCR-DGGE analysis of sorted fractions, and identification by cloning and

sequencing of the 16S rRNA genes revealed bifidobacterial populations with sequences with low similarity to characterized species in the database. This suggests the potential of as yet uncultured novel bifidobacterial species inhabiting the human intestine (Ben-Amor *et al.*, 2005; Vaughan *et al.*, 2005). Certain species such as *B. longum* and *B. infantis* were retrieved from all sorted fractions, while *B. adolescentis* was mostly recovered from the sorted dead fraction. Also quantitative hybridization with fluorescent rRNA probes combined with real time quantitative PCR targeting 16S ribosomal genes could provide insight in activity of bifidobacterial species in the human intestine by comparison of DNA and RNA patterns.

Another technique to link taxonomic identity to activity and function in microbial communities is microautoradiography (MAR) which determines the uptake of specific radiochemicals by individual cells (Lee *et al.*, 1999; Nielsen *et al.*, 2003). In combination with FISH, MAR-FISH allows monitoring of the radio-labelled substrate uptake patterns of the probe identified organisms under different environmental conditions (Ito *et al.*, 2002). The drawback of this technique is that only seven different probes can be used in a single experiment, keeping in mind that natural microbial communities can comprise thousands of species. And, as mentioned above, not all species are accessible to FISH probes. Recently MAR-FISH was extended by rendering it quantitative and by combining it with other approaches including microelectrode measurements or stable isotope probing (Wagner *et al.*, 2006). These approaches, when applied to the intestinal microbes, have the potential to identify those members involved in metabolism of specific dietary compounds. Upon addition of [¹³C] glucose to an *in vitro* model of the human colon, labeled RNA from glucose-consuming bacteria was detected after only 1 h of incubation, and molecular analysis of 16S rRNA genes indicated detectable differences in glucose use by the bacteria present (Egert *et al.*, 2006).

15.6 Functional genomics

Upon arrival of the post-genomic era, the focus of studies on gut microbiota and its members has shifted from composition and activity to include the study of the functional products of gene expression. Using emerging techniques, such as transcriptomics and proteomics, the molecular activity of the microbiota can be studied *in situ* on different levels.

To capture the immediate, ongoing and genome-wide response of organisms to the environment, microarrays are the method of choice. A nice example is provided by microarray analysis of *B. longum* NCC2750 which revealed the mechanisms underlying preferential use of glucose over lactose as a carbon and energy source. This is achieved by down-regulation of *glcP* (putative glucose transporter gene) expression in a lactose-dependent manner, thereby shifting the balance of uptake and metabolism between glucose and lactose (Parche *et al.*, 2006). The transcriptome of *B. longum* NCC2705 has also

been studied in relation to stress response using microarrays and several protein coding sequences potentially involved in oxidative stress defence mechanisms were identified as well as several different regulatory mechanisms by which the cells protect themselves from various stresses (Klijn *et al.*, 2005). Such studies may be used to devise novel ways of protecting the bifidobacterial cultures during manufacture and storage and further upon gastrointestinal transit.

As explained in the section 'Taxonomy and typing of bifidobacteria colonizing the human intestine', even microbes for which no genome sequence is yet available can be studied using so called smartman arrays, although a complete genome sequence of a related microorganisms will substantially facilitate interpretation of the data. Thus it is now feasible to study the transcriptome of the bifidobacterial community within the intestinal microbiota using bifidobacterial microarrays (Boesten *et al.*, unpublished). Using well-established methods good quality total RNA can be isolated from faecal samples (Zoetendal *et al.*, 2006c). Total RNA extracted from the microbiota in breast-fed infant faecal samples was hybridized to a microarray containing clone libraries of several bifidobacterial species spotted on glass slides (Klaassens *et al.* unpublished). Positive hybridization was observed to clones with insert sequences that showed high similarity to genes with a range of functions. Particularly interesting functions were carbohydrate metabolism and transporters, and vitamin metabolism. The annotated functions of the genes on the inserts could be related to the influence of the diet on the activity of the bifidobacterial community within the faecal microbiota.

To complement transcriptomics the functional complement of the genome can be studied using a proteomics approach. Figure 15.4 shows a picture of the proteome of *B. breve* visualized using two dimensional gel electrophoresis. Large-scale characterization of the entire protein complement of environmental microbiota at a given point in time is designated metaproteomics (Wilmes and Bond, 2004). So far there are two reports which illustrate the feasibility of metaproteomics to characterize complex bacterial ecosystems. One was a model sludge ecosystem which revealed several protein sequences using two dimensional gel electrophoresis and quadrupole time of flight mass spectrometry (Q-ToF MS) (Wilmes and Bond, 2004). The second study applied a combination of community genomics and proteomics in a low complex natural microbial biofilm and disclosed a substantial percentage of all the proteins encoded by the genome of the five most abundant organisms (Ram *et al.*, 2005). This example illustrates the feasibility of this approach in analyzing complex ecosystems. Metaproteomics can also be applied to the intestinal microbial ecosystem and the first characterization of the faecal metaproteome of newborns, with high faecal bifidobacterial content, indicated the presence of a bifidobacterial enzyme (Klaassens *et al.*, 2007). This indicates *in situ* activity of bifidobacterial species. Although it is still very challenging to approach the enormous proteome, as we are limited by the resolution of the technical properties of the proteomics techniques, more studies are

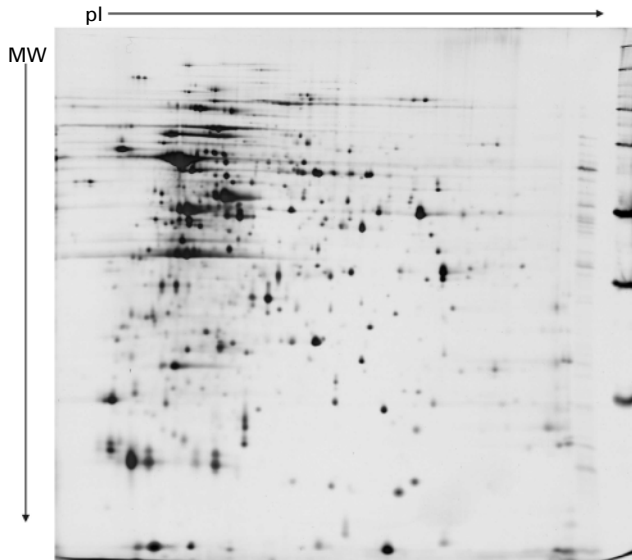


Fig. 15.4 Silver stained 2D gel of *B. breve* grown in MRS using IPG pH 4–7 and 12% acrylamide SDS PAGE gel.

underway. Currently poor database matches due to insufficient genome sequence information of the human intestinal microbes confound identification of the proteins. However, the ongoing construction of metagenomic libraries as explained below and sequence analysis of the human intestinal microbiota will enable meaningful identification in time (Gill *et al.*, 2006; Gordon *et al.*, 2005; Ley *et al.*, 2006).

The number of microorganisms that colonize the human epidermal and mucosal surfaces is estimated at 10^{13} – 10^{14} . This exceeds our population of human cells by a factor of 10 and in particular the colonic ecosystem is one of the most dense microbial communities on earth. This community is estimated to comprise 1000 species (Rajilić-Stojanović unpublished data) and may contain more than hundred times as many genes as the human genome. Many of these microbes rely on the human host for survival which offers a relatively non-hostile environment and supply of nutrients that is produced and consumed by the host and the microbes. However, considering the size of the microbiome, i.e. the sum of the microbial genomes, the host also depends on this co-evolving microbial gut community to a certain extent. Sequencing of the microbiome is a logical extension of the human genome project. Currently, 2000 partial or complete microbial 16S rRNA gene sequences of intestinal source are present in the database which is very useful for identification of species. The next step is to link the intestinal microbiota to functional insight in the total microbial genome and encoded functional attributes.

Recent studies showed the potential of functional screening of metagenomics of the human intestinal microbiota. In the majority of the studies clone libraries represented 16S rRNA genes to study the diversity of the target samples (Eckburg *et al.*, 2005; Breitbart *et al.*, 2003; Furrie, 2006; Manichanh *et al.*, 2006). A BAC library was constructed from the mouse colonic microbiota, which was functionally screened for β -endoglucanases (Walter *et al.*, 2005). Clones with β -glucanase activity as well as other putative genes were detected originating from probably uncultivated bacteria and *Bacteroides* species. Several efforts are underway to sequence the microbiome (Dore, 2006; Zoetendal *et al.*, 2006a). Sequencing of an extensive metagenomic library of the human distal gut revealed a high diversity in bifidobacterial genes when compared to the *B. longum* NCC2705 genome (Gill *et al.*, 2006). This suggests that the sequences in the metagenomic library were not derived from a single discrete strain, but instead, reflect the presence of multiple strains, as well as other *Bifidobacterium* phylootypes in the gut microbiota of the studied individuals. Although the metagenome is not directly linked to specific species but rather to the complete microbiome, it is a start to define the gene content and encoded functional attributes. Eventually, study of the actual activity of all annotated genes within the human microbial microbiome needs to be measured and linked to specific groups of healthy or diseased individuals or special diets.

15.7 Future trends

Understanding the mechanisms by which bifidobacteria affect the host is essential to support their possible role in functional foods. This requires more than the insight we have accumulated so far on the diversity, survival and general activity of bifidobacteria. At least three complementary lines of research need to be further developed. These include the development of (i) functional genomics to determine the global activity of bifidobacteria, (ii) genetic tools to allow for testing hypotheses of the bifidobacterial function, and (iii) models and real life studies that show the impact of bifidobacteria on the host and its intestinal microbiota.

Functional genomics approaches are developing rapidly as discussed above. However, insight into the global expression of bifidobacteria is only starting to develop. The public availability of complete *Bifidobacterium* genomes together with the metagenomic analysis of uncultured bifidobacteria will form the basis of advanced multi-species functional genomics approaches that address the transcriptome, proteome and metabolome of all members of this important group of intestinal bacteria. Included in this analysis is the use of Stable Isotope Probing (see above) that has recently been applied to notably the colonic microbiota and may establish the link between prebiotic substrates and bifidobacterial function.

Genetic tools for bifidobacteria are scarce especially in comparison to many other microbes of food industry interest. While plasmids are found in some bifidobacteria they are usually small in size (Sgorbati *et al.*, 1986). Hence, a dozen have been sequenced (see Table 15.3) and some have been used to construct plasmid vectors such as pMB1 (Rossi *et al.*, 1996) which led to a first generation of cloning vectors (Missich *et al.*, 1994). However, efficient gene transfer systems seem to be the bottleneck for further advancing the genetics of bifidobacteria and this is a serious limitation for further genetic approaches aiming at establishing cause-effect relations between genes and function.

Table 15.3 Plasmids found in bifidobacterial species

Strain	Accession number	Plasmid	Size (nt)	Reference
<i>B. longum</i>				
BK51	NC_006843	pTB6	3,624	(Tanaka <i>et al.</i> , 2005)
RW048	NC_004770	pNAC1	3,538	(Corneau <i>et al.</i> , 2004)
RW041	NC_004769	PNAC2	3,684	(Corneau <i>et al.</i> , 2004)
RW041	NC_004768	pNAC3	10,224	(Corneau <i>et al.</i> , 2004)
DJO10A	NC_004253	pDOJH10S	3,661	University of Minnesota, USA
DJO10A	NC_004252	pDOJH10L	10,073	University of Minnesota, USA
KJ	NC_002635	pKJ36	3,625	Food Science and Technology, Korea
KJ	NC_004978	pKJ50	4,960	(Park <i>et al.</i> , 1999)
NCC2705	NC_004943	pBLO1	3,626	(Schell <i>et al.</i> , 2002)
	NC_006997	pMG1	3,682	Research Center, BIFIDO Co., Korea
B2577	X84655	pMB1	1,847	(Rossi <i>et al.</i> , 1996)
B78	DQ452864	pBG2.2	2,197	Institute of Food Research, UK
<i>B. breve</i>				
NCFB 2258	NC_002133	pCIBb1	5,750	(O'Riordan and Fitzgerald, 1999)
VMKB44	NC_004443	pB44	3,624	University of Minnesota, USA
<i>B. pseudocatenulatum</i>				
VMKB4M	NC_003527	p4M	4,488	University of Minnesota, USA
<i>B. catenulatum</i>				
L48	NC_007068	pBC1	2,540	IPLA-CSIC, Spain
<i>B. asteroides</i>				
DSM 20089	Y11549	pAP1	2,140	Swiss Federal Institute of Technology, Switzerland

Finally, the impact of bifidobacteria on the host and its associated microbes is an important area for future studies. Notably the presumed interactions with the immune and neuroimmune systems are highly relevant as are other epithelial interactions with the host. This should not only be studied in adults but especially in neonates and this requires either novel or non-invasive methods as well as useful animal and other alternative models. The use of cell line models to determine interactions between bifidobacteria and their host has recently started and may provide one of such alternative models.

In conclusion, a sound framework for understanding the role of bifidobacteria in health and development has been realized – however, a continuing interest of academic researchers and industry scientists is needed to further develop bifidobacteria beyond this level and harvest the fruits of these bacteria that have been close to our heart since the beginning of our life!

15.8 Sources of further information and advice

Alimentary Pharmabiotic Centre website: <http://apc.ucc.ie>

Carbohydrate-Active Enzymes: <http://afmb.cnrs-mrs.fr/CAZY/geno/206672.html>

Draft genome sequence *B. longum* JGI: http://genome.jgi-psf.org/draft_microbes/biflo/biflo.home.html

Ribosomal database project, Michigan State University, USA: <http://rdp.cme.msu.edu>

Online database resource for rRNA oligonucleotide probes: <http://www.microbial-ecology.net/probebase/>

INFABIO: Diet, allergy, infection, bacteria, and the infant: <http://www.gla.ac.uk/infabio/>

PROEUHEALTH – The food, GI-tract functionality, and human health cluster: <http://virtual.vtt.fi/virtual/proeuhealth/>

EU and microfunction – Functional assessment of interactions between the human gut microbiota and the host: <http://www.eumicrofunction.be/>

‘Crown of life’ – Functional foods, gut microflora and aging: <http://www.crownalife.be/>

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15.10 References

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Genetics and functional genomics of probiotic bacteria: translation to applications

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16.1 Introduction and background

Several genomes of lactic acid bacteria (LAB) commonly used in food have been sequenced in the past several years (Table 16.1) including *Lactobacillus acidophilus* (supplement in starter cultures for fermented dairy products), *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lactobacillus bulgaricus*) (yogurt), *Lactobacillus plantarum* (plant, meat, and dairy fermentations), *Lactobacillus sakei* (French sausage), *Lactococcus lactis* (cheese starter), and *Streptococcus thermophilus* (cheese and yogurt starter). Comparative genomics with bacteria found in the gastrointestinal tract (GIT) including *Bifidobacterium longum*, *Lactobacillus gasseri*, *Lactobacillus johnsonii*, *Lactobacillus plantarum*, and *Lactobacillus reuteri* may result in the identification of genes important in probiosis and useful to the dairy industry. Genes identified in these GIT residents may provide insights on how to enhance growth, stimulate the immune system properly, inhibit pathogens, and promote adherence and survival in the GIT of probiotic bacteria.

16.1.1 Milk and dairy products – LAB species and genomes

The production of milk by female mammals provides a rich source of nutrients for newborn animals and is composed of a combination of macromolecules such as fat, protein, and carbohydrates in addition to other organic factors. LAB are present in animal milk and species such as *Lactobacillus reuteri* and have been cultured from human breast milk (Casas, 2000). LAB may reside as indigenous components of mammalian milk and growth in the GIT may be stimulated by complex carbohydrates and prebiotics provided in

Table 16.1 Summary of lactic acid bacterial genomes

Species	Reference	Genome size (bp)	Percent GC	Coding sequences	Coding density (%)
<i>Bifidobacterium longum</i>	(Schell, 2002)	2256646	60	1730	86
<i>Lactobacillus acidophilus</i> NCFM	(Altermann, 2005)	1993564	34.71	1864	87.9
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	(van de Guchte, 2006)	1864998	49.7	1562	73
<i>Lactobacillus johnsonii</i> NCC533	(Pridmore, 2004)	1992676	34.6	1821	89.3
<i>Lactobacillus plantarum</i> WCFS1	(Kleerebezem, 2003)	3308274	45.6	3052	84.1
<i>Lactobacillus sakei</i> 23K	(Chaillou, 2005)	1884661	41.25	1883	86.9
<i>Lactobacillus salivarius</i> UCC 118	(Claesson, 2006)	1827111	32	1717	84
<i>Lactococcus lactis</i> subsp. <i>lactis</i> IL1403	(Bolotin, 1999)	2365589	35.4	2310	86
<i>Streptococcus thermophilus</i> CNRZ1066	(Bolotin, 2004; Hols, 2005)	1796226	39	1915	84
<i>Streptococcus thermophilus</i> LMG18311	(Bolotin, 2004; Hols, 2005)	1796846	39	1890	84

milk. LAB have also been successfully added to milk and other dairy products, persisting in food matrices as potentially viable bacteria once consumed.

With the development of genetic tools during the past decade, successful manipulation of LAB has enabled investigators to explore gene function. The rapid proliferation of LAB genomic sequences has resulted in comprehensive genetic information about gene content and pathways in commercially important LAB. A prime example is the versatile organism, *L. plantarum*. The genome of *L. plantarum* was reported in 2003 and has yielded extensive information about biological pathways in LAB in a publicly available website (<http://www.lacplantcyc.nl>) (Kleerebezem, 2003).

Extensive comparisons among LAB genomes are generating fundamental insights regarding aspects of microbial metabolism that may be important for strain selection in numerous dairy food applications. LAB appear to have many transporters at the cell surface that facilitate uptake and utilization of carbohydrates and proteins. The increased understanding of metabolic pathways may yield improved strain selection and food processing strategies. Knowledge of the genomic sequence has yielded bio-informatic strategies for prediction of nutrient requirements in *L. plantarum* (Teusink, 2005). The curated database for *L. plantarum* (<http://www.lacplantcyc.nl>) contained 129 pathways and 704 predicted reactions. The actual experimental results that tested amino acid and vitamin requirements agreed with results predicted from knowledge of functional genomics and pathways (Teusink, 2005). These results support the future importance of systems biology approaches that will effectively combine functional genomics and biological pathway predictions to facilitate probiotic strain selection and utilization of strains in different food products (Teusink, 2006) (Fig. 16.1).

16.2 Genetics and genomics of probiotic bacteria

16.2.1 Genetic systems among lactic acid bacteria

Genetic approaches are important in order to correlate genes with specific functions and pursue functional genomics. Gene disruption or gene replacement strategies have been developed for LAB and have been especially useful with genetically amenable organisms such as *L. lactis*. Strategies generally require the utilization of homologous DNA sequences in order to apply integration systems or temperature-sensitive plasmid replicons. Temperature-sensitive plasmid replicons and chromosomal integration strategies using plasmid-borne genes have been used successfully to perform targeted mutagenesis in *L. acidophilus* and *L. reuteri* (Russell, 2001; Walter, 2005). Several strategies include the application of reprogrammed group II introns (Frazier, 2003), antisense RNA technologies (Sturino, 2002; Walker, 2000; Bouazzaoui, 2006), and tools to elevate recombination frequencies using lambda bacteriophage *red* (Vetcher, 2005).

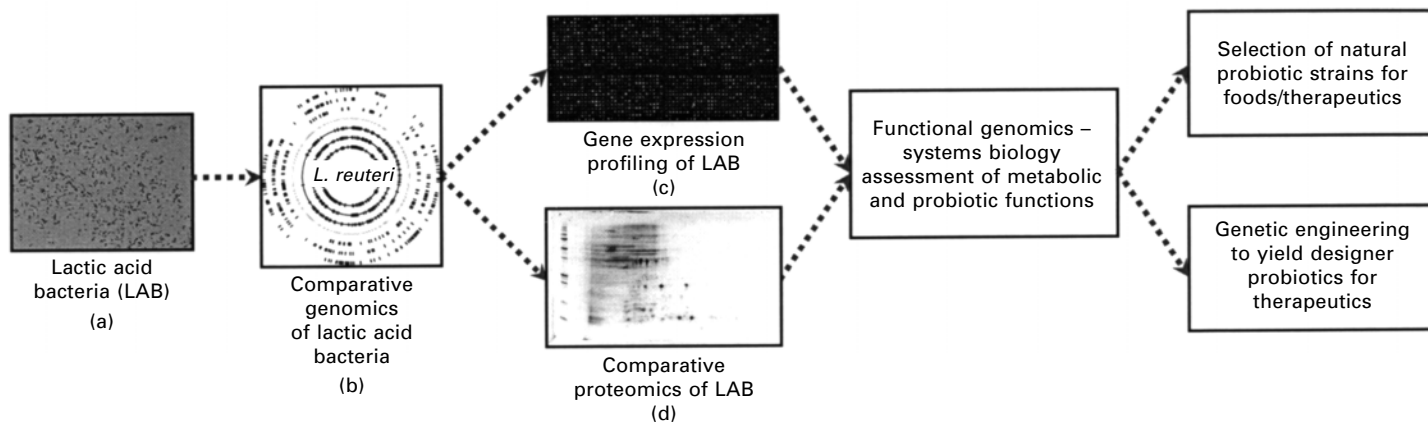


Fig. 16.1 Development and selection of probiotic strains based on systems biology approaches.

Gene expression is dependent on the presence of active promoter sequences in *cis* configuration just upstream of coding sequences, and recent progress has resulted in a more comprehensive understanding of different promoters in LAB. Promoters may be regulated by various mechanisms including negative catabolite repression (e.g., catabolic operons involved in carbohydrate metabolism such as the *lac* operon in *L. lactis*). In contrast to negative regulation, promoters may be selected for abilities to drive expression effectively by positive regulation due the presence of specific substrates. The nisin system is widely recognized as a strong, inducible system of gene expression in LAB (Mierau, 2005). Nisin-controlled gene expression systems have been used in heterologous LAB hosts such as *L. plantarum* and *L. reuteri* (Wu, 2006; Eichenbaum, 1998; Kleerebezem, 1997).

For many LAB, vectors are available for gene interruption, gene deletion and over-expression, but the transformation efficiencies and recombination frequencies for these organisms is still quite low. Data mining of LAB genomes revealed several potential reasons and solutions. First, genes in LAB with homology to the competence genes of *Bacillus* and *Streptococcus* species were found, and these genes may be important for increasing transformation efficiencies of selected probiotic strains (Hols, 2005). The limitations are apparent in *L. bulgaricus* since many genes in the competence pathway appear to be non-coding pseudogenes (van de Guchte, 2006). Second, LAB such as *L. bulgaricus* have restriction modification systems whose suppression may increase the transformation efficiencies and integration of heterologous DNA sequences (van de Guchte, 2006). Third, *B. longum* lacks the *recBCD* genes and therefore is missing key ORFs needed for homologous DNA recombination (Schell, 2002). By providing recombination machinery when engineering probiotic bacteria, site-directed mutagenesis could be improved by increasing the frequency of homologous recombination.

16.2.2 Gene regulation in lactic acid bacteria

Transcriptional regulators appear to control a large proportion of genes in LAB, while LAB chromosomes contain few two component-signaling systems and alternative sigma factors. Most transcriptional regulators for LAB and GIT residents are negative regulators and appear to be regulated by carbohydrates. *L. acidophilus* has 96 putative transcriptional regulators, but only one alternative sigma factor and nine two-component regulatory systems (Altermann, 2005). A prime example is *B. longum* in which approximately 70% of transcriptional regulators are predicted to be repressors, and many appear to be responsive to simple sugars and carbohydrates (Schell, 2002). *B. longum* may be able to adapt quickly to changes in nutrient availability due to the presence of repressors and may adjust its catabolic capabilities in different food or host environments.

Regular exposure to particular elements and nutrients may provide selective advantages for bacteria with specific gene regulatory features. Meat-dwelling *L. sakei* is routinely exposed to iron-rich substrates and reactive oxygen species in this particular food environment. As expected, *L. sakei* has three iron-dependent regulators that belong to the Fur family and is capable of regulating gene expression secondary to iron exposure (Chaillou, 2005). The Fur family of regulators is missing in various LAB including *L. johnsonii*, *L. brevis*, *L. casei*, *L. gasseri*, and *L. delbrueckii*, and may indicate the relative lack of importance of iron as an environmental cue for bacteria in the intestine and dairy products (Pridmore, 2004).

16.2.3 Protein secretion and cell surface proteins

Cell wall-associated and other secreted proteins play diverse roles in bacteriophage susceptibility, bacterial survival and persistence in the GIT, immunomodulatory capabilities, and autolysis. Protein secretion and anchorage may be important to the pharmaceutical and food industries for vaccine delivery, therapeutic applications, and nutrition. Many secreted and cell surface-associated proteins have been identified by mining the LAB genomes for genes with particular motifs and signal sequences, indicating the presence of different mechanisms of protein secretion and anchorage. A substantial fraction of extracellular proteins may be species-specific as in the case of *L. reuteri* (Bath, 2005). Evidence of Sec pathway-dependent signal sequences and genes encoding signal peptidases have been identified in LAB including *L. plantarum* (Kleerebezem, 2003) and *L. salivarius* (Claesson, 2006). Gene clusters encoding exclusively cell surface proteins (*cscA-D*) with Sec dependent secretion signals have been identified in Gram-positive bacteria including various LAB and may participate in metabolism of plant oligo- or polysaccharides (Siezen, 2006).

Cell-wall anchoring motifs such as N-terminal lipoprotein motifs and C-terminal LPxTG motifs (Boekhorst, 2004), suggesting anchorage to cell surface peptidoglycan, have been identified in several *Lactobacillus* genomes. In *L. plantarum* and *L. salivarius*, 22 and 10 ORFs, respectively, were identified with LPQTxE or LPXTG sortase recognition sequences (Kleerebezem, 2003; Claesson, 2006). Each genome contains a sortase gene, indicating that a subset of secreted proteins may be anchored to the peptidoglycan layer in LAB by sortase recognition motifs. Nearly 90% of putative extracellular proteins of *L. plantarum* and *L. johnsonii* are predicted to have a cell surface anchor (Boekhorst, 2004). Various anchor motifs found in LAB genomes include sortase-dependent anchors, lipoprotein anchors, choline-binding domains, peptidoglycan-binding domains, GW repeats, LysM domains, and WxL domains (Bath, 2005; van Pijkeren, 2006).

16.3 Functional genomics of macromolecular and nutrient metabolism

The dairy industry is dedicated to understanding the molecular biology necessary for starter cultures to convert milk into various metabolites since these reactions affect the nutrition and growth of organisms as well as the texture and flavor of food products. These reactions may also be important for understanding the molecular mechanisms of probiosis in mammalian hosts.

16.3.1 Carbohydrate metabolism

Based on comparative genomics and putative gene expression patterns of LAB, it is clear that these organisms are equipped to utilize a variety of mono-, di- and oligosaccharides. The coordinated regulation of expression of genes involved in sugar transport depended on sugars present in the local environment (Barrangou, 2006). Phosphoenolpyruvate (PEP): sugar transferase systems were important for the uptake of glucose, fructose, sucrose, and trehalose, whereas ATP-binding cassette (ABC) transporters were important for the uptake of raffinose and fructo-oligosaccharides (FOS) in *L. acidophilus* (Barrangou, 2006). The PEP:sugar phosphotransferase and ABC transporter systems appear to be important features of LAB that are particularly adept at sugar uptake and utilization. For example, *L. johnsonii* encodes 16 phosphotransferase-type sugar transporter systems, representing a relatively large number of sugar transporter systems when compared to other bacteria with similarly sized genomes (Pridmore, 2004). Despite being closely related species, *L. johnsonii* has 150 ORFs that are unique when compared to *L. gasserii* (Pridmore, 2004). Many of these genes are predicted to be involved in the utilization of unusual sugars and complex carbohydrates. Genes unique to each organism may be important for providing distinctive flavors and textures to food products. In addition to having a high number of genes for the metabolism of diverse sugars, genes predicted to be highly expressed based on the codon adaptation index are involved in sugar metabolism and include genes in the Embden-Meyerhof-Parnas pathway in *L. plantarum* (Kleerebezem, 2003).

The carbohydrates fermented by an organism are dependent on their niche. *L. johnsonii* primarily colonizes the ileum that is rich in mono- and disaccharides. Fermentation in *L. johnsonii* is restricted to mono-, di- and trisaccharides since it lacks many enzymes required for utilization of oligosaccharides present in the colon (Pridmore, 2004). Consistent with its ecologic niche of the lower GIT, *B. longum* includes more than 40 predicted glycosylhydrolases whose predicted substrates are di-, tri- and higher-order oligosaccharides (Schell, 2002). *B. longum* also has genes for proteins normally found in eukaryotes which include three α -mannosidases and an endo-N-acetyl-glucosaminidase (Schell, 2002). These enzymes may digest host glycoproteins and free host-derived sugars for nutrient utilization by LAB.

Bacteroides thetaiotamicron, like *B. longum*, is a commensal bacterium found in the human cecum and can induce epithelial cells to produce glycans that can be utilized by commensal bacteria (Hooper, 1999). *B. longum* and *B. thetaiotamicron* may use host mucins as a carbohydrate source when dietary sugars are scarce. Interestingly, when mono-associated mice were fed a carbohydrate-poor diet, gene expression in *B. thetaiotamicron* shifted towards pathways important for catabolism and utilization of host-derived sugars derived from mucus in the intestinal tract (Sonnenburg, 2005).

Several LAB lack transporter systems and pathways important for environmental carbohydrate utilization including *L. bulgaricus*, *L. sakei*, and *S. thermophilus*, which reflect their niche in carbohydrate-poor environments (van de Guchte, 2006; Chaillou, 2005; Hols, 2005). Many genes involved in sugar metabolism in *L. bulgaricus* and *S. thermophilus* appear to be pseudogenes, indicating that these organisms have lost the ability to ferment many sugars (Hols, 2005; van de Guchte, 2006). The genome of *L. sakei* 23K, isolated from French sausage, revealed few transport systems for sugars, and *L. sakei* may use inosine and adenine as primary carbon sources (Chaillou, 2005). Missing properties could be engineered in LAB such as the ability to produce high amounts of mannitol by *L. lactis*. Mannitol is a sugar polyol which may have beneficial properties for animal health including possible roles as an antioxidant, low-calorie sweetener, and osmoprotectant. A mannitol-hyperproducing *L. lactis* strain was engineered by disrupting two genes of the phosphoenolpyruvate (PEP) – mannitol phosphotransferase system (PTS) (Gaspar, 2004). This food-grade strain lacked a selectable marker and was generated by double-crossover recombination.

16.3.2 Amino acid metabolism

LAB are not capable of *de novo* synthesis of most amino acids, since many of the genes encoding enzymes for amino acid synthesis are missing or are predicted pseudogenes. LAB have several mechanisms to scavenge their surroundings for amino acids and peptides including the 90 kDa protease PrtH, amino acid transporter systems, numerous peptidases, and peptide transporter systems (Siezen, 1999). LAB have cell envelope-associated proteinases such as PrtH that degrade casein from milk into oligopeptides, amenable to importation by bacteria. PrtH or related family members are present in *L. acidophilus*, *L. bulgaricus*, *L. delbrueckii*, *L. gasseri*, *L. helveticus*, *L. johnsonii*, *L. lactis*, *L. paracasei*, and *L. rhamnosus*. *L. johnsonii* has more than 20 amino acid transporters, and many putative amino acid transporters are predicted to be highly expressed (Pridmore, 2004).

The exception is *L. plantarum*, an organism capable of surviving in a variety of environments including the GIT and the fermentation processes of plant, dairy and meat products. *L. plantarum* has the genes required for *de novo* synthesis of most amino acids, but it lacks the machinery to synthesize

branched amino acids. Even though it lacks PrtH and similar homologs, it does have oligopeptide uptake systems Opp and Dtp and nineteen genes for intracellular peptidases that provide capabilities to utilize exogenous amino acids (Kleerebezem, 2003). Analysis of the *B. longum* genome also revealed complete pathways for oligopeptide uptake and amino acid utilization in a bifidobacterial chromosome (Schell, 2002). *L. johnsonii* has a C1-like peptidase with homologs in phylogenetically distant *B. longum* and *Bacteroides fragilis* (Pridmore, 2004). This peptidase may digest a protein abundant in the ileum.

16.3.3 Nucleotides, cofactors, and vitamins

L. acidophilus, *L. johnsonii*, *L. lactis*, *L. plantarum*, and *L. sakei* can synthesize purines *de novo*, but only *L. plantarum* and *L. sakei* can make pyrimidines *de novo* (Schell, 2002; Boekhorst, 2004; Altermann, 2005; Chaillou, 2005). Many LAB cannot produce vitamins and cofactors for growth such as riboflavin, vitamin B6, nicotinamide, biotin and folate, explaining why the development of defined media is so challenging. *L. lactis* has been metabolically engineered to overproduce folate (vitamin B11) and riboflavin (vitamin B2) (Sybesma, 2004). This strain of a common starter culture could be used to enrich food and alleviate vitamin deficiencies.

16.4 Functional genomics of bacteria: environment interactions

16.4.1 Bacterial:bacterial interactions

Bacteria are typically part of complex, multi-species communities in different environments including soil, food, plants, and animals. Each member of the ecosystem may provide critical nutrients for their neighbors' survival and communicate via secreted molecules. *L. bulgaricus* and *S. thermophilus* are co-cultured to ferment milk into yogurt. *S. thermophilus* can make folate and its precursor p-aminobenzoic acid (PABA), while *L. bulgaricus* can only convert PABA into folate (van de Guchte, 2006). *L. bulgaricus* probably relies on *S. thermophilus* for PABA and folate to survive. In return, *L. bulgaricus* uses its extracellular protease, which *S. thermophilus* lacks, to digest casein into smaller peptides that can be imported as a source of amino acids for both bacteria (van de Guchte, 2006).

Quorum sensing is a mechanism for bacteria to communicate in a cell-density dependent manner via secreted molecules and coordinate gene expression (Bassler, 2006; Surette, 1998). Several types of quorum sensing molecules may be produced by bacteria, but auto-inducer-2 (AI-2) is hypothesized to act in cross-species communication (Bassler, 2006). LuxS, the protein responsible for AI-2 synthesis, is found in many bacterial lineages

including GIT residents and LAB (Kaper, 2005). Multiple *Lactobacillus* species contain the *luxS* gene and may use quorum sensing molecules to facilitate intercellular communication and survival in complex dairy food matrices (Kaper, 2005). AI-2 is known to contribute to biofilm formation and was detected in 10 of 12 filtered fecal samples from healthy individuals (Kaper, 2005). Interruption of the *luxS* gene resulted in enhanced biofilm formation in *L. reuteri*, although preliminary data from our studies indicate otherwise (S. Jones, pers. comm.) (Tannock, 2005). Differences in the role of LuxS with respect to LAB biofilm formation may depend on the genetic background of each particular bacterial strain. Surprisingly, little attention has been given to genes involved in AI-2 production or biofilm formation for LAB or GIT residents, even though biofilms are present in the oral cavity and GIT (Swidsinski, 2005a).

16.4.2 Colonization and adhesion factors

Probiotic bacteria may adhere to the GIT, and adherence may facilitate persistence and long-term colonization of indigenous organisms. Analyses of LAB genomes have revealed genes that may be important for adhesion including homologs to mucin-binding proteins (MUBs), fibronectin-binding proteins, and fimbriae. *L. reuteri* contains one MUB gene and a fibronectin-binding protein that are important for adhesion to mucin glycoproteins (Roos, 2002). Various LAB have genes containing conserved MUB domains, and these proteins may be important for interactions with intestinal mucins and carbohydrate-rich food matrices (Boekhorst, 2004). MUBs have been identified in *L. acidophilus*, *L. gasseri*, *L. johnsonii*, and *L. plantarum* (Altermann, 2005; Boekhorst, 2004). Fibronectin-binding protein homologs are present in *L. acidophilus*, *L. bulgaricus*, *L. gasseri*, *L. johnsonii*, and *L. plantarum* and may be important for adhesion and colonization in the host (Altermann, 2005; Boekhorst, 2004; van de Guchte, 2006). *L. johnsonii* has a fimbrial operon, and fimbriae-like structures can be seen on *L. johnsonii* exopolysaccharide-deficient mutants (Altermann, 2005). No homologs for MUBs, fibronectin-binding proteins, or fimbriae genes were found in *L. casei* or *L. sakei*, indicating that these bacteria may not colonize the gut or that other factors may substitute for adherence to mucosal surfaces including the GIT (Chaillou, 2005). *L. plantarum* has four predicted mannose adhesion genes that may bind to mannose residues on host cells (Kleerebezem, 2003). Comparative genomics between distantly related bacteria that occupy the same environment might identify genes that convey survival advantages.

16.4.3 Physiologic and processing-associated stress response pathways

The identification of stress response genes and pathways are a high priority in the dairy industry, since these pathways could be improved or induced in

order to promote survival of starter cultures. LAB are equipped to handle stresses such as changes in oxygen levels, temperature, salt concentrations, bile, and pH. LAB produce an array of enzymes to protect themselves from oxidative stress including thioredoxin, glutathione reductase, NADH oxidase, NADH peroxidase, pyruvate oxidase, thioreductases, catalase, and thiol peroxidase (Pridmore, 2004; Chaillou, 2005; Altermann, 2005; Boekhorst, 2004; van de Guchte, 2006; Kleerebezem, 2003).

LAB possess cold, heat, and alkaline shock proteins in addition to chaperones and proteases, and these factors may be important during the culturing or processing of dairy products. LAB produce copious amounts of acid and have several genes to maintain intracellular pH. H^+ transporting ATPases, ornithine decarboxylases, cation:proton antiporters, and systems to maximize uptake of or synthesize osmoprotectants may offer cross-protection to osmolar and acid challenges. *L. johnsonii* has three bile salt hydrolases and two bile salt transporters, which may be important for survival in the GIT since bile salt hydrolases can improve survival of *L. monocytogenes* in the GIT of guinea pigs (Pridmore, 2004; Dussurget, 2002). Bile salt hydrolases may catabolize bile salts in the intestine and reduce the environmental stress associated with the presence of bile.

16.4.4 Immune evasion and host interactions

Several factors have been identified in LAB and GIT bacterial residents that may be involved in immune evasion or host interactions. *L. acidophilus* and *L. johnsonii* have proteins with homologies to an IgA protease which may be important for evasion of the immune system by hydrolysis of host immunoglobulins (Altermann, 2005; Pridmore, 2004). Glycosyltransferases present in LAB may glycosylate serines to create mucin-like structures that may be significant to binding mucins or evading the host's immune system. *B. longum* has a protein with similarity to serpins, a family of protease inhibitors that contribute to immune evasion by myxoma viruses (Schell, 2002; Macen, 1993).

Bacteria such as EHEC are able to communicate with the host and control the expression of virulence factors via the AI-3/epinephrine/norepinephrine signaling molecules (Sperandio, 2003). The receptor for AI-3, QseC, was recently found in EHEC (Clarke, 2006). AI-3 is produced by various commensal bacteria including *Enterobacter cloacae*, *E. coli*, *Klebsiella pneumoniae*, and *L. reuteri* (Kaper, 2005). Studies of the effects of epinephrine or norepinephrine on LAB and other GIT residents may be insightful as a way of understanding how the host may secrete factors that promote intercellular communication with bacterial GIT residents.

16.5 Food and health applications of probiotics: translational aspects

16.5.1 Probiotics in milk and other dairy products

Probiotic bacteria may express specific genes resulting in production of different biological macromolecules when cultured in milk or dairy products. As an example, *L. bulgaricus* strains may produce different amounts and types of heteropolysaccharides when grown in milk (Bouzar, 1996). Heteropolysaccharides rich in arabinose content were produced by specific *L. bulgaricus* strains. Carbohydrates produced by LAB may act as prebiotics for other organisms. Prebiotics are defined as nondigestible food ingredients that may beneficially affect the host by selectively stimulating the growth or the activity of beneficial bacteria. Infants fed milk with fermented yogurt cultures including *L. casei* had increased yields of lactobacilli in feces and decreased amounts of enzymatic bacterial markers β -galactosidase and β -glucuronidase (Guerin-Danan, 1998). Interestingly, infants fed traditional yogurt yielded increased numbers of enterococci in feces and reduced percentages of branched-chain or long-chain fatty acids, markers of proteolytic fermentation (Guerin-Danan, 1998). The cell envelope protease PrtP is an important enzyme identified in *L. acidophilus* that catabolizes milk casein to many smaller proteolytic fragments for peptide and amino acid utilization (Altermann, 2005).

Probiotic bacteria such as *L. rhamnosus* GG diminished milk-induced inflammatory responses in hypersensitive individuals while stimulating immune responses in healthy individuals (Pelto, 1998). Probiotic *L. rhamnosus* GG reduced intestinal permeability caused by heterologous milk consumption of cow's milk by newborn rats (Isolauri, 1993). The common practice of heterologous animal milk consumption by humans highlights a potential benefit of probiotics if supplemental LAB similarly reduce intestinal permeability in susceptible human individuals. Consumption of prebiotic infant formula may result in a fecal microbiota that resembles that of breast-fed infants (Haarman, 2006). Modifications of formula or milk products with prebiotics or probiotics may result in dairy products with specific beneficial properties.

16.5.2 Probiotics in the oral cavity

Ninety-five percent of the general population has dental caries or periodontal disease (Caglar, 2005). Controlling these diseases has not been highly successful despite preventative therapies such as fluoride and vaccines against oral pathogenic bacteria. Probiotics are now being explored as a treatment option for alleviation or prevention of dental diseases.

Streptococcus mutans is a lactic acid bacterium that has long been recognized as a principal agent in dental caries. However, the presence of *S. mutans* is not sufficient to result in caries, indicating the involvement of other factors

in cariogenesis. Dental diseases may result due to changes in the complex microbiota present in the oral cavity. Replacement therapy or bacteriotherapy occurs when a pathogenic strain is replaced by a nonpathogenic strain (Caglar, 2005). Bacteriotherapy for *S. mutans* has resulted in human clinical trials with *S. mutans* BCS3-L1. This strain was modified to eliminate cariogenicity, promote colonization versus other endogenous *S. mutans* strains, and limit genetic transformation (Clancy, 2000; Hillman, 1998, 2000). Cariogenicity by *S. mutans* begins when the bacteria ferments dietary sugars into lactic acid. The decrease in pH due to the lactic acid dissolves the calcium phosphate in tooth enamel and can lead to tooth decay if not repaired. In order to reduce lactic acid production by *S. mutans*, lactate dehydrogenase (*ldh*) gene was replaced with the alcohol dehydrogenase gene from *Zymomonas mobilis*. Overproduction of the bacteriocin, mutacin 1140, allowed BCS3-L1 to inhibit other strains of *S. mutans* and enabled this engineered strain to preferentially colonize the oral cavity. A major concern regarding the use of engineered probiotics is the propensity of bacteria for genetic exchange and recombination. BCS3-L1 contains a point mutation in the gene for the competence peptide that lowers the transformation frequency. To further prevent genetic exchange and recombination with other bacteria, the *comE* gene that encodes the response regulator controlling competence in *S. mutans* was also deleted. The increasing knowledge base of genes and functional genomics will facilitate new developments that may make probiotic engineering more palatable to regulatory agencies by limiting genetic exchange.

Lactic acid bacteria have been considered detrimental to oral health since they ferment sugars into lactic acid, but lactic acid production may not be the whole story. Unlike *S. mutans*, *L. reuteri* produces lactic acid, but its presence does not result in the release of calcium from hydroxylapatite (Nikawa, 2004). This difference may be caused by variations in bioavailability of lactic acid and other factors that may counteract or neutralize the presence of lactic acid. Also, many lactic acid bacteria are consumed in dairy products that have excellent buffering capacity and contain calcium to enhance remineralization of the enamel. Probiotic research in the oral cavity has focused on *Bifidobacterium bifidum*, *L. acidophilus*, *L. casei*, and *L. reuteri*. Some strains of *Lactobacillus* and *Bifidobacterium* may lower the propensity of individuals to initiate caries formation, reduce the overall risk of dental caries, and act as a prophylactic agent for *Candida* spp. infection (Ahola, 2002; Sookkhee, 2001). These effects are usually accompanied by reduced numbers of cariogenic *S. mutans* and pro-inflammatory *Candida* spp. in the oral cavity (Ahola, 2002).

Oral lactobacilli may adhere to enamel, but the same organisms may not routinely colonize the oral cavity, indicating that long-term colonization may not be necessary to alleviate oral diseases. For example, *L. rhamnosus* (LGG) does not colonize the oral cavity for long periods, but treatment with this strain reduced the risk of caries in children (Yli-Knuuttila, 2006; Nase, 2001). In the future, oral probiotics development may shift to include

endogenous lactobacilli including *L. crispatus*, *L. fermentum*, *L. gasseri*, *L. paracasei*, *L. plantarum*, *L. reuteri*, *L. rhamnosus*, and *L. salivarius* that are capable of colonizing the oral cavity and providing long-term disease prophylaxis (Yli-Knuuttila, 2006).

16.5.3 Probiotics in the gastrointestinal tract (GIT)

Probiotics in functional foods including dairy products may have important effects on intestinal physiology and immunity. Although knowledge of molecular mechanisms of probiosis is quite limited, insights into probiotic:host interactions in the GIT are being gathered and may have implications for health promotion and disease prevention.

We are just beginning to understand the spatial topography and localization of commensal bacteria in the mammalian intestine. Studies of the GIT microbiota using PCR-based methods and colony counts are difficult and rarely provide consistent results. Fluorescent *in situ* hybridization (FISH) is providing fundamental insights and can overcome limitations of PCR-based methods and bacteriologic culture. Recent, informative intestinal FISH studies used Carnoy's fixative, which retains the structural integrity of the mucus layer, and yielded detailed images of microbial communities in intestines of healthy and diseased animals and humans. One study examined the location and composition of the GIT microbiota using healthy mice and murine colitis models (Swidsinski, 2005a). In this study, each segment of the intestine differed in the type and location (fecal mass, interlaced layer, mucosa, or in the crypts) of the bacteria. Commensal Gram-positive bacteria consistent with either *Enterococcus* or *Lactobacillus* spp. were found in the interlaced (mucus-associated) layer adjacent to intestinal epithelial cells. These findings suggest that LAB may colonize intestinal regions adjacent to the mucosa, and this location may enable intimate probiotic:host interactions. The spatial distribution of endogenous bacteria was altered in the diseased state, and biofilms were observed *in vivo*. A second study described increased numbers of adherent bacteria at the mucosal surface in humans with inflammatory bowel disease (IBD) (Swidsinski, 2005b). Biofilm composition changed when comparing non-diseased and diseased states. *Bacteroides* spp. was the dominant genus found in the biofilms of patients with IBD, while biofilms were only detected occasionally in healthy controls. The fecal stream exhibited ample bacterial diversity, but only *Bacteriodes* spp., *Brachyspira*, *Enterobacteriaceae*, *E. coli*, *Enterococcus faecalis*, *Eubacterium rectale*, and *Fusobacterium prausnitzii* were found to be adherent. Characterization of the indigenous microbiota and their location in the GIT will enhance selection of potential probiotics for different food and medical applications.

The genus *Lactobacillus* includes more than 100 different species (www.bacterio.cict.fr/l/lactobacillus.htm), and has received attention as a possible source of probiotic agents and protein delivery systems for the mammalian intestine (Tannock, 1997; Seegers, 2002; Neu, 2003). To date,

fewer than 20 species have been found consistently in the mammalian gastrointestinal tract. In the mouse, a subset of *Lactobacillus* species have been characterized in the gastrointestinal tracts of healthy animals (Madsen, 1999; Peña, 2004). Only a restricted subset of *Lactobacillus* species has defined probiotic activity in animal and human studies. Several *Lactobacillus* species stably colonize and persist in the mammalian intestine (de Champs, 2003; McCarthy, 2003; Valeur, 2004). In contrast to *E. coli* and *L. crispatus*, specific *L. bulgaricus* and *L. casei* clones significantly reduced pro-inflammatory cytokine responses *ex vivo* in human intestinal tissue explants obtained from patients with Crohn's disease (Borruel, 2002, 2003). These results suggest that defined probiotic *Lactobacillus* clones may reduce inflammation in the intestinal mucosa of patients with IBD by direct immunosuppression. The murine interleukin-10 (IL-10)-deficient mouse colitis model has provided additional insights about probiotic *Lactobacillus* spp. as potential prophylactic or treatment modalities in Crohn's disease. The replenishment of intestinal IL-10 by recombinant *L. lactis* ameliorated disease in IL-10-deficient mice, indicating the importance of IL-10 in controlling intestinal inflammation and pointing to possible therapeutic applications of engineered LAB (Steidler, 2000). The modification of microbial ecology in the human intestine and introduction of specific probiotic bacteria into a complex microbiota may prevent recurrence or diminish inflammation in human IBD (Rastall, 2005).

Whether a probiotic strain is active in specific regions of the GIT may be an important factor in selection of candidate probiotic strains. Cellular activity can be measured via reporter gene technology such as luciferase expression systems. When a four-day-old culture of *L. casei* DN-114 001 was inoculated into mice harboring a human microbiota, *L. casei* produced luciferase in the ileum, but maximal levels of bioluminescence were detected in cecal samples (Oozeer, 2004). In contrast, luciferase was not produced in proximal regions of the gastrointestinal tract such as the stomach, duodenum, or jejunum. Reporter genes may be used to assess whether strains express important molecular features in specific locations or environments. Such reporter assays may be promoter-dependent since different promoters will vary in activity and may skew results accordingly (Oozeer, 2005). Multiple bacterial genes may be induced in the mammalian gastrointestinal tract following administration of probiotic strains, and *in vivo* gene expression technology (IVET) has facilitated our understanding of genes selectively induced in animals. Following colonization with *Lactobacillus plantarum*, 72 genes were induced in the murine intestine when compared to *Lactobacillus* genes expressed during routine bacteriologic culture (Bron, 2004). Specific *L. reuteri* genes were induced in the mouse gastrointestinal tract and may encode factors that modulate intestinal function (Walter, 2003). These genes, and others that may not be differentially expressed *in vivo*, may confer probiotic functions in the intestine. Bacteria can be modified to express anti-inflammatory cytokines such as IL-10 to alleviate intestinal pathology such as IBD (Steidler,

2000; Braat, 2006). By learning where commensal bacteria are located and active in the GIT, selection of therapeutic strains will improve, and insights can be gained on how changes in the normal microbiota affect disease progression and outcome.

16.6 Conclusions and future trends

The rapid expansion of genetic tools and genomic sequencing of LAB have yielded vast amounts of molecular information in a short time span of several years. Commercial applications of LAB and other beneficial bacteria in functional foods including dairy products will continue to evolve rapidly with the proliferation of knowledge of bacterial biological systems. Functional genomics will immediately impact the science of natural strain selection in the food industry. By comparing gene content and gene expression profiles in various environmental conditions including animal models, natural LAB strains may be evaluated for specific beneficial or metabolic functions and high throughput screening may be feasible. Rational selection based on particular biologic features will be possible using highly parallel investigations of gene content and gene expression of entire bacterial chromosomes. Engineered probiotics may eventually be developed for specific food applications, although the pharmaceutical industry will likely capitalize first on genetically-modified organisms for medical applications. The bottom line is that explorations of whole bacterial systems at the genomic level will ultimately advance the fields of functional foods and probiotics at a rapid pace unimaginable before the advent of functional genomics.

16.7 Sources of further information and advice

Books

Probiotics & Prebiotics: Scientific Aspects. Edited by Gerald W. Tannock (Caister Academic Press, 2005).

Websites

<http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi>

Compilation of completed microbial genomes (including LAB genomes) in the Entrez Genome Project of the National Center of Biotechnology Information (NCBI), USA.

http://genome.jgi-psf.org/mic_home.html

Compilation of draft microbial genomes (including LAB genomes) at the Joint Genome Institute and US Department of Energy.

<http://www.isapp.net/>

Website of the International Scientific Association for Probiotics and Prebiotics (ISAPP). The site contains important links and news pertinent to academia and industry.

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Characterizing probiotic microorganisms

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17.1 Introduction

In selecting a probiotic microorganism for use in foods, the strain must be identified at the genus, species and strain levels (Saarela *et al.*, 2000). Proper characterization of a strain is important for safety and quality assurance, for understanding the interactions of gut microbiota, and for identifying and differentiating putative probiotic strains. In addition, proper characterization is important to maintain consumer confidence. Product labels often list invalid names of organisms or misidentify the species the product contains (Biavati *et al.*, 1992, Yaeshima *et al.*, 1996, Reuter, 1997, O'Brien *et al.*, 1999, Hamilton-Miller *et al.*, 1999, Mattarelli *et al.*, 2002, Masco *et al.*, 2005). At the very least, organisms should be accurately identified to the species level on product labels.

Characterization to the strain level has several potential applications. Understanding the complex interactions among microorganisms in the intestinal ecosystem requires methods of differentiating a strain of interest from other strains of the same species contained in the indigenous microbiota. Strain differentiation techniques also aid in assessing survival of a probiotic organism through the gastrointestinal system. The ability to uniquely identify a strain also supports the licensing or intellectual property rights of the manufacturer and lends credibility to statements made about the potential health benefits of consuming a particular product containing a strain with demonstrated probiotic effects. The health effects associated with probiotic microorganisms are strain-specific. According to the FAO/WHO guidelines for probiotic use (FAO/WHO, 2002), specific health benefits observed in research employing a specific strain may not be extrapolated to other, closely-related, strains.

Therefore, reliable techniques for identification of organisms at the strain level are required.

17.2 Definitions

To create a framework for this discussion, definitions for some of the terms used throughout are provided. A **species**, as described by Colwell *et al.* (1995) and *Bergey's Manual of Systematic Bacteriology* (Brenner *et al.*, 2001), is a group of strains that are highly similar to each other and collectively have certain distinguishing characteristics. A species is the only taxon defined phylogenetically by the International Committee for Systematic Bacteriology and is described as 'strains with approximately 70% or greater DNA-DNA relatedness and with 5°C or less ΔT_m ' (Wayne *et al.*, 1987). A **genus** is defined by *Bergey's Manual of Systematic Bacteriology* (Brenner *et al.*, 2001) as 'one or more species with the same general phenotypic characteristics, and which cluster together on the basis of 16S rRNA sequences'. A **subspecies** is a group of strains within a species that consistently cluster on the basis of phenotypic or genotypic characteristics (Wayne *et al.*, 1987, Brenner *et al.*, 2001). Developing a definition for a **strain** is a greater challenge. A strain has been defined as 'any culture knowingly defined from the original strain' (De Vos and Trüper, 2000) and as the 'descendents of a single isolation in pure culture...ultimately derived from an initial single colony' (Brenner *et al.*, 2001). However, for a discussion of probiotic strain characterization, these definitions are too stringent. These definitions imply some knowledge of the source and history of an isolate is prerequisite to its evaluation. Therefore, a more 'operational definition' of strain, based on those proposed by Tenover *et al.* (1995) and Dijkshoorn and Towner (2001), would be 'a strain is an isolate that can be differentiated from other isolates of the same genus, species, and subspecies by at least one phenotypic or genotypic characteristic'. The concepts of strain-level characterization and identification will be discussed in more detail at the conclusion of this chapter.

Several terms are often used interchangeably when discussing bacterial characterization and are defined here as well. **Classification** refers to the grouping of organisms according to their similarities or relatedness based on an established set of properties, and is not always related to taxonomy. **Identification** places an organism within a classification scheme and usually involves naming the organism. Identification also refers to the recognition of previously undescribed groups (i.e., identification of a new species). **Differentiation** refers to the separation of organisms at any taxonomic level based on an established set of criteria. **Typing** refers to grouping of organisms based on relatedness and may be operationally defined based on the method of differentiation (electrophoresis pattern type, ribotyping pattern, etc.). Typically, typing refers to the differentiation of isolates below the species level (Dijkshoorn and Towner, 2001). **Fingerprinting** refers to grouping

organisms based on the similarities of multivariate data (e.g., electrophoretic banding patterns).

While many different types of bacteria (commonly including members of *Lactobacillus*, *Lactococcus*, *Bifidobacterium*, *Enterococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, and *Sporolactobacillus*) and yeasts (*Saccharomyces cerevisiae*) have been identified as putative probiotic organisms (Holzapfel *et al.*, 1998), this chapter will focus primarily on the methods used to identify bifidobacteria at the genus and species levels and the methods used to differentiate individual bifidobacteria strains. Early molecular methods and their limitations will be briefly discussed and nucleic acid-based techniques will be examined in more detail.

17.3 ‘Classic’ techniques

17.3.1 Fructose-6-phosphate phosphoketolase

In 1965, the pathway of hexose fermentation in bifidobacteria was elucidated by Scardovi and Trovatelli (1965). The key enzyme in this pathway is fructose-6-phosphate phosphoketolase (F6PPK; EC 4.1.2.22), which cleaves hexose phosphate into erythrose-4-phosphate and acetyl phosphate. Further actions of transaldolase and transketolase result in xylulose-5-phosphate and acetyl-phosphate, which are converted into acetic and lactic acids in a theoretical ratio of 3:2. After its identification in *Bifidobacterium*, the presence of F6PPK became the central means of identifying members of this genus. Some yeasts were also known to produce F6PPK (Schramm *et al.*, 1958), but at low levels compared to those observed in bifidobacteria. With the advent of 16S rDNA sequence-based phylogenetics (Stackebrandt *et al.*, 1997) and the identification of *Gardnerella vaginalis* and the taxonomic re-assignment of this species to the *Bifidobacteriaceae* (Greenwood and Pickett, 1980, Gavini *et al.*, 1996), the presence of F6PPK – in the truest sense – is now the key to identifying members of this family. However, *Gardnerella* can be differentiated from *Bifidobacterium* morphologically (*Gardnerella* is Gram-negative or Gram-variable) or by mole% G+C (*Gardnerella* exhibits 43% G+C). Additional genera in this Family have also been identified: *Scardovia* and *Parascardovia* (Jian and Dong, 2002) (formerly represented by *Bifidobacterium inopinatum* and *Bifidobacterium denticolens*, respectively), and *Aeriscardovia* (Simpson *et al.*, 2004). These genera may be differentiated from *Bifidobacterium* by morphological characteristics, fermentation patterns, DNA base composition, the ability to grow aerobically, or by sequence comparison of conserved genes. Few isolates from these genera have been identified and each consists only of the type species. Therefore, most researchers will assign a strain to the genus *Bifidobacterium* if it is an anaerobic, Gram-positive rod with F6PPK activity or will employ a nucleic acid-based method – most commonly, PCR amplification using *Bifidobacterium* genus-specific primers or hybridization of a genus-specific nucleic acid probe.

17.3.2 Carbohydrate fermentation assays

Before the prevalence of nucleic acid-based methods, carbohydrate assays were the most frequently used means of identifying and differentiating bifidobacterial species. Various researchers proposed classification schemes based on fermentation patterns and growth characteristics of collections of bifidobacteria (Dehnert, 1957, Reuter, 1963, 1971, Mitsuoka, 1968, Scardovi, 1984, Crociani *et al.*, 1994). In these assays, carbohydrates were added to tubes of a carbohydrate-free basal medium, inoculated with washed preparations of bifidobacteria, and then incubated under defined conditions. Modern-day kits simplified the procedure by providing small test strips with individual wells, each containing a defined amount of a carbohydrate, which is rehydrated with a carbohydrate-free basal medium containing a pH indicator into which the cells have been suspended (e.g., API® 50 CH kits, Biomérieux). The ability of a strain to ferment a particular carbohydrate is detected by a color change of the indicator within a defined time period.

Caution should be used when interpreting fermentation results. A negative result does not necessarily mean a strain lacks the ability to utilize a specific carbohydrate. Rather, the result indicates the strain did not produce sufficient acid to result in a decrease in pH within the defined period under the specific conditions of the assay. Despite attempts to minimize variability in the protocols, carbohydrate assays are also greatly influenced by the age and preparation of the inoculum, as well as the time, temperature, and conditions of the incubation (anaerobic/aerobic). For example, in our lab, changing the composition of the medium from which the cells were harvested prior to being resuspended in the carbohydrate-free basal medium produced different fermentation results.

Results of carbohydrate assays are also subjective and often difficult to interpret. When the carbohydrate assay is performed with tubes of liquid media instead of strips of small test wells, the pH of the media can be measured at the end of the assay to definitively score positive results. However, even with efforts to maximize reproducibility, carbohydrate assays alone are simply too variable and lack the accuracy needed to differentiate bifidobacterial species (Mitsuoka, 1984, Scardovi, 1984, Gavini *et al.*, 1991).

17.3.3 Peptidoglycan and cell wall fatty acid analysis

Other classic phenotypic methods have also been employed to differentiate bifidobacteria. Determination of the composition and structure of cell wall peptidoglycan was a traditional characterization method for Gram-positive bacteria (Kandler, 1970) and was used to classify and identify bifidobacterial species (Scardovi *et al.*, 1979b, Lauer and Kandler, 1983, Lauer, 1990). Cell wall fatty acid analysis, another classic method, was also useful to early researchers attempting to differentiate bifidobacteria from lactobacilli and related organisms. However, while fatty acid determination may be performed quickly, may be compared to a database of analyzed reference strains, and may provide assistance in assigning strains to species, the fatty acid composition

varies greatly with the composition of the growth medium, the growth temperature and the culture's age and therefore is not a useful discrimination tool for individual strains or an accurate identification tool for species, relative to more current methods.

17.3.4 Whole-cell protein electrophoresis and isozyme patterns

In conjunction with carbohydrate fermentation patterns and peptidoglycan composition, protein electrophoresis has been used to characterize strains of bifidobacteria. Electrophoresis has either consisted of separation of whole-cell soluble proteins or separation of constitutive, native proteins followed by staining for specific enzymatic activity. The mobilities of homologous proteins in the gel are related to protein polymorphisms, which in turn relate to the phylogenetic relationships among the strains.

Biavati *et al.* (1982) examined electrophoretic patterns of soluble cellular proteins obtained from 1,094 strains of bifidobacteria and correlated them with previously published DNA-DNA hybridization results. The presence of a 'genus band' was used to identify bifidobacteria and to confirm the existence of new species. Additionally, identification of bifidobacteria based on electrophoretic patterns obtained with the type strains for the species was more reliable for identification of strains than carbohydrate fermentation patterns, which were too variable to provide accurate separation.

In 1971, Scardovi *et al.* used the mobility of isozymes of F6PPK following starch gel electrophoresis to characterize 19 strains of bifidobacteria. Although F6PPK mobility was not useful for differentiating species of *Bifidobacterium*, a relationship between electrophoretic type and source of the isolate was established (Scardovi *et al.*, 1971a). Elaborating on this technique, Scardovi and Sgorbati used starch gel electrophoresis to differentiate among isozymes of transaldolase, transketolase, 6-phosphogluconate dehydrogenase (6PGD), aldolase, and fructose-6-phosphate phosphoketolase for 49 strains of *Bifidobacterium*. The appearance of identical zymogram patterns among some strains led to a proposal to merge some of the species and identified strong similarities among other groups (Scardovi and Sgorbati, 1974).

Subsequent work by Scardovi and other researchers employed isozyme electrophoresis, in conjunction with DNA homology, carbohydrate fermentation patterns, and peptidoglycan types to characterize strains. The results of this work have played an important role in our understanding of the phylogeny of bifidobacteria, identifying similarities among groups of strains, differentiating among species, and identifying new species (Scardovi and Sgorbati, 1974, Trovatelli *et al.*, 1974, Scardovi *et al.*, 1979a, 1979b, Biavati *et al.*, 1982, Lauer and Kandler, 1983, Biavati and Mattarelli, 1991, Biavati *et al.*, 1991).

A more recent application of enzyme electrophoresis in 1994 compared isozymes of β -galactosidase to enzyme activities and carbohydrate fermentation patterns. Several isozymes were identified, each unique to a particular *Bifidobacterium* species (Roy *et al.*, 1994). When comparing the electrophoretic

method to the more common phenotypic characterization schemes, the dairy-related bifidobacteria could more readily be differentiated from the non-dairy strains by electrophoresis.

Protein electrophoresis and isozyme typing have been useful in characterizing bifidobacteria, but require preparation and analysis of numerous samples to accurately evaluate relationships among strains. In addition, these methods are neither rapid nor highly discriminatory.

17.4 Nucleic acid-based techniques to identify bifidobacteria

Nucleic acid-based techniques are of increasing importance in differentiating and typing bacteria and providing means for evaluating inter- and intra-species relatedness (Busch and Nitschko, 1999). The advantages of using molecular techniques to identify microorganisms based on their nucleic acid sequences include applicability to all bifidobacteria, and the methods are more objective, more sensitive, and often less time-consuming than the traditional phenotypic analyses. Though some researchers and commercial manufacturers still employ phenotypic characterization (commonly, carbohydrate fermentation patterns) as the basis for their identification schemes, individual strains within species will vary in terms of carbohydrate utilization. This is one reason for the discrepancy often noted by researchers using DNA-based techniques to identify commercial isolates of bifidobacteria.

17.4.1 DNA-DNA homology and mole percent G+C content

The introduction of DNA base composition determination and DNA-DNA hybridization, two classic typing assays now standard in the descriptions of bacterial taxa, greatly impacted bifidobacteria characterization. Bifidobacteria, with a high (>55%) mole percent G+C (Werner *et al.*, 1965), were clearly separated from the lactic acid bacteria and were officially recognized as a separate genus in the 8th edition of *Bergey's Manual of Determinative Bacteriology* (Rogosa, 1974). DNA-DNA hybridization indirectly measures nucleotide sequence similarity of two entire genomes. DNA is extracted from two bacteria samples and denatured into single strands at high temperatures. DNA from both samples is then incubated together at lower temperatures, allowing the formation of heteroduplexes from complementary sequences which are used to determine DNA relatedness among the two organisms. DNA-DNA hybridization has the advantage of measuring overall genomic relatedness and is not greatly influenced by growth conditions, phenotypic variability, or mutations.

Scardovi's research group extensively applied DNA-DNA hybridization to bifidobacteria, concluding they were distinct from lactobacilli and were comprised of different species (Scardovi *et al.*, 1970). They were able to

confirm previous species designations, identify similarities among strains, and recognize new species among isolates having varied phenotypic characteristics. Their group increased our understanding of the relationships among bifidobacteria, using techniques beyond basic morphology, biochemical properties and carbohydrate fermentation patterns. DNA-DNA hybridization allowed identification of bifidobacteria which were being isolated from a wide variety of ecological niches and also allowed identification of strains and species atypical of the usual classification schemes.

DNA-DNA hybridization, considered the taxonomic standard for classifying and identifying bacterial species, has found limited use in the majority of current research settings. The technique requires a large collection of reference strains, isolation of large quantities of DNA, and involves significant amounts of labor, time and expense. However, characterization of bifidobacteria based solely on phenotypic attributes is difficult because of the large phenotypic variability among strains, the subjectivity involved in the interpretation of results, a finite number of observable phenotypes, a lack of reproducibility, and a dependence on culturing and assay conditions.

An additional challenge to characterization is the interpretation of results – regardless of method used – relative to the current taxonomic structure of bifidobacteria. Our understanding of the phylogenetic relationships among bifidobacteria is constantly evolving. With the development of novel characterization methods and the isolation of diverse strains, new phylogenetic relationships among groups of strains are identified and taxa may be merged or separated. As recent examples within the *Bifidobacteriaceae* family, *Bifidobacterium denticolens* and *Bifidobacterium inopinatum* were transferred to *Parascardovia denticolens* and *Scardovia inopinatum*, respectively (Jian and Dong, 2002), and *Aeriscardovia aeriphila* was identified (Simpson *et al.*, 2004).

More relevant to the food and probiotic supplement industries was the ‘continuum’ described in 1971 between *B. longum* and *B. infantis* (Scardovi *et al.*, 1971b). The strong similarity between these two species and with *B. suis* was the subject of much discussion and research (Scardovi *et al.*, 1979a, Lauer and Kandler, 1983, Bahaka *et al.*, 1993, LeBlond-Bourget *et al.*, 1996, Miyake *et al.*, 1998, Kagermeier-Callaway, 2000, Biavati, 2001, Jian *et al.*, 2001, Klein, 2001), finally resulting in the merger of *B. infantis* and *B. suis* as biotypes of *B. longum* (Sakata *et al.*, 2002). *Bifidobacterium lactis* was identified in 1997 (Meile *et al.*, 1997) and is the most frequently identified species in probiotic dairy products (Bonaparte and Reuter, 1997, Masco *et al.*, 2005). Its exact taxonomic position and relationship to *B. animalis* have been studied and debated (Cai *et al.*, 2000, Klein, 2001, Ventura *et al.*, 2001b, Ventura and Zink, 2002, 2003, Berthoud *et al.*, 2005). Currently, *B. lactis* has been classified as *B. animalis* ssp. *lactis* (Masco *et al.*, 2004).

Because of significant expansion in the *Bifidobacteriaceae* family and changes among *Bifidobacterium* species, methods for the accurate identification of genus and species, as well as for strain differentiation and characterization

were needed, which were more accurate and reliable than phenotypic methods. A variety of nucleic acid-based methods were developed which have increased the ability to accurately identify isolates and to assess the phylogenetic relationships of bifidobacteria. In addition to increased accuracy, these methods do not depend on the ability to culture the organism, allow the study of the entire bacterial genome rather than only the fraction related to phenotypic expression, and typically exhibit greater discriminatory power and reproducibility.

17.4.2 16S rRNA sequencing

While DNA-DNA hybridization is the superior method for establishing bacterial species, 16S rRNA sequencing can be used to differentiate species. Strains with 16S rRNA sequence similarities <97% belong to different species, though sequence similarities >97% do not necessarily represent the same species (Stackebrandt and Goebel, 1994). The availability of PCR, universal primers, automated sequencing services, and access to online sequence databases have expanded the popularity of this technique. In addition, the technique is culture-independent, allowing characterization of bacteria that can not be grown in the laboratory. 16S rRNA sequencing has been extensively applied in the phylogenetic study of prokaryotic organisms and has been used to evaluate relatedness among bacteria above the genus level (Stackebrandt and Woese, 1981, Woese, 1987, Stackebrandt and Goebel, 1994, Stackebrandt, 2002). Examples of characterization at this level include defining the hierarchy of the *Actinobacteria* class and separation of *Bifidobacterium* and *Gardnerella* genera within the *Bifidobacteriaceae* (Stackebrandt *et al.*, 1997). Although *Gardnerella* is closely related to bifidobacteria, shares high 16S rRNA sequence similarity, and was once a member of the *Bifidobacterium* (Embley and Stackebrandt, 1994), the genera were separated by the lower (43 mole%) G+C content of *G. vaginalis* (Greenwood and Pickett, 1980).

Frothingham *et al.* obtained partial 16S rDNA sequences from seven bifidobacterial species and revealed similarities of 94–99% (Frothingham *et al.*, 1993). Comparison of the entire 16S rDNA sequence from 31 bifidobacterial species also revealed similarities of greater than 91% (Miyake *et al.*, 1998), indicating a high degree of homogeneity in this group and suggesting the usefulness of 16S rDNA as a target for genus and species identification. When interpreting species identification based on 16S sequencing, it is important to consider the high degree of 16S sequence similarity exhibited by bifidobacteria, the number and diversity of the reference strains found in the sequence database, and the discriminatory power represented by a partial sequence compared to the entire 16S region. For example, when a 500-bp region of the 16S rRNA gene was sequenced from commercial and reference strains and used to identify bifidobacteria (Yeung *et al.*, 2002), the type strains of *B. animalis* and *B. infantis* were identified as *B. lactis* and *B. suis*, respectively.

17.4.3 Genus-specific identification of bifidobacteria with nucleic acid probes and primers

Sequencing of 16S rDNA identified both regions of high conservation and variability. These regions have been targeted in design of probes and PCR primers varying in terms of their level of specificity for classification – from universal primers for eubacteria to genus, species, subspecies, and strain identification of bifidobacteria. Lists of some of the genus- and species-specific probes and PCR primers used to identify bifidobacteria have been compiled (Matsuki *et al.*, 2003, Satokari *et al.*, 2003).

Langendijk *et al.* (1995) developed *Bifidobacterium* genus-specific probes, based on the 16S rRNA sequence, and used whole-cell FISH (fluorescence *in situ* hybridization) to quantify bifidobacteria in human fecal samples. For rapid identification using PCR, Matsuki *et al.* (2002) developed PCR primers for four groups of bacteria commonly associated with the human GI tract, including *Bifidobacterium*, based on comparing 16S rRNA sequences. Their *Bifidobacterium* genus-specific primers reacted with *G. vaginalis*, but had the advantage of being able to identify bifidobacteria from total fecal DNA without cultivation.

Another genus-specific hybridization probe, Im3, based on the 16S rRNA sequence of bifidobacteria was developed by Kaufmann *et al.* (1997). The probe hybridized strongly with 30 strains of *Bifidobacterium* and weakly with *G. vaginalis* and a *Propionibacterium* strain. However, when Im3 was paired with primer Im26, only *Bifidobacterium* strains yielded positive PCR results. Kaufmann *et al.* included universal primers as positive PCR controls in their assessment of primer specificity. Without universal primers, a negative PCR result may be interpreted as inadequate cell lysis, a lack of quality DNA, or PCR failure. Considering the difficulties often associated with bifidobacterial cell lysis, these are all valid possibilities and should not be discounted when negative PCR results are obtained from crude cell lysates and universal primers have not been included.

17.4.4 Species-specific identification of bifidobacteria with nucleic acid primers

Yamamoto *et al.* (1992) sequenced 16S rRNA from *B. adolescentis*, *B. bifidum*, *B. breve*, *B. infantis*, and *B. longum* to develop probes specific for these species, although there was some cross-reactivity with species of non-human origin. A similar result was also observed with primers designed for *B. breve*, *B. infantis*, and *B. longum* identification which either cross-reacted with other species or yielded false-negative results (Roy *et al.*, 1996). Matsuki *et al.* (1998) used 16S rRNA sequencing to create species-specific primers for *B. adolescentis*, *B. angulatum*, *B. bifidum*, and *B. breve* and group-specific primers for *B. pseudocatenulatum/catenulatum* and *B. infantis/longum*. Specificity analysis revealed some cross-reactivity between the *B. adolescentis* primers and *B. ruminantium* and between *B. longum* primers and *B. suis*.

This research was further expanded with the development of species-specific PCR primers for *B. infantis*, *B. dentium*, *B. gallicum*, and *B. longum*, which also reacted with *B. suis* (Matsuki *et al.*, 1999). These PCR primers were employed to identify bifidobacteria in fecal isolates, results were then confirmed by DNA-DNA hybridization.

17.4.5 Multiplex PCR

Screening of isolates with multiple PCR primers in separate reactions involves much time and many reagents. Development of multiplex PCR methods, combining multiple PCR primers into a single reaction with one set of amplification conditions, overcomes this limitation. Primers are designed to target different sequences, resulting in amplicons of varying sizes that allow for simultaneous identification. Ventura *et al.* were able to simultaneously identify *Bifidobacterium* genus and *B. lactis* species using primers targeting the 16S rRNA and 16S-23S ITS sequences (Ventura *et al.*, 2001b). Universal primers, resulting in amplicons from all eubacteria, were included to confirm the presence of adequate quality DNA in the sample.

A nested multiplex PCR reaction was developed to identify *B. adolescentis* and *B. dentium* (Bonjoch *et al.*, 2004). Genus-specific PCR was performed first, amplifying the 16S rRNA gene. This reaction mixture was then used with primer sets developed previously (Matsuki *et al.*, 1999), specific for the two bifidobacteria species. This technique was used to characterize bifidobacteria from human and animal waste water.

Dong *et al.* (2000) developed multiplex PCR primers to identify *B. bifidum*, *B. adolescentis*, *B. infantis*, *B. breve*, and *B. longum* based on 16S rRNA sequences. The reaction mixture included one forward primer and five reverse primers, one for each species. The specificity of the multiplex PCR was slightly less than that obtained through a series of single-primer PCRs – weak positive results occurred with *Gardnerella* and *B. minimum* and non-specific banding occurred when multiple species were present in the same sample.

Germond *et al.* (2002) developed two sets of multiplex PCR primers based on 16S rRNA sequences, using Im3 as the reverse primer, to identify ten species of bifidobacteria – *B. adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. dentium*, *B. infantis*, *B. longum*, *B. pseudocatenulatum*, and *B. lactis*. The two PCR mixtures were performed in series, with the results of the second reaction resolving ambiguous results related to nonspecific banding of the first reaction. The specificity of the primers was limited to species from human sources, as some of the primers also targeted sequences found in species isolated from animals and sewage (*B. subtilis*, *B. minimum*, *B. denticolens*, *B. inopinatum*, and *B. cuniculi*). The multiplex PCR conditions identified bifidobacterial species from single colonies and identified multiple species that were the most abundant among the total bifidobacteria in fecal samples. For species of bifidobacteria in fecal samples

that were less abundant, single primer pair reactions were needed for positive detection.

Mulli   *et al.* (2003) developed conditions for three multiplex PCRs to identify 12 bifidobacterial and related species in human fecal samples based on 16S rDNA sequences – *B. bifidum*, *B. breve* and *B. infantis*; *B. angulatum*, *B. catenulatum/pseudocatenulatum*, *B. dentium*, and *B. longum*; and *B. adolescentis*, *B. scardovii*, *B. gallicum*, *Parascardovia denticolens*, and *Scardovia inopinata*. There was some cross-reactivity with *B. suis* and the *B. infantis* and *B. longum* primers, as well as *B. catenulatum* with the *B. angulatum* primers.

A multiplex PCR reaction was developed by Kwon *et al.* that was able to identify eight species of bifidobacteria in a single reaction – *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, *B. longum/suis*, *B. lactis*, *B. infantis*, and *B. pseudolongum* (Kwon *et al.*, 2005). The primer mixture consisted of two *Bifidobacterium* conserved primers, six species-specific primers and two group-specific primers based on 16S and 23S rRNA sequences and the ITS region. The primers were designed such that *B. longum* and *B. lactis* yielded two amplicons each – one for the species and one for the group (*longum/infantis* or *animalis/lactis*, respectively). Therefore, if an isolate was identified as a member of the group but displayed only one amplicon, it was identified as the other species from the pair, *B. infantis* or *B. animalis*, respectively. With minimal non-specific banding and cross-reactivity, this unique approach to primer design allowed the identification of eight species of bifidobacteria in a single reaction, the most species of any method to date.

Because of the cross-reactivity of primers, non-specific banding, and false negative results, when the species of an isolate is unknown, multiplex PCR may be used as an initial screening. However, depending on the primers and PCR conditions selected and the level of accuracy desired, it should then be followed by a single PCR to accurately confirm the species identification. Identifying species from among a mixture of multiple species also represents a challenge. Depending on the relative concentrations of the species targeted by the multiplex reaction, underrepresented species may not be identified and single primer pair reactions should be performed if a greater level of sensitivity is desired.

17.4.6 Strain-specific identification of bifidobacteria with nucleic acid primers

Strain-specific primers were developed based on the 16S rRNA sequence from *Bifidobacterium* sp. LW420 by Kok *et al.* (1996). Sequence comparisons identified the strain as possibly *B. lactis*, which also correlated with the observation that the strain was acid- and oxygen-tolerant. These strain-specific primers were then used to identify LW420 among fecal samples obtained from infants fed formula containing this strain. The specificity of the primers was determined by screening a collection of 11 species of bifidobacteria.

However, other bona fide strains of *B. lactis* were not included in the comparisons, although one strain of *B. animalis* was included. Following the current taxonomic designation of *B. lactis* and *B. animalis* as subspecies, it appears the two primers developed are probably subspecies-specific. Similarly, Wang *et al.* developed primers specific for *B. adolescentis* and *B. longum* to identify these organisms among human and animal fecal samples (Wang *et al.*, 1996). While the primers for the two species did not react with any of the eleven other anaerobic genera found in the human gastrointestinal tract included in the screening, the bifidobacteria primers were only evaluated against one other *Bifidobacterium* strain, a *B. infantis*. Further evaluation of the primers against a range of bifidobacterial strains would be required to assure the primers were truly strain-specific.

Two sets of strain-specific primers were designed based on sequence comparisons of the 16S-23S rRNA ITS region to identify *B. infantis* Y1 and *B. breve* Y8, strains used in the manufacture of a commercial pharmaceutical product, VSL-3 (Brigidi *et al.*, 2000). The specificity of the primers was assayed by screening a collection of more than 60 strains of bifidobacteria from five species (*B. longum*, *B. infantis*, *B. breve*, *B. adolescentis*, and *B. bifidum*). When the ITS sequence of *B. infantis* Y1 was compared to those available in a database, high homology was revealed to the type strains of *B. lactis* and *B. animalis*; therefore, seven *B. animalis* strains were also included in screening of the *B. infantis* Y1-specific primers. Ultimately, it was possible to specifically detect *B. breve* Y8 and *B. infantis* Y1 in commercial products and in human fecal samples.

One of the limitations to strain identification using PCR is the high level of sequence similarity among bifidobacteria. Most strain-specific primers are designed by sequencing a gene or region in the strain of interest, and then comparing this to similar sequences found in a database and identifying unique regions that may serve as sites for primer design. In theory, this is a logical approach and results in primer sequences that are unique to the strain. However, considering the diversity of bacteria in the environment, it is more appropriate to conclude the primers are only specific for the strain of interest relative to the small subset of sequences from strains that have been deposited in the database. For practical reasons, the best approach to confirm specific strain identification is to screen PCR primers against closely related strains of the same genus and species and, when analyzing bacterial populations, to be able to differentiate a specific strain from among indigenous microbiota. While comparisons to sequences available in databases may give an indication as to the specificity of primers, the greater the number of related strains screened with a set of primers, the stronger the conclusion of their specificity.

17.4.7 Gene sequencing

A challenge in using 16S rRNA to differentiate bifidobacteria is the high degree of sequence homology displayed among species. Therefore, sequence

analysis of other genes has been utilized as a technique to characterize closely related strains. An ideal target would provide a sufficient level of sequence variation to aid in strain differentiation but also would be conserved enough for development of PCR primers and for inferring phylogenetic relationships.

The 16S-23S rRNA ITS (internally transcribed spacer) region is universally present in bacteria and is more variable than the 16S rRNA sequence. ITS sequences from bifidobacteria correlated well with the phylogenetic relationships established using 16S rRNA sequences and with DNA-DNA hybridizations, while exhibiting greater discriminatory power (LeBlond-Bourget *et al.*, 1996). The ITS regions displayed strong similarities among strains of a species, suggesting the usefulness of this region for species identification. Comparisons of the ITS regions from strains of *B. animalis* and *B. lactis* revealed two 8-bp insertions unique to *B. lactis*, which were used to develop *B. lactis*-specific PCR primers. ITS sequencing was also able to differentiate among strains of *B. lactis* (Ventura and Zink, 2002).

However, the ITS region is not an accurate phylogenetic marker, as its sequence is too variable. Additionally, there may be heterogeneity among multiple ITS regions within the chromosome of an isolate. Therefore, sequences of other conserved proteins have been evaluated for strain differentiation and identification. While extensive gene sequence information is not currently available for many bifidobacteria, the number of genes sequenced and the diversity of strains examined is continually increasing and should prove to be useful in the future. Many of the gene sequences targeted for species identification have been housekeeping genes, included *recA* (Kullen *et al.*, 1997b), *tuf* (Ventura *et al.*, 2003a), *grpE* and *dnaK* (Ventura *et al.*, 2005), *ldh* (Roy and Sirois, 2000), *xfp* (Yin *et al.*, 2005), and pyruvate kinase (Vaugien *et al.*, 2002). As an example of their high discriminatory power, gene sequence comparisons have been able to differentiate between closely related strains of *B. animalis* and *B. lactis* – *recA* and *tuf* (Ventura and Zink, 2003), *groEL* and *atpD* (Masco *et al.*, 2004), *grpE* and *dnaK* (Ventura *et al.*, 2005), *ldh* (Roy and Sirois, 2000), and pyruvate kinase (Vaugien *et al.*, 2002). Sequences of *hsp60* have been used to differentiate among strains of *Bifidobacterium* species and *Gardnerella* (Jian *et al.*, 2001) and to identify new genera in the *Bifidobacteriaceae* family (Jian and Dong, 2002, Simpson *et al.*, 2004).

17.5 Nucleic acid-based techniques to type bifidobacteria

17.5.1 Ribotyping

Ribotyping has been used to estimate polymorphism within the 16S or 23S rRNA genes in bifidobacteria. During ribotyping, DNA is extracted and cleaved with a frequently-cutting restriction enzyme. The fragments are electrophoresed and then allowed to hybridize to a labeled rRNA gene probe. The resulting pattern is then used to differentiate bifidobacteria. A modification

to this basic technique involves embedding the cells in low-melting point agarose prior to lysis and DNA extraction (McCartney and Tannock, 1995).

Ribotyping is more effective in characterizing bacteria that contain multiple copies of the rRNA genes, like bifidobacteria (Bourget *et al.*, 1993, Yoon *et al.*, 1999, Satokari *et al.*, 2001a, Schell *et al.*, 2002), rather than a single copy. Ribotyping demonstrates high reproducibility among laboratories, is applicable to all bacteria, and does not require knowledge of the genomic sequence. However, this technique is labor- and time-intensive. Automated ribotypers are available, along with a library of patterns of reference strains, which allow for ease of processing and identification of a large number of samples. When comparing a pattern from an isolate to a library, it is important to consider the number and diversity of reference strains representing the genera or species of interest.

Sakata *et al.* (2006) compared species delineations to species identification from an automated ribotyping device. Clusters of the *EcoRI* ribotype patterns did not correlate well with *Bifidobacterium* species, suggesting that although ribotyping may be useful in discriminating among bifidobacteria strains, the technique was not suitable for species identification. The discriminatory power of ribotyping may vary with the restriction enzyme selected and the specificity of the probe. More than one restriction enzyme may be needed to differentiate closely related strains (McCartney and Tannock, 1995, McCartney *et al.*, 1996, Kimura *et al.*, 1997). While 16S rRNA probes are most commonly used, bifidobacteria have been differentiated using 23S rRNA probes as well (Mangin *et al.*, 1994, 1995, 1996, 1999). Ribotyping has been used to track a specific bifidobacteria strain after human consumption (Mangin *et al.*, 1994) and, in combination with PFGE, has been used to assess the variability of bifidobacteria populations in humans (McCartney *et al.*, 1996, Kimura *et al.*, 1997, Mättö *et al.*, 2004). Ribotyping has also been used with RAPD-PCR and other molecular characterization methods to conclude that *B. infantis* and *B. suis* should be included as biotypes in the species *B. longum* (Sakata *et al.*, 2002).

17.5.2 RFLP

Restriction fragment length polymorphism (RFLP) is one means of assessing the variability in genes without sequence determination. A segment of DNA is amplified by PCR, subsequently digested with a restriction enzyme, and then electrophoresed through an agarose gel, yielding a unique pattern of fragments. The banding pattern varies with the primers and the amplicon size, the actual variation in the amplified sequence, the restriction enzyme used, and the number of copies of the sequence in the chromosome (as may be the case with multiple 16S rRNA operons). 16S rDNA-RFLP, or amplified rDNA restriction analysis (ARDRA), was used to uniquely identify a probiotic strain from among the indigenous microbiota of human subjects using the restriction enzyme *HaeIII* (Kullen *et al.*, 1997a). With this technique, it was

possible to monitor the appearance and disappearance of the ingested strain in fecal samples. Depending on the variability of the amplified sequences, it may be necessary to screen a number of restriction enzymes or to include patterns from more than one digest to characterize strains. If the sequence of the amplified gene is known, *in silico* digests may be performed using computer software to aid in restriction enzyme selection. Two enzymes, *AluI* and *TaqI*, were required to differentiate between bifidobacterial species of human and animal sources with ARDRA (Dalcenserie *et al.*, 2004). Only 12 of 16 bifidobacterial groups and/or species could be differentiated by ARDRA with *Sau3AI* (Ventura *et al.*, 2001a). By adding *BamH1* patterns, it was possible to differentiate 14 of the species – except for *B. animalis/lactis* and *B. longum/suis*. However, because of recent taxonomical changes unifying each of these groups, this method does provide accurate discrimination at least to the species level. Similar results were obtained when Roy and Sirois used three restriction enzymes (*BamH1*, *Sau3AI*, and *TaqI*) to differentiate the species of *B. infantis*, *B. longum*, and *B. animalis* with ARDRA, but could not differentiate the type strain of *B. lactis* from *B. animalis* (Roy and Sirois, 2000).

Venema and Maathius (2003) were able to classify bifidobacterial species common to the human GI tract using six restriction enzymes. This allowed for identification of 14 species, including *B. longum* and *B. suis*, as well as *B. animalis* and *B. lactis*. However, this increase in discriminatory power – to the subspecies level – makes the assay cumbersome for large numbers of isolates and results in increased assay costs because of the need for large amounts of restriction enzymes.

One advantage of ARDRA is that, as a PCR-based method, it may be fairly rapid (if few restriction enzymes are used), allowing many samples to be processed in a single day. However, because of the high 16S rRNA sequence similarity among bifidobacteria, the discriminatory power is lower than other typing methods. Thus, strains of different species may display similar ARDRA patterns.

To discriminate among bifidobacteria, other DNA sequences have been targeted to identify regions with more variation. Ventura and Zink (2003) compared the *tuf* and *recA* gene sequences of *B. lactis* and *B. animalis* strains, each theoretically digested with two restriction enzymes. Analysis of the 16S-23S rRNA ITS region revealed greater sequence variability than either the 16S rDNA or *tuf* and *recA* sequences, suggesting the ITS region may be able to distinguish between these two closely related groups of strains. Using *Sau3AI*, it was possible to differentiate between the ITS-RFLP patterns of *B. animalis* and *B. lactis* strains. All sequence analysis and RFLP patterns revealed high homogeneity among the *B. lactis* strains and strong similarity between the two groups, ultimately leading the authors to suggest *B. animalis* and *B. lactis* be separated at the subspecies level.

17.5.3 REP-PCR

With the introduction of PCR came the development of a new group of bacterial typing methods. The basis of these methods is the amplification of polymorphic DNA through the selection of primers whose annealing sites are variable in number and location around the chromosome. The advantages of these methods, as with traditional PCR, include they are rapid, require equipment and reagents which are typical of most research labs, and are widely applicable. They also share the disadvantages associated with traditional PCR, including differential or incomplete amplification of strains (for a review of PCR biases, see von Wintzingerode *et al.* (1997)).

REP-PCR (repetitive extragenic palindromic-PCR) targets highly conserved, non-coding, repetitive chromosomal elements which are 38-bp sequences containing a stem-loop structure with a 5-bp variable loop. REP-PCR was introduced to differentiate among strains of bacteria ((Versalovic *et al.*, 1991); review (Versalovic *et al.*, 1994)). Similarly, ERIC-PCR is based on ERIC (enterobacterial repetitive intergenic consensus) sequences, conserved 126-bp elements. Other conserved repetitive sequences targeted by PCR include BOX elements, which were among the first repetitive elements identified in Gram-positive microorganisms, and interspersed polytrinucleotides, including (GTG)₅. When the PCR primer annealing sites are on opposite DNA strands and are within a few thousand bases of each other, amplification occurs. The resulting amplicons vary in size and are separated by electrophoresis to create a unique banding pattern.

Although ERIC sequences were originally identified in the *Enterobacteriaceae*, they have also been identified among other prokaryotes. Shuhaimi *et al.* (2001) first identified ERIC elements in bifidobacteria and other Gram-positive probiotic microorganisms. It was possible to differentiate among five species of bifidobacteria, and between *B. longum* and *B. infantis*, and among strains of *B. pseudocatenulatum*. With the same ERIC-PCR primers, Ventura and Zink (2002) were also able to differentiate *B. lactis* and *B. animalis*. Ventura *et al.* (2003b) were also able to apply ERIC-PCR to the differentiation of 26 species of bifidobacteria, including strains from culture collections and fecal samples, and were able to use ERIC-PCR patterns to identify bifidobacteria isolated from food products.

BOX-PCR was used to differentiate reference strains of bifidobacteria (Gómez Zavaglia *et al.*, 2000). These patterns were then compared to those obtained from isolates obtained from fermented milk products to identify species, which correlated well with those obtained by whole cell protein SDS-PAGE. Using BOX-PCR for species identification, the majority of 58 food and dietary supplements evaluated were found to contain *B. animalis* ssp. *lactis* (Masco *et al.*, 2005).

Masco *et al.* (2003) evaluated 35 strains of seven species of dairy-related bifidobacteria (*B. adolescentis*, *B. animalis*, *B. breve*, *B. bifidum*, *B. infantis*, *B. lactis*, and *B. longum*) with primers for ERIC, BOX, (GTG)₅, and REP sequences. BOX-PCR was selected for further study because it was the most

discriminatory, yielding more than 20 bands and exhibiting the greatest inter-strain variation, and was able to differentiate among species, subspecies, and strains. It was possible to separate the groups of *B. longum/infantis/suis* and *B. animalis/lactis*. There was good correlation between identification with BOX-PCR and identification with species-specific primers. Also, multiple strains of each species were clustered together, except for strains of *B. asteroides* and *B. pseudolongum* ssp. *pseudolongum*.

The sensitivity and reproducibility of REP-PCR (REP, ERIC, BOX, etc.) depends on a number of factors, including the method of preparing the template DNA. While crude cell lysates may be used, and in fact, some research has demonstrated reproducibility is independent of template preparation, accurately quantified and purified genomic DNA was recommended for optimum reproducibility by Versalovic *et al.* (1994). Primer length and mole% GC content are also factors that contribute to pattern reproducibility.

17.5.4 AP- and RAPD-PCR

AP-PCR (arbitrarily primed-PCR) and RAPD-PCR (randomly amplified polymorphic DNA-PCR) are similar in concept to the amplification of DNA sequences interspersed around the chromosome, except that AP- and RAPD-PCR employ a single primer with a completely arbitrary sequence and PCR conditions of reduced stringency (Welsh and McClelland, 1990, Williams *et al.*, 1990). The lower stringency conditions allow the primers to anneal at multiple locations despite imperfect matches to the template DNA. As with REP-PCR, annealing sites on opposite strands that are close enough together result in the amplification of the region in between. Amplicons of various lengths then represent a banding pattern characteristic of the strain. There is no strict consensus delineation of the terms AP- and RAPD-PCR and these terms are often used interchangeably, although a system of nomenclature has been proposed (Vanechoutte, 1996). Both methods usually involve single-primer reactions, although AP-PCR typically refers to methods that use primers of greater lengths (perhaps 18–25 bases) while RAPD-PCR involves much shorter primers (~6–10 bases) (Towner and Grundmann, 2001). According to the original method description, AP-PCR also employed two cycles of low stringency (annealing temperature 40°C for 5 minutes) followed by ten cycles of higher stringency (annealing temperature 60°C for 1 minute) with primers of 20 or 30 bases in length (Welsh and McClelland, 1990). RAPD-PCR employs reduced stringency during all amplification cycles (Williams *et al.*, 1990).

Because the amplification conditions of AP- and RAPD-PCR employ lower annealing temperatures than typical PCR, these methods are very sensitive to slight alteration in procedure and suffer from a lack of reproducibility. Because of this sensitivity to variations in reaction conditions, it is important to include reference strains as positive controls for comparisons.

Towner and Grundman (2001) emphasized the need to adhere to a standardized protocol, beginning with preparation or isolation of the DNA for amplification through the conditions of staining and destaining the gel, which is especially important when comparing patterns among strains on different gels. An overview of the factors which influence pattern reproducibility includes template DNA, primer design, primer:template DNA ratio, reaction conditions or reagent sources, transposable elements and plasmids.

AP-PCR was used to differentiate between the indigenous microbiota of four subjects fed yogurt containing a strain of *B. bifidum* (Chen *et al.*, 1999). Two primers were included in the study, one of which was a primer specific for ERIC elements and the other an arbitrary primer 20 bases in length. The first four cycles of PCR included a low stringency annealing temperature (37°C).

RAPD-PCR was used to assess the variability of bifidobacteria isolated from rats fed kidney beans (Fanedl *et al.*, 1998). The RAPD patterns of the 15 isolates were compared to those of the type strains of 20 species of bifidobacteria. The unknown isolates did not form a coherent cluster, nor did they cluster with any of the type strains. These results indicated a large degree of genetic variability among the isolates, and therefore species identification was not possible. Eighty primers were initially screened and seven were selected that yielded between one and ten fragments. A low (36°C) annealing temperature was used and an extension time of two minutes was selected to produce bands no larger than 2 kb, with most bands being smaller than 750 bp. These screening criteria may have resulted in the selection of primers that were more useful for strain differentiation rather than species identification. However, the discriminatory power of this technique is easily modified. Varying the sequence or length of the PCR primers and/or the stringency of the annealing conditions might have produced banding patterns that would have been useful for species identification.

Five single-primer RAPD-PCR reactions, selected from among one hundred primers on the basis of discriminatory power, were able to differentiate among culture collection strains and commercial isolates (Vincent *et al.*, 1998). It was possible to separate strains of *B. adolescentis*, *B. bifidum*, and *B. breve* into groups. It was also possible to group strains into clusters of *B. infantis/longum* and *B. animalis/lactis* and then to divide each into their respective subclusters.

RAPD-PCR has been employed to characterize the genetic variability among strains of *B. longum*, *B. infantis*, and *B. suis* using three 10-base long primers and a low (30°C) annealing temperature (Sakata *et al.*, 2002). RAPD-PCR was more discriminatory than ribotyping, was able to differentiate among these species, and also indicated high levels of similarity among the three species. RAPD-PCR was also used with other molecular methods to demonstrate intraspecies genetic heterogeneity of bifidobacteria isolated from human fecal samples (Mättö *et al.*, 2004).

A variation of AP-PCR, termed TAP-PCR (triplicate arbitrarily primed-

PCR) was developed for strain differentiation and identification (Cusick and O'Sullivan, 2000). TAP-PCR capitalized on the sensitivity of AP-PCR to varied reaction conditions by creating a master mix of template DNA and the PCR amplification mixture containing a single 18-base primer specific for a conserved region of 16S rRNA. The mixture was then split into three aliquots, each of which employed a different annealing temperature (38, 40, or 42°C). The resulting amplicons were electrophoresed in adjacent wells. Bands appearing in at least two of the three lanes were considered less sensitive to varied reaction conditions and are included in the pattern analysis. With this technique it was possible to differentiate among species and strains. The added advantage of this technique was increased reproducibility compared to typical AP-PCR. However, a large collection of bifidobacteria strains, representing a variety of species, should be characterized with TAP-PCR to better assess the specificity and applicability of this method.

17.5.5 PFGE

PFGE (pulsed-field gel electrophoresis) is a widely-used and highly discriminatory molecular typing method, based on the comparison of restriction-digested total cellular DNA fragment patterns (Basim and Basim, 2001). In PFGE, cells are embedded in agarose plugs and treated with enzymes and detergents to lyse the bacteria. The chromosomal DNA is then digested with rare-cutting restriction endonucleases recognizing 6- or 8-bp sequences. Slices of the agarose plugs are inserted into a gel and electrophoresis is performed while the electric field changes direction and the pulse times continually increase. The resulting banding pattern is related to polymorphisms around the chromosome. The choice of restriction endonuclease depends largely on the G+C content of the strains to be characterized and enzymes should be selected that yield an optimum number of bands for pattern differentiation and for maximum discriminatory power. Comparing PFGE patterns obtained with multiple single-enzyme digests can increase the discriminatory power of the method, but the additional reagents are costly. Using PFGE, DNA molecules as large as 12 Mb can be separated. This technology has been used to physically map chromosomes, to estimate chromosome size, to aid in the precise selection of cloning fragments, to estimate double-strand breaks in DNA, to fingerprint bacteria, and to estimate relatedness among bacterial strains.

PFGE has been applied to strains of *Bifidobacterium* by various researchers to examine strain relatedness. Bourget *et al.* (1993) used PFGE to estimate chromosome size and compared the restriction patterns of five strains of *B. breve* obtained from culture collections. Digestion with *Xba*I, *Dra*I or *Spe*I yielded unique restriction patterns for each strain, except for ATCC 15698 and CIP 6466 which produced identical patterns when digested with *Dra*I and *Xba*I. This observation of strain-specific patterns first suggested the feasibility of PFGE in distinguishing and differentiating closely related strains of bifidobacteria.

McCartney *et al.*, and subsequently Kimura *et al.*, used PFGE and ribotyping to assess differences in populations of lactic acid bacteria and bifidobacteria within and between fecal samples taken from human subjects over time (McCartney *et al.*, 1996, Kimura *et al.*, 1997). Isolates displaying different PFGE patterns were considered unique strains and therefore could be differentiated at the strain level. Over a one-year period, McCartney *et al.* identified a subject that displayed a 'simple' gut microbiota containing five strains of bifidobacteria and lactobacilli, while another subject displayed a 'complex' microbiota consisting of more than 30 strains.

Expanding on McCartney's work, Kimura *et al.* identified individuals as having either simple (less than four strains of bifidobacteria) or complex gut microbiota (up to ten strains). Surprisingly, isolates of bifidobacteria from fecal samples from each of two different subjects were identified as being the same strain. The conclusions drawn by these researchers about microbiota composition, complexity, and stability in these subjects depended upon the assumption that strains with identical PFGE and ribotyping patterns were identical strains. While it is true that isolates with different PFGE profiles and ribotypes may reasonably be considered different strains, the reverse is not necessarily true. For example, Crittenden *et al.* did not observe strain-specific PFGE patterns among four distinct strains of *B. lactis*. The strains displayed the same *Xba*I and *Spe*I patterns, which suggested a lack of heterogeneity among these isolates. However, despite having similar PFGE profiles, phenotypic differences related to amylase activity were observed (Crittenden *et al.*, 2001). Other researchers have similarly used PFGE to differentiate closely related strains of bifidobacteria from various sources (Bonaparte and Reuter, 1997, Grand *et al.*, 2003, Roy *et al.*, 1996, Simpson *et al.*, 2003, Ventura and Zink, 2002).

To compare the discriminatory power of some nucleic acid-based methods used for the differentiation of bifidobacteria, Mättö *et al.* characterized isolates by ribotyping, RAPD-PCR, and PFGE (Mättö *et al.*, 2004). An equal number of ribotypes and PFGE types were observed, greater than the number of RAPD types. However, six of the isolates included in the study were not analyzed by PFGE, probably because of insufficient cell lysis. These strains were, however, characterized by ribotyping and RAPD. The discriminatory power of PFGE, as revealed by the relative number of pattern types compared to ribotyping and RAPD-PCR, may have increased if the strains not lysed by PFGE were discarded from the comparison of the methods, or if a different restriction enzyme was included in the survey.

In general, PFGE is a more discriminatory method than RAPD-PCR, which is more discriminatory than ribotyping or other common typing methods (O'Sullivan and Kullen, 1998, Busch and Nitschko, 1999). In applying PFGE to bifidobacteria, this technique 'is perhaps the most discriminatory technique at the strain level, even if it has no value in taxonomic identification' (Biavati and Mattarelli, 2001). In fact, for the differentiation of probiotic strains, PFGE is considered the 'gold standard' (FAO/WHO, 2002).

PFGE was applied by Simpson *et al.* (2003) in their analysis of 160 strains of bifidobacteria isolated from pigs. They were able to divide the isolates into 15 distinct profiles, comprising seven major pattern types. Other analyses, including RAPD-PCR, cell morphology, whole-cell protein electrophoresis, 16S rRNA sequence analysis and DNA hybridization, were used to evaluate the groupings established by PFGE. Results from these other methods supported the PFGE analysis and led Simpson *et al.* to conclude the seven PFGE types represented four distinct species, including two previously unreported species that shared less than 92% similarity in their 16S rDNA sequences to known *Bifidobacterium* species.

Roy *et al.* (1996) used PFGE with *Xba*I or *Spe*I to differentiate strains of *B. animalis*, *B. bifidum*, *B. breve*, *B. infantis*, and *B. longum* obtained from culture collections and commercial suppliers. It was possible to differentiate strains belonging to *B. infantis* and *B. longum*, two species traditionally difficult to separate using more traditional methods. It was also possible to identify unique patterns for many of the other strains evaluated and to identify closely related strains. Within a species, some of the culture collection strains did not exhibit unique patterns, and the PFGE patterns were not useful in definitively identifying species; however, many of the PFGE profiles were strain-specific. They also identified similar profiles between some of the culture collection and commercial strains and identified similar profiles among commercial strains, suggesting PFGE could be used as a typing method to differentiate bifidobacteria at the strain level or to identify the original source of commercial starters. Their work, and later work by Bonaparte and Reuter (1997), revealed identical PFGE patterns between *B. animalis* ATCC 27536 and eleven and four commercial strains of *B. animalis*, respectively.

PFGE is one of the most discriminatory methods and is very reproducible. However, PFGE is culture-dependent, requires a substantial investment of equipment, and is intensive in terms of time, labor, and reagents. Most published PFGE protocols used with bifidobacteria require five to seven days to complete. Recently, our lab developed a rapid method, requiring only 24 hours between obtaining a broth culture and obtaining a digital image of a gel (Briczinski and Roberts, 2006). This method was specifically developed for bifidobacteria and was assessed with 34 strains of bifidobacteria, representing seven species commonly employed in the dairy industry. Banding patterns and intensities were similar to those obtained with a longer, traditional method. With the logistical constraints of any PFGE method, we have found that no more than 30 strains may easily be prepared per day. While our rapid method does allow for a faster result to be obtained and is ideal for a research setting, the PFGE method is still quite labor-intensive and is not likely suited for routine analysis in a commercial setting or for high sample throughput.

17.6 Nucleic acid-based techniques to characterize bifidobacterial populations

17.6.1 T-RFLP

Terminal restriction fragment length polymorphism (T-RFLP) is a nucleic-acid based technique used to quantitatively assess the diversity of a microbial population (Liu *et al.*, 1997). This technique varies from basic RFLP in that polymorphisms in amplified gene sequences are represented only by the sizes of the 5' and/or 3' terminal restriction fragments. With T-RFLP, rRNA genes are amplified from the total DNA in a sample using universal primers, one or both of which is labeled. The resulting amplicons are digested with a frequently-cutting restriction enzyme, usually with a 4-base pair recognition sequence. Fluorescently labeled primers enable detection of either 5' or 3' terminal fragments from among all of the restriction fragments. As only the labeled terminal restriction fragments are assayed, T-RFLP produces patterns with fewer bands and would have lower discriminatory power compared to traditional RFLP analysis if fragment sizes were merely estimated on a typical electrophoresis gel. For accurate characterization of the bacterial population, precise measurement of the number of nucleotides in each fragment employs an automated DNA sequencer with the ability to resolve fragments that differ by as little as one base pair. By precisely measuring the fragment lengths and relating them to reference strains, it is possible to identify and semiquantify bacteria, to obtain a unique 'community fingerprint', and to estimate the microbial diversity in a sample.

T-RFLP has been used to characterize bacterial populations from soil (Dunbar *et al.*, 2000, Hackl *et al.*, 2004), saliva (Sakamoto *et al.*, 2003b), vaginal (Coolen *et al.*, 2005), and fecal samples (Nagashima *et al.*, 2003, Wang *et al.*, 2004, Jernberg *et al.*, 2005). T-RFLP has also been used to characterize the microbial diversity of human fecal samples. When genus-specific primers were employed, the technique was able to differentiate species of bifidobacteria (Sakamoto *et al.*, 2003a). This same technique was also used to assess the diversity and distribution of bifidobacteria in breast-fed and/or bottle-fed infants (Sakata *et al.*, 2005).

The primary challenge to use of T-RFLP is the universality or specificity of the primers employed. If the goal is to represent the diversity of the entire microbial population, it must be recognized the 'universal' primers have been developed based on 16S rRNA sequence databases, which may not be representative of the complete bacterial population. Additionally, total microbial diversity is most likely underestimated, as groups of bacteria occurring in small numbers may not be represented proportionally after PCR, and multiple copies of the PCR target in the genomes of certain organisms may also skew the distribution. For example, no bifidobacteria were detected by T-RFLP in the microbiota of elderly subjects (Hayashi *et al.*, 2003). While bifidobacteria populations decline with age in humans, the authors propose this was most likely related to a very low occurrence (rather than a complete absence) of bifidobacteria in the fecal samples.

17.6.2 PCR-T/DGGE

Another powerful method for characterizing diversity of a microbial population is PCR-TGGE (temperature gradient gel electrophoresis) or PCR-DGGE (denaturing gradient gel electrophoresis). After PCR is performed, typically with primers that target variable regions of 16S rRNA, the amplicons are separated via electrophoresis through a gel with increasing denaturation conditions (either temperature or a denaturant, such as urea or formamide). As the amplicons move through the gel, the double-stranded DNA products will begin to melt at a specific denaturation point. As it melts, the product's conformation changes and its migration slows. To prevent complete separation of the products into single strands, one of the PCR primers contains a 'GC clamp' of 30–50 bases at the 5' end. The melting behavior of the PCR products is sequence-dependent, making it possible to separate products whose sequences differ by as little as one base pair. However, it is important to note that two amplicons with the same mole% G+C along their length may exhibit the same migration distances despite different DNA sequences.

PCR-T/DGGE generates a fingerprint unique to the bacterial population. This can be used to characterize changes in a bacterial population over a period of time or after a change in diet or some other intervention. The varying intensities of the bands in the pattern may be used to semiquantitatively assess the relative concentrations of groups of bacteria in an ecosystem. To identify isolates in a sample, the bands are compared to the migration of reference strains or the bands may be excised and sequenced. Limitations of this method are similar to those of other PCR-based methods, including inconsistent cell lysis and DNA extraction, amplification inefficiency and PCR biases, and the specificity of the primers used to characterize the population.

The diversity of the bacteria in fecal samples was assessed by amplifying a segment of V6-V8 16S rRNA and using PCR-TGGE (Zoetendal *et al.*, 1998). The banding patterns were unique to each individual and were fairly consistent over a period of six months. Fifteen of the most prominent bands were sequenced and, interestingly, only two of them shared more than 97% similarity to those in sequence databases, the majority representing undescribed bacterial species. Similar results, with the majority of strains identified by PCR-DGGE exhibiting less than 97% similarity to identified species, have been obtained with colon biopsy samples (Nielsen *et al.*, 2003) and infant fecal samples (Favier *et al.*, 2002). The detection limit of this method has been estimated at 10^9 per 10^{11} cells in a 1-gram fecal sample (Zoetendal *et al.*, 1998) or 10^4 cells of a pure culture per mL (Temmerman *et al.*, 2003b).

To increase the selectivity of this technique, *Bifidobacterium* genus-specific primers were applied with PCR-DGGE to obtain species-specific patterns for most strains of bifidobacteria, except for closely related species of *B. infantis/longum*, *B. pseudocatenulatum/catenulatum*, and *B. breve/dentium* (Satokari *et al.*, 2001a). This method of characterization was also used to monitor changes in the population of bifidobacteria in fecal samples (Satokari

et al., 2001a, Sakamoto *et al.*, 2003a) and to monitor the establishment of bifidobacterial species in infants (Favier *et al.*, 2002, 2003, Satokari *et al.*, 2002, Vlková *et al.*, 2005). This approach was also employed to identify intragenomic heterogeneity among multiple *rrn* operons in the type strain of *B. adolescentis* (Satokari *et al.*, 2001a). The use of genus-specific primers with PCR-DGGE was also used to monitor changes in bacterial populations when subjects were fed prebiotic oligosaccharides and/or the probiotic strain *B. lactis* BB-12 (Satokari *et al.*, 2001b). A nested PCR technique was developed for use with DGGE to identify less abundant bifidobacterial populations among total fecal DNA. Genus-specific primers were used to select for bifidobacteria, the resulting PCR products were purified, and then universal primers for 16S rRNA were used to amplify variable regions of the genes for differentiation by DGGE (Nielsen *et al.*, 2003, Temmerman *et al.*, 2003a, Masco *et al.*, 2005, Vlková *et al.*, 2005). PCR-DGGE has also successfully been used to identify the species of probiotics in yogurt and pharmaceutical products, comparing them to identification by culture-dependent methods and evaluating label declarations (Fasoli *et al.*, 2003, Temmerman *et al.*, 2003b, Masco *et al.*, 2005).

As an alternative target to 16S rRNA, the transaldolase gene was amplified using conserved primers with PCR-DGGE, and it was possible to differentiate among species of bifidobacteria (except for *B. catenulatum/angulatum*) and to identify subtypes among some species (Requena *et al.*, 2002). The advantage of transaldolase as a PCR target is that it is only present once in the chromosome and contains conserved regions for primer development.

17.7 Future trends

DNA microarrays, or 'gene chips', represent a powerful method for comparing entire genomes of related strains and for evaluating gene expression from an organism exposed to different environmental conditions. Numerous specific DNA fragments are fixed to a glass surface and allowed to hybridize with cDNA or DNA sequences from a strain of interest. This method has the advantage of high throughput screening once the initial experiment is designed and equipment has been setup. DNA microarrays have applications of detecting genome variability, characterizing the composition of a bacterial population, and strain identification. They represent a novel alternative to determining the entire genomic sequence of a group of related strains.

Multilocus sequence typing (MLST) is a recent approach to molecular typing that is based on the principles of multilocus enzyme electrophoresis (MLEE) (Maiden *et al.*, 1998). While MLEE analyzes the electrophoretic mobilities of specific enzymes and associates different migratory patterns with variation among the isolates, MLST utilizes the variation found within several specific genetic sequences as a means of identifying allelic profiles in bacterial isolates. The sequences from a number of gene fragments are

obtained and unique sequences of each gene are considered individual alleles. The alleles exhibited by each of the fragments are combined to represent a unique profile or type. This method considers the variation from multiple genes and therefore is highly discriminatory, is highly reproducible, exploits common molecular biology reagents and sequencing services, and is electronically portable (Enright and Spratt, 1999, Spratt, 1999). MLST has been most commonly used as an epidemiological tool to track bacterial pathogens (Enright and Spratt, 1998, Kotetishvili *et al.*, 2002, Adiri *et al.*, 2003, Viscidi and Demma, 2003, Helgason *et al.*, 2004, Robles *et al.*, 2004, Zhang *et al.*, 2004), but has not been used with bifidobacteria. This method has great potential for unambiguous strain-level differentiation of probiotic strains in the future.

17.8 Conclusions

A variety of molecular methods have been used to characterize and identify bifidobacteria. Generally, phenotypic methods of strain characterization are limited by the fact strains exhibit varying attributes under conditions of the assay, and often yield ambiguous or inconsistent results within and among species. Recent molecular methods are nucleic acid-based, and are typically more discriminatory and reproducible. However, an 'ideal' method for strain identification would be able to type all strains and yield unambiguous results at the genus, species, and strain levels, would display high levels of stability, discriminatory power, reproducibility, and concordance with other typing methods. It also would not require great technical skill or be time-, cost-, or reagent-intensive. No one method can possibly fulfill all of these criteria. A polyphasic or consensus approach is best, which includes a combination of genomic and phenotypic methods to characterize and differentiate strains (Vandamme *et al.*, 1996).

As defined at the beginning of this chapter, a strain is an isolate that can be differentiated from other isolates of the same genus, species, and subspecies by at least one phenotypic and/or genotypic characteristic. Strain differentiation depends on recognizing unique properties capable of discriminating at this level. Some nucleic acid-based methods reviewed in this chapter may prove useful in separating closely related bifidobacteria strains. However, strain differentiation and strain identification are not the same. An often overlooked but very important fact is that typing methods rely on identifying *differences* between isolates. The results of a particular method or a combination of typing methods with very high discriminatory power are only capable of determining whether or not a strain is different *relative to another strain or group of strains*. This concept of *relative* strain identity is not the same as *absolute* strain identity. It is impossible for a typing method to determine that strains are *identical*, just that they are 'not different'.

There are two factors affecting the relative specificity associated with a

strain-specific identification scheme: (1) the number and type of different phenotypic and genotypic assays used to differentiate the strain of interest and (2) the number of other isolates included in the screening. A large number of closely related strains, at least of the same species, should be compared and be clearly differentiated from the target strain. Only one different phenotypic result or gene polymorphism separates strains, but to create a strain-specific classification scheme involving every possible phenotypic assay and DNA-based typing method is simply not feasible. As Dijkshoorn and Towner (2001) stated, for unequivocal strain identification, 'a complete DNA sequence would form the ultimate reference standard for recognizing sub-types within a species, but short of achieving this ideal, any typing technique relies on finding detectable differences between isolates'. Until entire genome sequencing is routine, the challenge is to identify powerful typing methods capable of providing a comfortable level of relative strain identity.

17.9 References

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Methods to improve the viability and stability of probiotics

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18.1 Introduction – importance of improving the viability and stability of probiotics

Probiotic functional foods face high technological demands since probiotics, live microorganisms which when administered in adequate amounts confer a health benefit on the host (Anon., 2002), have to retain their viability during all the production steps and even in the gastrointestinal (GI) tract of the consumer. Surveys of probiotic products on the market have unfortunately revealed common quantitative and qualitative deficiencies especially regarding labelling and viability of probiotic strain(s) (Temmerman *et al.*, 2003a, b; Fasoli *et al.*, 2003; Theunissen *et al.* 2005). These observations indicate that probiotic production technologies as well as processing and formulation steps applied are often non-optimal.

The aim of industrial probiotic production is to produce in an affordable manner high-quality, safe end-products with long enough shelf-life. For the food industry an easy formulation of probiotic preparations into products is of concern. Thus probiotic preparations are usually provided as highly concentrated DVI (direct vat inoculation) products, which can be directly used at the probiotic food production site (Gomes and Malcata, 1999; Saarela *et al.*, 2000). For the successful production of high-quality probiotic products a good knowledge on the strain-specific characteristics is necessary. Viability losses of probiotic microbes unavoidably occur during their processing and formulation steps (Fig. 18.1), but with a strong know-how of the production strain these losses can be minimised. A common feature for most probiotic strains is that they are specialised in living in the human or animal GI tract, which makes them well adapted to that special environment but poorly adapted

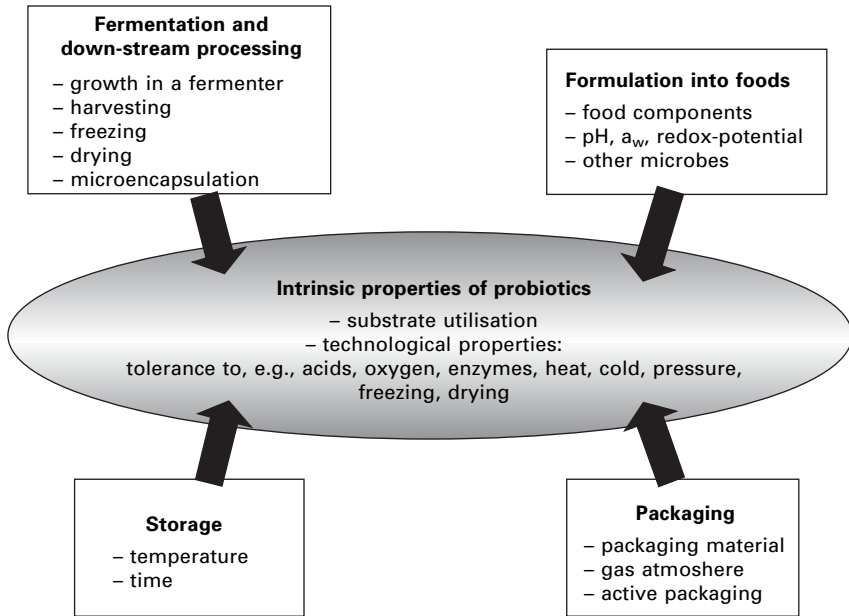


Fig. 18.1 Factors that affect probiotic viability and stability.

to other environments. Microbes that survive and thrive in the GI tract can therefore be difficult to propagate and down-stream process in both small and large scale. Typical problems in probiotic technology are difficulties in obtaining high cell concentrations during growth, and retaining viability during down-stream processing, formulation and in the end product.

18.2 Determining the viability of probiotics

Plate count technique, which is based on reproduction of bacterial cells on agar plates, is the traditional method used for quality assurance of probiotic products. Culture-based analysis enables determining the number of bacterial cells able to grow on the medium applied and their identification. To gain more insight into the actual physiological state and metabolic activities of the cells, fluorescent stains, which enable the detection of viable, damaged, and dead bacterial cells, can be used. Typically a dual approach including staining of viable cells with one dye followed by counterstaining of dead or all cells with another stain in, order to obtain the total cell number, is applied in viability studies (Breeuwer and Abee, 2000). Fluorescent cells are then detected with fluorescence microscopy, fluorometer, or flow cytometer (FCM). Fluorescence microscope, although slower than fluorometer or FCM analysis, enables investigating cell morphology and clumping. Fluorometry method on the other hand allows obtaining the results in less than one minute after

the staining, and with this method 96 samples can be detected simultaneously (Alakomi *et al.*, 2005), which makes this method suitable for routine viability studies. Viability stains (fluorescent probes) can be applied to fresh cells, dried probiotic cell preparations and probiotic food products (Auty *et al.*, 2001; Bunthof and Abee, 2002; Alakomi *et al.*, 2005; Maukonen *et al.*, 2006).

Fluorescent probes detect differences in, e.g. membrane permeability or enzyme activity of cells thus rendering fluorescent techniques basically different from culture which relies on microbial reproduction. Moreover, plate count may underestimate the number of viable cells due to the viable but nonculturable state of bacteria, which is caused by cell stress or injury (Camper and McFeters, 1979). The drawback of applying fluorescent probes in probiotic viability studies is that they do not allow the identification of the bacteria, and they are not applicable for sample materials which contain other bacterial species (e.g., starter bacteria) or which cause heavy background staining. Furthermore, probiotic bacteria show variable staining with different fluorescent probes. It therefore has to be kept in mind that fluorescent techniques are not universal (i.e., not all probes suit to all probiotic species or all probiotic products) and successful application necessitates careful tailoring of the method for targeted probiotic bacterial species and product types (Maukonen *et al.*, 2006).

18.3 New methods to improve the viability and stability of probiotics

Probiotic viability and stability can be enhanced at several stages including strain selection and improvement of strain properties, production conditions, formulation, and packaging and storage (Fig. 18.2). Whenever possible, a probiotic strain should be chosen based on its health-benefiting properties as well as on technological robustness. Since both functional and technological properties can vary remarkably within a genus and even within a species, technological properties of potential probiotic strains should be considered at an early stage of the probiotic product development. However, owing to the inherent limitations in the adaptability of GI-originating bacteria to survive outside their natural environment, maintaining their viability and stability during processing is often a demanding task.

18.3.1 Optimising the growth in a fermenter

Growth of a probiotic strain in a fermenter is the first production step affecting the viability and stability of the final probiotic product. Probiotic growth (medium components, pH, temperature) is nowadays typically optimised using statistical process parameter evaluation, experimental design and process optimisation. Utilising these tools enables reducing the number of tests to be performed during the growth optimisation step. Statistical tools have been

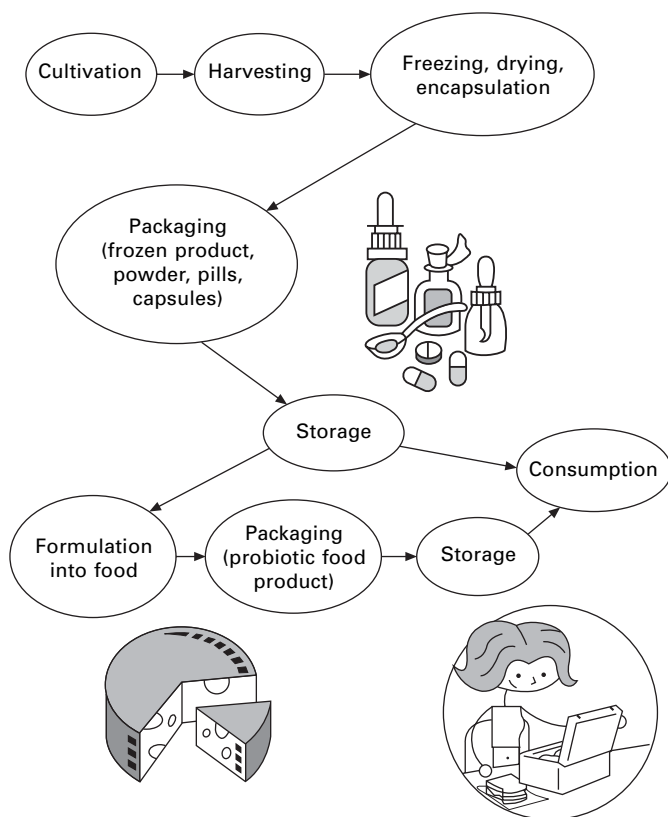


Fig. 18.2 Stages of probiotic production.

applied in the optimisation of the growth of, e.g., *Lactobacillus casei* (Oh *et al.*, 1995; Ha *et al.*, 2003), *Lactobacillus salivarius* (Juarez Tomas *et al.*, 2002), *Lactobacillus rhamnosus* (Liew *et al.*, 2005), *Bifidobacterium longum* (Kiviharju *et al.*, 2005), and *Bifidobacterium animalis* subsp. *lactis* (Mättö *et al.*, 2006). The growth performance of a bacterial strain is typically followed by measuring base consumption during pH-controlled fermentation and by determining the obtained cell mass after the fermentation. To obtain the number of living cells the cell mass is then commonly further analysed with plate count.

Since it is well known that bacterial cells in stationary growth phase are more robust than cells in exponential growth phase, cell mass harvesting in batch fermentations is typically started when cells enter the stationary phase (this information can be obtained by determining the growth curve for the bacterial strains and by following the base consumption during pH-controlled fermentation). Another way to affect cell physiology during growth is, instead of performing a free-cell batch fermentation, to immobilise and grow cells in a suitable carrier matrix in a continuous culture. It is well known that free-

swimming (planktonic) cells have different physiology compared to cells living in a biofilm (O'Toole *et al.*, 2000), and this knowledge can be utilised to affect strain properties during growth in fermenters (Doleyres and Lacroix 2005). Immobilised cells typically show altered morphology, membrane composition and metabolism, and increased tolerance to antimicrobial compounds compared to free cells (Doleyres and Lacroix, 2005). In the study of Doleyres *et al.* (2004) gel bead immobilised *Bifidobacterium longum* cells were more tolerant to hydrogen peroxide, simulated gastric and intestinal juices, and to various antibiotics than free cells grown in a continuous culture. Additionally, immobilised cell technology allows achieving high stability and volumetric productivity of the cultures (Doleyres *et al.*, 2002).

18.3.2 Utilising cells' stress responses

Technological properties of a probiotic strain might be improved by activating the cells' stress response machinery either by turning on their stress genes during processing with suitable sublethal treatments (van de Guchte *et al.*, 2002), or by genetically modifying the strain to make it more robust (e.g., by transfer, multiplication and/or additional activation of genes responsible for stress response). The profound difference with these two approaches is that activation of stress genes by stress-treatment is transient and therefore the treatment has to be performed immediately prior to harvesting and downstream processing, whereas genetic modification may enable better initial stress-tolerance of the strain.

Probiotic cells' natural stress responses have been utilised, e.g. to improve their tolerance to adverse conditions such as low pH (Park *et al.*, 1995; Lorca *et al.*, 1998; Lorca and de Valdez, 2001; Maus and Ingham, 2003; Saarela *et al.*, 2004) (Fig. 18.3), heat (Kim *et al.*, 2001; Desmond *et al.*, 2002; Ananta and Knorr, 2004) and drying (Desmond *et al.*, 2002; Prasad *et al.*, 2003).

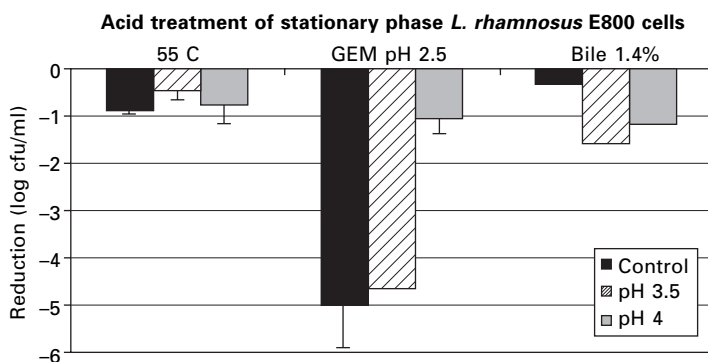


Fig. 18.3 When *Lactobacillus rhamnosus* E800 cells were acid treated (pH 4), cell viability at pH 2.5 in growth medium (GEM) improved markedly (about 10 000 x). However, acid treatment did not improve the heat and bile tolerance of the strain.

Using suboptimal pH during or at the end of fermentation can induce the stress response in the probiotic culture and can help it to survive better (Lorca and de Valdez, 2001; Maus and Ingham, 2003; Silva *et al.*, 2005). Cells that enter into stationary phase develop a general stress-resistance and are thus more resistant to various types of stresses (including down-stream processing and storage) than cells in the log phase (Brashears and Gilliland, 1995; Lorca and de Valdez, 1999; van de Guchte *et al.*, 2002).

Stress tolerance of *Lactobacillus* strains has also been improved by overproducing stress response genes. In the study of Derzelle *et al.* (2003) adaptation of *Lactobacillus plantarum* NC8 to cold-shock, stationary phase or freezing could be improved by overproducing CspL, CspC, or CspP, respectively, while Desmond *et al.* (2004) could improve the solvent (butanol) tolerance of *Lactobacillus paracasei* NFCB 338 by overproducing GroESL. Overproduction of GroESL also improved the spray-drying and freeze-drying survival of *L. paracasei* NFCB 338, but it had no enhancing effect on the storage stability of the bacterial powders (Corcoran *et al.*, 2006). Over-expression of BetL in *Lactobacillus salivarius* 118 using a nisin-inducible promoter has been shown to improve the strain's tolerance towards various stresses, increasing its osmo-, cryo-, baro- and chill tolerance and survival after freeze- and spray-drying (Sheehan *et al.*, 2006). BetL is involved in betaine accumulation in the cells which is well known to have a protective role against environmental stresses in various bacteria (Kempf and Bremer, 1998).

18.3.3 Utilising fibres and prebiotics as protectants and carriers for probiotics – synbiotics with multifunctions?

During the past few years utilisation of fibres and prebiotics as carriers and protectants for probiotic cells has gained increasing interest. These kinds of products combining the health benefits of both probiotics and prebiotics/fibres will have added value if the fibre can protect probiotic cells during down-stream processing, formulation and/or storage and thus improve their viability and stability.

Fresh cell applications

There are a few studies where the effect of prebiotics on the stability of fresh probiotic cells either during cold-storage or in adverse conditions has been investigated. In the study of Shin *et al.* (2000) it was shown that adding large quantities (5%) of galactooligosaccharide (GOS) and especially fructooligosaccharide (FOS) to growth medium (skim milk) enhanced the survival of two *Bifidobacterium* strains during cold-storage. The results of Saarela *et al.* (2003) studied the effect of prebiotic lactose derivatives on the functional and technological properties of four *Lactobacillus* strains. They concluded that although prebiotic lactose derivatives did not largely affect the properties of studied probiotics, lactulose showed some potential in

protecting the technologically least robust strain (a *Lactobacillus salivarius* strain) against bile and during cold storage. In the study of Helleland *et al.* (2004) Litesse® (modified polydextrose) supplementation did not affect the growth performance or cold storage stability of probiotic strains (*Bifidobacterium animalis* subsp. *lactis* Bb-12, *L. rhamnosus* GG, *Lactobacillus acidophilus* La-5 and 1748) in milk- or water-based cereal puddings. However, in a recent study by Martinez-Villaluenga *et al.* (2006) RFOs (raffinose family oligosaccharides) addition (2%) in milk improved the storage stability of *B. animalis* subsp. *lactis* Bb-12 and *L. acidophilus* La-5 and in the study of Saarela *et al.* (2006) oat flour (with 20% β -glucan) addition improved the storage stability of *L. rhamnosus* E800 in low pH (3.5) apple juice. These results indicate that prebiotics/fibres may have potential as probiotic protectants but the applications are fibre, probiotic and application specific.

Dry cell preparations

Probiotic cells in products have been stabilised by freezing, drying (freeze-dried or spray-dried) or encapsulation. To enhance the survival of probiotics during these steps, protectants are used. For the cryoprotection of LAB a large selection of compounds has been used. Among the most commonly used cryoprotectants in freeze-drying are skim milk with or without supplements and various disaccharides (for a review see Hubalek, 2003; Carvalho *et al.*, 2004). Some of these compounds can also be used as thermoprotectants during spray-drying.

Only few studies have been performed where fibres and prebiotics as cryo- or thermoprotectants have been investigated. In the studies of Corcoran *et al.* (2004) and Ananta *et al.* (2005) polydextrose and inulin supplementations in the carrier medium (reconstituted skim milk, RSM) did not enhance *L. rhamnosus* viability during spray-drying or powder storage. When these carriers were used alone, the spray-drying survival was clearly inferior to that of the control carrier (RSM). Also the storage stability at 37°C was poorer for inulin carriers than for RSM (polydextrose was not included in stability studies) (Corcoran *et al.* 2004). In the study of Saarela *et al.* (2006) the capability of different fibre preparations to protect the viability and stability of *L. rhamnosus* during freeze-drying, storage in freeze-dried form and after formulation into foods was studied. In freeze-drying trials and in dry food applications wheat dextrin and especially polydextrose proved to be promising carriers for the *L. rhamnosus* strains: freeze-drying survival and storage stability of the powders at 37°C were comparable to the control carrier (sucrose) (Fig. 18.4) and also the stability of *L. rhamnosus* in chocolate-coated breakfast cereals was good. Since in the study of Corcoran *et al.* (2004) and Ananta *et al.* (2005) both different control carrier and different drying techniques were used, the results of these studies and the study of Saarela *et al.* (2006) are difficult to compare. However, it can be concluded that in non-milk-based applications prebiotic fibres can be promising protectants for probiotic cells during drying.

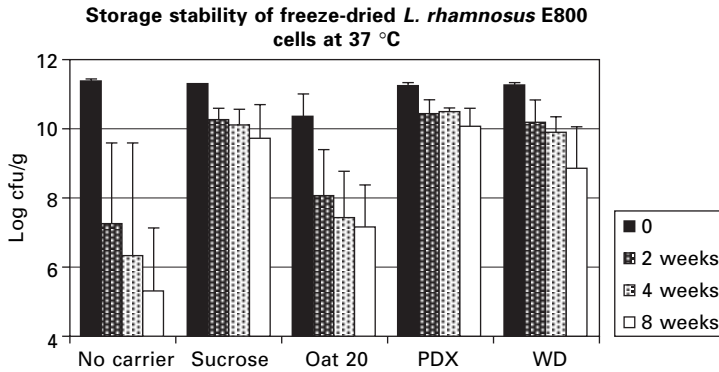


Fig. 18.4 Storage stability of freeze-dried *L. rhamnosus* E800 at 37 °C was good when sucrose, polydextrose (PDX) and wheat dextrin (WD) were used as cryoprotectants; oat flour (with 20% β -glucan) was a poorer protectant than sucrose, PDX or WD but it still showed a clear protection of the cells (compared to no carrier).

For the probiotic encapsulation carriers/supporting material used includes alginate, carrageenan, cellulose acetate phthalate, chitosan, gelatine, gum arabic, and starch (Krasaekoopt *et al.* 2003). Basic encapsulation techniques are extrusion and emulsion, but cells can also be encapsulated during freeze- and spray-drying. Probiotic encapsulation is further described in Chapter 19.

18.4 Increasing the range of probiotic foods

Probiotic foods have become the fastest growing European food market with an annual growth of over 10% in the EU (Anon., 2004). An extensive range of probiotic foods are on the market in different European countries, most of these being produced by the dairy industry. Today probiotics are also incorporated into widening range of other types of food products such as cereal-based products. Increasing the range of probiotic foods is, however, limited by several factors. These include possible unsuitable food matrix (low pH, competing microbes, inhibitory components such as NaCl) and non-optimal storage conditions (e.g., room temperature which occasionally can reach values well over 30°C). The fact that by definition probiotics have to retain their viability during product formulation, storage and consumption (Anon., 2002) sets great demands for probiotic viability and stability. Probiotic viability can be enhanced by several ways (see above and Chapter 19) and this will eventually enable widening the range of foods with probiotics. Also, since probiotic bacteria show large variation in their robustness (e.g., regarding tolerance to oxygen and low pH; Mättö *et al.*, 2004) selecting a strain with good technological properties will be a key factor when novel food applications for probiotics are looked for.

18.5 Future trends

Spray-drying has gained increasing interest as a way of producing starter and probiotic powders. Compared to traditionally used freeze-drying, spray-drying enables large-scale economic production of bacterial powders (Silva *et al.*, 2005) and this makes it an attractive drying technique. The disadvantage of using spray-drying is, however, the additional heat stress step that the bacteria have to endure. To obtain stable powders (moisture content max. 4%) high temperatures which are detrimental to bacterial activity and viability have to be applied during the spray-drying process. Combining spray-drying with fluidized beds enables reducing the spray-drying outlet temperature which in turn will improve probiotic viability. This has been shown by Gardiner *et al.* (2002) with *Lactobacillus paracasei* and by Simpson *et al.* (2005) with bifidobacteria. In the future spray-drying combined to a fluidized bed will thus probably be the drying method of choice at least for the more heat-tolerant bacterial species.

The rapidly increasing number of published lactic acid bacterial genome sequences will in the future also enable utilising this sequence information in the studies related to probiotic technology (Klaenhammer *et al.*, 2005). If genome sequence information is available for the probiotic species of interest, this can be utilised, e.g. to study the gene expression (transcription) profile of the strain during fermenter growth. This will enable better control and optimisation of the growth than is currently possible. Transcription profiling during various production steps will allow following important genes for probiotic survival during processing (e.g., stress and acid tolerance genes) and identifying novel genes important for the technological functionality of probiotics (Klaenhammer *et al.*, 2005; Klijn *et al.* 2005).

Increasing knowledge of genes important for the technological functionality and rapid development of the toolboxes for the genetic manipulation of *Lactobacillus* and *Bifidobacterium* species will in the future enable tailoring the technological properties of probiotic strains. Overcoming technological hurdles related to genetic strain improvement will, however, not lead to wide application of tailored strains in probiotic food products unless consumer attitudes towards the products containing these strains become more favourable (Lähteenmäki *et al.*, 2002; Grunert *et al.*, 2004). Furthermore, with genetically engineered strains aimed at human/animal consumption, safety issues are of utmost importance and have to be separately considered for each modified strain (Ahmed, 2003). Options for specific genetic engineering include classical strain improvement (CSI; sequential random mutagenesis and screening) and genome shuffling (Patnaik *et al.*, 2002). These techniques enable targeting multiple (unknown) genes simultaneously and using even genetically poorly characterised strains (Patnaik *et al.*, 2002). Yet another practical option for improving the technological properties of probiotic strains is – instead of trying to improve the properties of existing strains – to isolate new strains/species that would naturally have the desired properties from different environments such as animal gastrointestinal tract and food or plant materials.

18.6 Sources of further information and advice

Scientific articles on probiotic technology are published in several journals, the most important of these being *International Dairy Journal*, *International Journal of Food Microbiology*, and *Journal of Applied Microbiology*.

Recent review articles (in addition to those mentioned in the references) on probiotic technology include the following:

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Microencapsulation for delivery of probiotics and other ingredients in functional dairy products

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19.1 Introduction

Microencapsulation is defined as the technology of packaging solid, liquid and gaseous materials in miniature sealed capsules that release their contents at controlled rates over prolonged periods of time under the influence of certain processing and environmental triggers (e.g., shear, temperature, enzymes, pH, fermentation, etc.). The industrial production of foods often requires the addition of ingredients, which are used to control flavour, colour, texture and preservation. There are many instances where it is advantageous to encapsulate ingredients. Controlled release of flavours is a particularly important advantage of encapsulation. Such a goal can be achieved by enabling the release of ingredients with matrixes having variable diffusion or degradation properties, as well as release by swelling or melting (Madene *et al.*, 2006). Other examples of the usefulness of ingredient encapsulation are presented in Table 19.1.

The areas which have probably most widely used capsulated ingredients are the prepared foods industry, the bakery industry and the ingredient suppliers themselves. The use of encapsulated ingredients in the dairy industry was rather limited in the past, with enzyme addition for accelerated cheese ripening being the most studied application. However, the advent of functional foods is increasing the use of encapsulated ingredients (Angustin, 2003). Currently, at least two commercial dairy products contain encapsulated bioactives (probiotics and omega-3 oils), but there could be more since some specialty dairy beverages contain multiple vitamin and mineral supplements. And this could only be the beginning, since, in addition to probiotics and omega-3 oils, a variety of bioactive compounds could be added to dairy products for

Table 19.1 Some examples of encapsulated ingredients and their applications in foods (Jackson and Lee, 1991; Shahidi and Han, 1993)

Reason for encapsulation	Examples	
	Ingredient	Food application
Slow release during manufacture	Organic acids (lactic, citric)	Meat – sausages
Slow release during cooking at home	Flavours	Microwaveable foods
Slow release in mouth	Sugars; flavours; acids	Chewing gum; candy coating
Slow release in the GI system	Probiotics	Nutraceuticals
Protection against high temperature oxidation	Ascorbic acid	Bread – dough conditioning
Increased stability during cooking	Vitamins	Pasta, prepared foods
Provides a powder form	Flavours – essential oils	Prepared foods
Powder particle size control – prevents stratification, better flow properties	Colours	Dry blends
Colour masking	Vitamin A; minerals	MSG table salts
Taste masking	Omega-3 oils	Milk, yogurt
Reduces reactions between ingredients	Minerals, oils, vitamins	Dry blends
Increased retention in the cheese matrix during manufacture	Enzymes	Cheese ripening

the purpose of developing such functional foods: vitamins, minerals, antioxidants, peptides, phytosterols, polyphenols, bioflavonoids and fibres. The aim of this chapter is to examine encapsulation technologies applied to bioactives added to dairy products, and why encapsulation can be useful. Since probiotics have been at the forefront of the development of functional dairy products, emphasis will be made on these ‘bioactive ingredients’.

19.2 Challenges in the addition of probiotics and bioactive compounds to dairy products

There must be a reason to encapsulate. Encapsulation of probiotics, and of many chemicals, often requires a specific processing step which complicates the manufacture of the ingredient and increases its costs. Therefore, encapsulated ingredients are typically used only if a problem exists in the preparation of the functional food. The development of functional foods with probiotics poses at least seven challenges (Champagne *et al.*, 2005):

- 1 ingredient selection: proper strain
- 2 quantity needed to have the biological effect. Can it be done?
- 3 toxicity issues
- 4 stability of the probiotic to processing steps
- 5 stability of the probiotic during storage
- 6 evaluation of the population in the product
- 7 effect on sensory properties.

In the past, companies which added a probiotic culture to fermented milks selected the strains on the basis of their ability to grow in milk, their ability to compete with the starter cultures, and their stability during storage. As scientific evidence of the health effects of probiotics accumulates, it appears that the strain itself may be a critical component of the functionality of the product. Therefore, strains are now selected not so much on their technological properties but rather on their ability to confer a health effect. Unfortunately, the number of strains which have demonstrated health properties is rather limited, and companies are now faced with the challenge of finding ways to ensure viability. Indeed, with respect to probiotics, the viability of the cells is a critical issue. Although there are instances where nonviable cells have shown biological activity (Kim, 2004; Liyan *et al.*, 2005), it is generally considered best to have viable cells in the product. During processing of dairy foods, the following situations can be detrimental to the viability of probiotics:

- inhibitory compounds produced by starter cultures (fermented milks)
- heating – pasteurization (most dairy products)
- freezing (ice cream, frozen desserts)
- oxygen (stirring during cheese manufacture or packaging)
- ingredients (salt in cheese; flavours in yoghurt or flavoured milks)
- drying (powders).

And the detrimental effects of acid (cheese, yoghurt), oxygen (yogurt, powdered blends) or humidity (powdered blends) during storage add to those encountered during processing.

Although in appearance the problems associated with probiotics would seem very different from those of chemicals; in practice, the same challenges often occur. Thus, as for probiotics, some bioactive chemicals are sensitive to acid, oxygen or heat; and the effects on sensory properties are always a concern whatever the bioactive ingredient added.

19.3 Microencapsulation methods for delivery of probiotics and other ingredients in functional dairy products

There are numerous methods of microencapsulation. To our knowledge, the commercial products currently available are based on gel particles, spray

coating, spray drying, extrusion and emulsions. Therefore, the focus will be made on these approaches. Other valuable techniques for microencapsulation such as liposomes, coacervation, or molecular inclusion (cyclodextrins) do not yet appear to have extensive use in the preparation of bioactives destined for the preparation of functional dairy foods, either because of cost or the requirement of non food-grade compounds in the process.

19.3.1 Microentrapment in gel beads

In this section, the production of solid matrixes which contain the bioactive compound rather evenly distributed will be examined. It could be argued that this process is not true microencapsulation because some particle sizes can reach 2 mm in diameter, and we do not obtain a capsule with a membrane coating having a different composition from that of the core. Thus, bioencapsulation (when applied to probiotics), matrix encapsulation or microentrapment could also be used to describe this process. Nevertheless, the particles obtained can often achieve the delay release and protection properties required.

Extrusion and emulsion methods for probiotics

Various polymers can be used to prepare ionic gel particles (Doleyres and Lacroix, 2005), but alginate, carrageenan and pectin are the most frequent. The technique relies on the ability of these polymers to form gels in presence of minerals, principally calcium and potassium. In all cases the polymers thus present multiple free carboxylic radicals on their chains, and the gelling ions form bridges between the molecules to form gels.

Three methods of producing alginate beads which carry probiotics are most commonly used (Fig. 19.1). The most popular is the alginate extrusion process in a calcium chloride solution. In some cases, starch (Dembczynski and Jankowski, 2002; Sultana *et al.*, 2000; Muthukumarasamy *et al.*, 2006) pectin and whey proteins (Guérin *et al.*, 2003) have been blended with alginate in order to improve the matrix in the subsequent applications. There seems to be little effect of CaCl_2 on cell viability during microentrapment, but high alginate content, high cell loading and large particles appear to be preferable when the products are subsequently challenged in simulated gastric environments (Chandramouli *et al.*, 2004). There are various scale-up methods as well as industrial suppliers of extrusion bead-forming equipment (Champagne, 2006), and the production can be contracted out.

The oil emulsion processes are less simple but easier to scale up (Krasaekoopt *et al.*, 2003). They have the advantage of enabling the production of smaller beads (Gouin, 2004), which can be critical in preventing sensory changes in the functional foods enriched with the probiotic-containing beads.

Coating of the alginate beads can also be carried out to improve their protective properties. The beads are simply dipped in a solution containing a cationic polymer, such as chitosan (Fig. 19.1), gelatin or poly-L-lysine

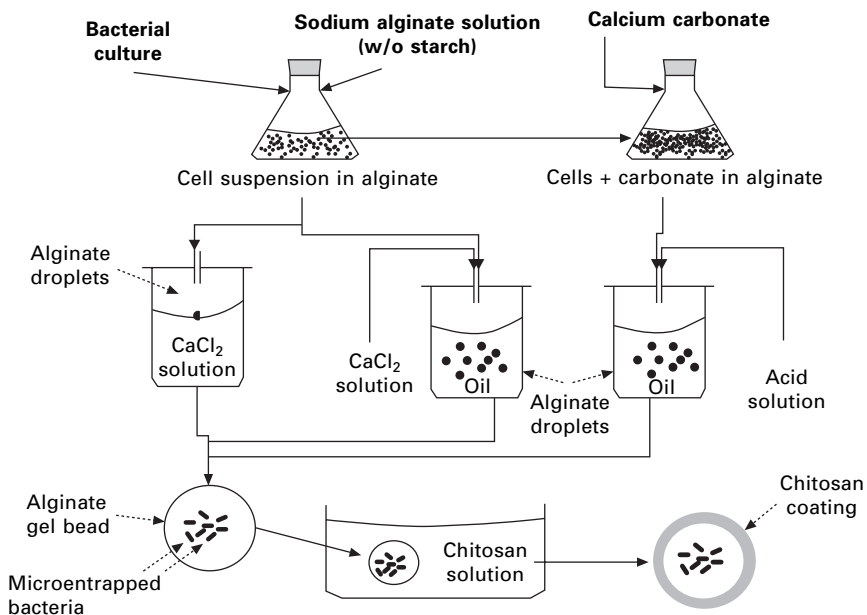


Fig. 19.1 Several techniques used to microentrap probiotic bacteria in alginate gel beads.

solutions (Krasaekoopt *et al.*, 2004; Groboillot *et al.*, 1993). Other coating or bead modification methods for alginate have been attempted (Krasaekoopt *et al.*, 2003; Le-tien *et al.*, 2004), but important losses in viability have resulted in some instances (Hyndman *et al.*, 1993; Larish *et al.*, 1994).

Some protein-based microentrapment techniques are also carried out by ionic gelification. Gelatin, caseins (Chung *et al.*, 2005) or whey proteins serve as substrates. Limited data suggest that alginate gels offer better protection to probiotics than other carbohydrate-based or protein-based gels (Muthukumarasamy *et al.*, 2006; Kailasapathy and Sureeta, 2004) but more data are needed on the effect of the matrix.

Spray-chilling technology

The production of gel beads can also be achieved by spray chilling, which is considered the least expensive encapsulation technology (Gouin, 2004). In spray chilling, a molten matrix of hydrogenated or fractionated vegetable oils with low melting point (32–42°C) containing the bioactive compound is atomized through a nozzle into a vessel. This process is similar to spray drying with respect to the production of fine droplets. However, it is based on the injection of cold air in the vessel to enable solidification of the gel particle, rather than hot air which dries the droplet into a fine powder particle. The liquid droplet thus solidifies and entraps the bioactive product. The matrix is consequently a thermo-gelling product. The particles are very small

and it does not appear that this technology has been used for probiotics. However, it is useful in entrapping lipids, and has been applied for omega-3 oils and it is used for the encapsulation of solid food materials, such as vitamins, acidulants and solid flavours.

19.3.2 Encapsulation by spray coating

Although there are much less scientific data on the effect of spray coating for probiotics than with microentrapment in gels, this technology has been the most extensively applied commercially to these cultures. Many of the process details are proprietary, but the extensive industrial interest in this technology is reflected by the patent activity in the field (Durand and Panes, 2001; Ubbink *et al.*, 2003; Shin *et al.*, 2002). The technology is very useful in protecting the bioactives against the detrimental effect of the gastric environments, and is therefore successfully applied for the nutraceuticals market. Preliminary data suggest that it is also promising for the protection of probiotics added to foods.

In spray coating, the core material needs to be in a solid form, although not necessarily completely dried. The core material is kept in motion in a specially designed vessel, either by injection of air at the bottom or by a rotary action of some sort (vessel or pale) (Fig. 19.2). A liquid coating material is sprayed over the core material and solidifies to form a layer at the surface. The coating material can be injected from many angles (Fig. 19.2), and this influences the properties of the coating. In food applications the

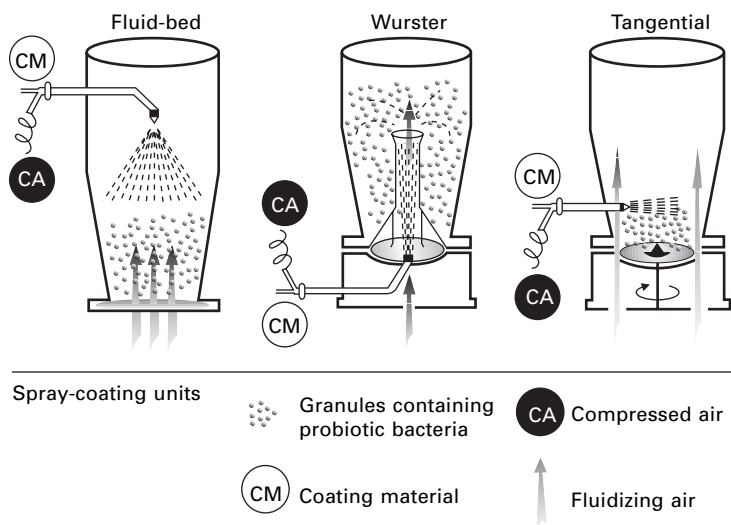


Fig. 19.2 Various designs of spray-coating processes.

coating is mostly lipid-based, but a wide variety of compounds can be used (Ubbink *et al.*, 2003)

- lipids:
 - waxes (paraffin, beeswax, carnauba, candellila, shellac)
 - fatty acids (oleic, stearic, palmitic) and their salts
 - hydrogenated or hardened oils
 - mono or diglycerides, and their derivatives
 - fats of animal origin (milkfat)
 - phospholipids.
- proteins: gluten, casein
- carbohydrates: cellulose and its derivatives, carrageenan.

The temperature of this coating material is typically kept between 60 and 120°C during spraying while the temperature of the fluidizing air can vary between 10 and 50°C (Durand and Panes, 2001). The process conditions (mostly air temperature and spraying rate) must be adjusted to prevent a strong increase in the temperature of the core material, particularly if probiotics or other temperature-sensitive bioactives are being processed. Initial particle size ranges between 100 and 1000 µm in diameter, and the coating will ultimately represent between 30 and 80% of the weight of the product.

Depending on the particle size to be coated, various equipments are preferable. For instance, the pellet-coating process (Ubbinck *et al.*, 2003) is recommended for particles having a volume of at least 0.02 cm³.

Spray-coating is particularly well suited for coats having multiple layers. For example, a first layer of lipids can be applied in order to confer a protective property for the probiotic bacteria in the core, while a second layer, protein or other, can be used in order to adjust the specific gravity or stabilizers for the product remaining in suspension in beverages (Shin *et al.*, 2002).

19.3.3 Multiple emulsions and liposomes

Oil-in-water (O/W) and water-in-oil (W/O) emulsions are prepared by homogenizing an oil phase and an aqueous phase together in the presence of an emulsifier. The type and concentrations of the various ingredients used to formulate these emulsions, the homogenization and other processing conditions employed determine their physicochemical properties and stability (McClements, 2005). In the emulsion preparation, various matrix materials can be used, and examples include pectin, gum arabic, modified starch and maltodextrins. Pectin can be hydrated in water and the oil-based material is directly added to the gum solution, while mixing. The mixture is then homogenized to form an emulsion, which can also be spray dried to yield a powder.

W/O/W and O/W/O multiple emulsions are complex systems, termed <<emulsions of emulsions>>. For example, W/O/W emulsions consist of

small water droplets dispersed in large oil droplets, which in turn, are themselves, dispersed in an aqueous phase. They are normally prepared using a two-step process, using conventional homogenization techniques. A schematic representation of the two-step process in the formation of a double emulsion is described by Garti (1997) Garti and Benichou (2004). Hydrophilic-lipophilic balance (HLB) values of the emulsifiers need to be carefully considered. For example, for the production of the W/O/W, the emulsifiers for the internal interface must be hydrophobic and hydrophilic for the external interfaces. Multiple emulsions have a number of potential advantages over simple emulsions, such as the controlled release, protection of labile ingredients, ability to entrap both hydrophilic (Guimberteau *et al.*, 2001) and lipophilic compounds such as retinol (Yoshida *et al.*, 1999), reduction of fat content, segregation of incompatible ingredients, and encapsulation in a single system of two substances whose efficiency and stability require two different pH values (Tedajo *et al.*, 2001). However, stabilization for food systems remains an issue due to droplet coalescence, or diffusion of the water molecules from the internal aqueous phase to the bulk aqueous phase (Garti and Benichou, 2004). Chen *et al.* (1999) used the W/O/W multiple emulsion to increase the stability of milk immunoglobulin G (IgG) against strong acid and alkali, which is able to prevent the growth of infection agents.

Liposomes are formed when films of phospholipids are dispersed in aqueous media. Multilamellar vesicles, single compartment vesicles and macrovesicles are the three forms of liposomes. The multilamellar vesicles are aggregates which contain several alternating layers of phospholipids bilayers and water. Preparation involves the evaporation of a chloroform solution of phospholipid, cholesterol and other hydrophobic compounds into a film that coats the evaporation flask. The liposomes are then produced when an aqueous core solution is mixed with the lipid film (Reineccus, 1995). Liposomes are mainly used for delivery of oil or water soluble materials; such as omega-3 fatty acids, yeasts, enzymes (Hawker, 1995).

Emulsions are mostly applied to chemical bioactives, but a few processes have involved probiotics. One method involves mixing a probiotic cell suspension with a mixture of sesame oil bodies in sesame oil (Hou *et al.*, 2003).

Combining processes can generate interesting approaches. Picot and Lacroix (2003a and 2003b) have combined emulsification and spray drying to develop a low-cost microencapsulation method adaptable to large-scale production. In this method, a micronized powder containing the probiotic bacteria is added to a hydrophobic phase, which can be anhydrous milk fat. The hydrophobic phase containing the dry material to be encapsulated is added to a hydrophilic phase, in this case a 10% whey protein isolate solution. An oil-in-water emulsion is prepared using a dynamic loop mixer, and the emulsion is then spray dried. The product contains a core of probiotic powder having a first coating layer of milk fat and a second coating layer of whey proteins, generated during spray drying. A similar approach has been proposed by

Crittenden *et al.* (2005) using different homogenization systems and different emulsifiers.

19.3.4 Spray drying

Spray drying is extensively used in the production of microencapsulated ingredients. A liquid suspension of the bioactive product is sprayed at the top of a large vessel, creating a mist of fine droplets, in conjunction with hot air. The droplets dry into solid particles.

Spray drying has had limited use for probiotics in the past because of the high temperatures used during the process which would generate high viability losses. Indeed, the inlet air can typically be at 120–160°C, and is still at 50–80°C at the outlet. However, there is intense activity with this process and success has been achieved with probiotics by using 3 strategies:

- 1 The drying is only partially carried out in the spray dryer. Thus the product is dried to 10–20% solids with the spray dryer, which is enough to obtain a solid particle. This particle is then is dried to completion on a fluid-bed dryer, which uses lower air temperatures for drying.
- 2 Protective compounds are added to the cell suspension prior to spray drying (Ross *et al.*, 2005), or the probiotics are emulsified in an oil phase (see Section 19.3.3).
- 3 The cells are adapted prior to spray drying.

The reader is referred to Chapter 18 where extensive details are given on how probiotics can be spray-dried.

19.3.5 Extrusion

Extrusion was proposed almost 20 years ago as a method of stabilizing lactic cultures (Kim *et al.*, 1988). In this process, dried cultures are mixed with bulking agents (cellulose, glycerol) and small amounts of water to form wet granules. This mixture is processed in an extruder with screens having pores about 1 mm in diameter. The wet particles can subsequently be air dried or spray coated. Refinements of this method have appeared where ingredients designed for enteric protection have been added in the bulking agents blends (Simmons *et al.*, 2004). The disadvantage of this method is the low ratio of probiotic bacteria to protective compounds. Thus, in extrusion, the initial probiotic culture represents less than 10% of the final product, while in spray coating, for example, it can represent 50% of the mass. Products obtained from extrusion have rather low populations, typically in the 10^9 – 10^{10} CFU/g range. This is acceptable for the nutraceutical market, but might be a bit low as a supplement to foods. Indeed, many uncoated freeze-dried probiotics are marketed with 10^{11} CFU/g populations.

19.3.6 Co-crystallization

Co-crystallization is a process whereby active compounds are added to solutions containing high concentrations of carbohydrates. As temperature is lowered and over-saturation occurs, crystallization of the carbohydrates occurs. The bioactives are entrapped in the crystals as they are forming (Chen *et al.*, 1988). This technique has not been successful for probiotics (Champagne *et al.*, 1995).

19.4 Using microencapsulation for the delivery of probiotics and other ingredients in functional dairy products

With respect to probiotics, the most often reported benefit of microencapsulation is enhanced viability during storage (Table 19.2). Encapsulation can promote the enrichment of dairy products with many other bioactive compounds. This section examines the benefits of encapsulation on various dairy products.

19.4.1 Methods other than encapsulation for high viability of probiotics in foods

As mentioned previously, adding probiotics to foods offers many challenges. Encapsulation will provide many opportunities to address these problems. But before these elements are presented, it must be remembered that

Table 19.2 Advantages of microentrapment of probiotics in alginate or carrageenan gel beads for the incorporation of probiotics into dairy products

Advantage	Product	Reference
Increased viability during storage	Ice milk	Kebary <i>et al.</i> 1998
	Frozen yoghurt	Shah and Ravula 2000
	Unfermented milk	Trueslstrup-Hansen <i>et al.</i> 2002
	Yoghurt	Adhikari <i>et al.</i> 2000; Anjani <i>et al.</i> 2004; Hussein and Kebary 1999
	Powders	Kailasapathy and Sureeta 2004; Capela <i>et al.</i> 2006; Lee <i>et al.</i> 2004
Increased survival to freezing	Ice milk	Kebary <i>et al.</i> 1998; Sheu <i>et al.</i> 1993; Sheu and Marshall 1993
Continuous systems of inoculation	Cheese, yoghurt	Doleyres and Lacroix 2005
Growth in presence of oxygen in broth	Starters	Talwalkar and Kailasapathy 2003
Concentrated without centrifugation	Starters	Champagne 2006

Table 19.3 Examples of strategies other than encapsulation which improve viability of probiotics in dairy products (adapted from Champagne *et al.*, 2005)

Dairy product	Strategy
Yoghurt and fermented milks	Only use <i>Streptococcus thermophilus</i> in starter Reduce starter inoculation level Inoculate with ruptured cells of the starter Use <i>Str. thermophilus</i> strains having oxygen scavenging properties Add the probiotics with the starter rather than at the end at packaging Pre-incubation with probiotics only; delay inoculation with starters Packaging under nitrogen atmosphere Pack in containers having high oxygen diffusion barriers Add antioxidants (e.g., ascorbic acid) Add enzymes (lactase, catalase) Add ingredients or flavours which contain growth factors for probiotics Stop fermentation at a higher pH
Cheese	As for yoghurt: lower starter inoculation level, probiotic only starters, growth factors Cottage: grow the probiotic in the cream dressing Cheddar: add probiotics to the milled and salted curds Cheddar: add a probiotic which can grow during cheese ripening Gouda and others: increase processing temperatures to favour probiotics Goat cheese: lower salt content
Frozen yoghurt	Increase pH
Milk	Remove oxygen (vacuum, electroreduction)

encapsulation technologies generally add a step in the process of producing probiotics, which increases costs. Encapsulation is therefore only one of the possible tools when food manufacturers have to improve the viability of probiotics in their products. Examples of other approaches in dairy products are found in Table 19.3. In fermented products, there are many other alternatives to microencapsulation, most adaptations being related to the management of the starters. However, in frozen and dried dairy products, microencapsulation is definitely the most favourable approach.

19.4.2 Manufacture or inoculation of dairy starters and probiotics

Many dairy processors rely on suppliers for both their starter and probiotic cultures. The production of dairy cultures involves steps that are sometimes stressful for lactic cultures: fermentation under agitation, concentration by centrifugation or ultrafiltration, freezing or drying. Producing concentrated cultures in alginate or carrageenan gel beads is an alternative to the traditional process, if viability drops are unacceptably high during processing (Champagne, 2006). In this technology, bacteria are microentrapped in gel particles of 1–

2 mm which are then added to the growth medium. The beads can occupy from 5 to 20% of the medium. Bacterial growth occurs in the gel beads and the population can reach levels well above 10^{10} CFU/g of gel. These concentrated cultures are then recovered without requiring centrifugation or filtration. The approach also enables an alternate method of drying, since lower-costing air drying (fluidized bed or other) can be used instead of freeze drying. However, this immobilized cell technology (ICT) suffers from a number of drawbacks:

- the need for a bead production step in the process
- lower overall biomass yields for most cultures
- solid state of the culture which can affect texture in foods and beverages.

In the past, cultures which did not survive well to the technological stresses of the traditional process were simply discarded from the product line. However, in the future, culture producers will have to examine alternatives such as this ICT, if they must market a very sensitive strain having valuable demonstrated health effects.

Cells encapsulated in alginate or carrageenan gel beads can also be used for continuous inoculation of milk. In this approach, milk is continuously injected into a bioreactor containing the bead-entrapped cultures. Cell release occurs from the surface, and milk exits the bioreactor towards the production vat inoculated with the culture. This ICT takes advantage of cell release from the beads, which is a disadvantage when creating biomass. The ICT inoculation process has the advantages of high cell productivity and rather stable strain ratios after a certain operation time (Doleyres and Lacroix, 2005). Furthermore, immobilized cells may ultimately have different properties as free cells do, such as increased resistance to inhibitory compounds (antibiotics, sanitizers). Understanding the mechanism of stress in the ICT system may lead to the development of cultures with improved capacity to survive and function under industrial and digestive tract conditions (Doleyres and Lacroix, 2005).

19.4.3 Yoghurt

Probiotics

In yoghurt, the most widely studied encapsulated probiotics are alginate or carrageenan-entrapped cultures. Many teams report increased viability of probiotics during storage of yoghurt when microentrapped in gel beads (Table 19.3). However, the results of some of these studies are conflicting (Picot and Lacroix, 2004).

The reason for these discrepancies is probably found in the study of Talwalkar and Kailasapathy (2003) where it was observed that the benefits of microentrapment occur when there is oxygen in the medium. Microentrapment thus appears to provide a microenvironment having reduced oxygen levels, which prevent viability losses to oxygen-sensitive strains

(Talwalkar and Kailasapathy, 2004) rather than protect the cells against the detrimental effects of the acid environment in yoghurt. It can be hypothesized that the discrepancies in the literature would be due to various oxygen sensitivities between the strains (microentrapment being ineffective when the strains are not sensitive to oxygen) or to variable dissolved oxygen levels in the products due to differences in experimental procedures.

A second reason for the discrepancies might be due to improvements in the alginate matrix. Thus, addition of starch in the alginate core or coating the bead with chitosan (Fig. 19.1) appear to more systematically improve the viability of the cultures (Sultana *et al.*, 2000; Krasaekoopt *et al.*, 2006).

Gel beads are not the only form of microencapsulation of probiotics for incorporation into yoghurt. In one application, coated particles 1–3 mm in diameter (presumably obtained by spray coating) are added to a liquid yoghurt (Shin *et al.*, 2002). The particles have a first coating of lipids in order to confer protection in the gastro-intestinal tract, and a second coating to adjust their specific gravity. In this approach, the particles remain in suspension in the liquid product. To obtain similar specific gravities, adjustments are required to the particles' second coating or, more probably, to the liquid yoghurt formulation. Presumably, the culture would have enhanced stability during storage, but no data are given in this respect.

The use of microentrapment is generally limited to the probiotic cells, and the starter cultures are typically inoculated as free cells. In one study, the yoghurt starters were also encapsulated in alginate beads and added to the milk blend to carry out the fermentation. In this situation, acid production rate is significantly slower (Maragkoudakis *et al.*, 2006), and this has been observed as well with cheese starters (Champagne *et al.*, 1992). The presence of the beads also negatively affected the sensory properties of the product, confirming previous data (Adhikari *et al.*, 2000 and 2003).

Nevertheless, at least one commercial product has probiotic-containing capsules in yoghurt (Yoplait-Mexico), but the technology used is not identified. The presence of the particles is clearly recognized in the mouth. Thus, this is an example where encapsulation is used not only for viability benefits but also in product differentiation. Presumably, the effect on sensory properties can become desirable if the consumer is forewarned and expects the presence of the beads.

Omega-3 oils

The n-3 polyunsaturated fatty acids (n-3 PUFA) have been implicated in reducing cancers and the risks of heart disease as well as inflammatory and immune disorders. Owing to the high number of unsaturated double bonds, n-3 PUFA are prone to oxidation and their shelf life is limited to six months when stored at 4°C in closed containers under N₂.

Spray drying of an emulsion containing the omega-3 oil with starch as an encapsulation matrix has been used to prolong its shelf life and also allows their use in large varieties of food systems such as infant formula (Anderson,

1995). Microencapsulated fish oil obtained by spray drying an emulsion was as effective as the daily intake of fish oil gelatine capsules in providing the dietary levels of n-PUFA (Wallace *et al.*, 2000). Additionally the shelf life of the encapsulated omega-3 could be extended by more than two years (Anderson, 1995).

Microencapsulated fish oil (up to 18% w/w) could be used to fortify instant powdered milk based protein-carbohydrate products (Roche, Switzerland). Fermented milk, yoghurt and processed cheese could also be fortified with fish oil in a milk-carbohydrate matrix (patent-pending MicroMax Technology (Sanguani and Augustin, 2001)).

19.4.4 Cheese

Probiotics

In cheesemaking, a fraction of the bacterial cells are lost in the whey. There appears to be a higher retention of the microencapsulated probiotics in the cheese matrix, as evidenced by higher counts of microentrapped cells in the first weeks following production. However, contrary to yoghurt, microencapsulation of probiotics in alginate beads does not seem to improve their stability during storage or ripening (Gobbetti *et al.*, 1998), and may even be detrimental (Kailasapathy and Masondole, 2005; Godward and Kailasapathy, 2003a).

Microentrapment in alginate beads has been shown to protect lactic cultures against bacteriophage attacks (Stenson *et al.*, 1987). Phage infection is not a problem currently in the industrial use of probiotics, even if they are involved during the fermentation. However, if they ever do, either in industrial or intestinal environments, microentrapment could be examined as a potential solution. Cells entrapped in alginate gels are also less active in proteolysis of milk caseins (Stenson *et al.*, 1987). Microentrapment could thus be beneficial in preventing off-flavours due to proteolytic activities of the probiotic cells in dairy products.

Vitamins and minerals

The water-soluble and lipid-soluble vitamins' stability can be improved by encapsulating them into appropriate matrices, since they are relatively unstable to oxygen content, enzymes, oxidizing agents, pH, etc.

Efficient encapsulation of the water-soluble vitamins requires a good physical barrier to prevent them from leaking away into the food products. Spray chilling and spray cooling which involve dispersing these compounds in a molten fat or wax and spraying the dispersion through nozzles at refrigeration temperature are the appropriate procedures to protect the water soluble components in solid foods such as cereals and bread.

However, for the liquid foods systems, encapsulation of vitamin C in liposomes offers the best protection. For example, ascorbic acid incorporated in liposomes demonstrated a half life of 100 d compared to 18 d at 4°C for

a pure solution of ascorbic acid (Kirby *et al.*, 1991). DeMan *et al.* (1986) demonstrated that vitamin D delivered in liposomes yielded higher levels of vitamin D in cheese immediately after manufacturing, compared to vitamin D delivered in an emulsion form or homogenized in the cream. However, the retention of vitamin D in liposomes decreased by 40% after seven months storage compared to approximately 15% for the two others techniques. This work suggests that the efficiency of the various encapsulation techniques also needs to be compared over prolonged storage period to assess their potential.

The lipid soluble vitamins such as vitamins A, D, E and β -carotene are generally easier to encapsulate than the water soluble forms. Spray drying of the emulsion containing such vitamins, fatty acids or flavour oils is the most commonly employed method of protection. The powder can also be used to produce tablets. O₂-permeability of the encapsulation matrix selected (e.g., maltodextrin, starch, gum arabic, pectin) has a major impact on the oxidative stability of these vitamins (Goubet *et al.*, 1998). The diffusion of the oxygen through the capsule barriers depends upon the state of the polymer mixture selected. According to the glass transition (T_g) theory, at low water availability (A_w) and temperature, the polymers are in a rigid glassy state, so the gas diffusion is limited. At higher A_w and temperature, above the glass transition temperature, these polymers exist into a liquid-like rubbery state, with higher molecular mobility, thus allowing gas diffusion.

Bioavailability of iron is negatively altered by interactions with food ingredients (phytates, tannins, polyphenols). Additionally, iron acts as a catalyst of oxidative reactions with fatty acids, and vitamins, which can reduce the product shelf life and the nutritional quality of food systems such as milk. Encapsulation of iron will significantly reduce these interactions and also the off-flavour development. The bioavailability of the readily water-soluble iron (ferrous gluconate and lactate) is superior to the poorly water soluble iron such as ferrous fumarate. The iron solubility will determine the encapsulation procedure to be employed. Use of liposomes is the most popular method to incorporate iron into milk based products. A study conducted on ferrous sulphate encapsulated in lecithin liposomes (Uicich *et al.*, 1999; Bocci *et al.*, 1997) confirmed that the thermal process and six-month storage did not decrease iron availability of the iron fortified milk.

Incorporation of soluble calcium (CaCl₂) into milk decreases the milk's stability to heat treatment and affects the overall product's flavour. The use of microcrystalline cellulose based ingredient co-pressed with calcium carbonate and carboxymethyl cellulose can overcome these problems (Ayling, 1999). By encapsulating the calcium salt in a lecithin liposome, the interactions of calcium with milk proteins at high temperature could be minimized. With this technique, it was also possible to fortify 100 g of soya milk with up to 110 mg Ca, reaching levels equivalent to those of cow's milk (Hirotsuka *et al.*, 1984). For the fortification of foods products with other minerals, such as Mn, Mg, Zn, there is little information available on the problems associated with them and the most appropriate technique of incorporation.

19.4.5 Ice cream and frozen desserts

Numerous studies show that probiotics entrapped in alginate or carrageenan beads have greater viability following freezing in various dairy desserts (Table 19.2). This is particularly evident when cryoprotectants, such as glycerol, are added in the gel particles (Sheu *et al.*, 1993; Sultana *et al.*, 2000). Microencapsulation enables the use of such protective compounds in the beads, without having to add them to the ice milk mix, where they would affect sensory properties. Therefore, the microenvironment which is provided by beads seems to be a critical aspect of the success of the application. This is in line with the data for yoghurt where, for that application, the oxygen microenvironment is involved. Presumably, maintaining this microenvironment with fresh beads requires processing controls. It can be expected that addition of the fresh cryoprotectant-enriched beads must be done immediately prior to freezing, in order to limit the diffusion of the protective ingredient out of the bead. The smaller the bead, the higher the surface to volume ratio, and the greater diffusion would occur. It is not surprising, therefore, that the viability benefits of microentrapment to freezing are greater with large beads (Sheu *et al.*, 1993). Unfortunately, large beads will affect texture (Sheu *et al.*, 1993; Kabary *et al.*, 1998) and a compromise needs to be found; Sheu *et al.* (1993) recommended beads having 30 µm diameter.

Although most of the data involve fresh beads, some studies have examined the use of freeze-dried beads (Shah and Ravula, 2000; Godward and Kailasapathy, 2003b) which, arguably, might be more representative of industrial practices. With freeze-dried cultures, the benefit of microencapsulation in survival to freezing did not occur. This was presumably due to the absence of high levels of cryoprotectants in the alginate gel particles; no beneficial microenvironment was generated in the beads with the procedures used. Data also show that cells in fresh beads survive better than those in freeze-dried products. These results suggest that companies wishing to add freeze-dried probiotics to their frozen desserts would benefit from rehydrating the microentrapped cultures prior to use, and possibly in solutions having cryoprotective compounds.

19.4.6 Unfermented milks

Probiotics

Losses in viability of probiotic bacteria during storage are much lower in unfermented milk than in yoghurt because of the higher pH. Nevertheless, for some strains sensitive to oxygen, significant drops in viability occur (Bolduc *et al.*, 2006). It has been shown that lower oxygen levels are encountered inside gel particles (Talwalkar and Kailasapathy 2004), and this could potentially explain why bifidobacteria microentrapped in alginate gels have proven to be more stable than free cells during incubation at 4°C in 2% fat milk (Truelstrup-Hansen *et al.*, 2002).

A number of commercial unfermented milk products contain probiotics, and to our knowledge the cultures are added after pasteurization. It is always a concern to add ingredients after the heat treatment because of the potential contamination with pathogens. It was therefore examined whether microencapsulation in gel beads would enhance the survival of the cultures to heating. Although some protection was conferred by microentrapment when the cultures were pasteurized at 63°C for 30 min, viability losses were high at 70°C for 1 min (Kushal *et al.*, 2005). At this point, even if milk provides some protection to the cells during heating, it appears that the protection by microentrapment in alginate beads is insufficient to enable a pre-pasteurization inoculation.

Other bioactives

Probiotics are at the forefront of the development of most functional dairy foods, but the exception is unfermented milks. A variety of bioactive compounds have been added to milk: minerals, vitamins, enzymes, oils and sterols.

Ascorbic acid incorporated in liposomes with vitamin E exerts a synergistic antioxidant effect. This combination can be added to ensure the protection of food emulsions (Reineccius, 1995). It is used as an emulsifier system in the emulsion, and adsorbs at the water-oil interface, so the antioxidants can act at the site where the oxidative reactions can occur (Pothakamury and Barbosa-Canovas, 1995).

19.4.7 Powders

Probiotics

Adding probiotics to milk-based powders destined to be heated, such as for sauces, is arguably useless because of the viability losses encountered. However, a certain number of powdered dairy blends are consumed directly (Probio'Stick) or freshly after rehydration (instant skim milk, infant formulas and instant pudding blends). Therefore, some studies have examined the stability of encapsulated probiotics in dried milk.

Results show that freeze-dried cultures microentrapped in gel particles are more stable during incubation at room temperature (Table 19.2). However, spray coating a freeze-dried culture is also effective in this goal (Siuta-Cruce and Goulet, 2001). With respect to the spray-coated cultures, it can be hypothesized that the lipid coating could constitute a barrier to water and oxygen entrance in the particle.

In one study, the microencapsulation was carried out using spray-drying technology (O'Riordan *et al.*, 2001). Microencapsulation was detrimental to the stability of bifidobacteria during storage at 19–24°C. Therefore, not all microencapsulation technologies are successful in enhancing viability of cells during storage of dried products.

19.4.8 The gastrointestinal system

Dairy products are valuable carriers of probiotics (Ouweland *et al.*, 2003). This is partially related to the buffering capacity of milk, and data show that non-encapsulated probiotics survive better passage through the gastrointestinal system if they are consumed in cheese and unfermented milk rather than in a powder (Saxelin *et al.*, 2003). With respect to powders, encapsulation by spray coating of particles dramatically improves the survival of probiotics to the gastric environment. Therefore, the question arises as to the possibility that microencapsulation could not only improve the survival of probiotics in the dairy foods as such, but also in the stomach afterwards.

Data are conflicting with respect to the effect of microencapsulation of probiotics in alginate or carrageenan beads on survival to gastrointestinal (GI) environments. Many studies show a positive effect (Le-Tien *et al.*, 2004; Guérin *et al.*, 2003; Iyer and Kailasapathy, 2005), but other turn out negative (Sultana *et al.*, 2000; Trueslump-Hansen *et al.*, 2002). The reasons for these discrepancies can obviously be linked to differences between strains, but they could also be due to the method of bead production, the coating method (Lee *et al.*, 2004; Siuta-Cruce and Goulet 2001; Krasakoopt *et al.*, 2004), the size of the bead particle (Chandramouli *et al.*, 2003) or the cell loads (Chandramouli *et al.*, 2004). It remains to be seen if the protective effects observed in these assays, where particles are used directly in the simulated GI systems, can also extend to those which are consumed through a dairy product.

19.5 Future trends

Microencapsulation provides the opportunity to create microenvironments. Since some probiotics do not grow well in milk due to low lactase or protease activities, co-encapsulation of the probiotic bacteria with prebiotics (Iyer and Kailasapathy, 2005) could enable enhanced populations in the fermented dairy products.

In cheesemaking, many compounds are lost in the resulting whey. Therefore, microencapsulation has been proposed for the enhanced recovery of enzymes or folic acid in the cheese matrix. As for prebiotics, co-encapsulation could provide the opportunity to add multiple bioactive compounds.

With respect to enzymes and cheese, encapsulation has traditionally been done to enhance the delivery of ripening enzymes. The techniques could be extended to enzymes which would have health-promoting properties. Examples could include bile salt hydrolases, and linoleate isomerases.

Nanotechnologies are not well adapted to probiotics because of the size of whole bacteria, but they could be used to deliver bioactive fractions of the cultures (Iyer and Kailasapathy, 2005). Furthermore, they could be used for the encapsulation of other low-molecular weight bioactives. The advantage is obviously less impact of the microcapsules on texture of the dairy foods.

Industry has typically tried to prevent changes in sensory properties when adding microencapsulated bioactives. But this might change in the future. As mentioned earlier, a yoghurt beverage is currently on the market which advertizes the presence of bifidobacteria in capsules which can easily be observed and tasted. It can be expected that this approach will expand, and that encapsulation will provide a means with which industry could more clearly advertize the presence of the bioactive compounds in their products.

Phytosterols, lutein, glucan, polyphenols and many other ingredients with health benefits can also be added to food systems. However, many of these nutraceutical compounds still do not exist in purified form. Their incorporation in food matrices will require innovative bioencapsulation procedures to ensure their stability over storage. Additional research for new wall materials and microencapsulation techniques are still required for value added ingredients to be incorporated into various food systems.

19.6 Sources of further information and advice

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Assessing the safety of probiotics with regard to antibiotic resistance

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20.1 Introduction

The lactic acid bacteria (LAB) comprise a taxonomically diverse group of Gram-positive, non-spore-forming rods and cocci (Gasser 1994). The conventional definition of the LAB group includes the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Enterococcus* and *Streptococcus*. The genus *Bifidobacterium*, though phylogenetically unrelated to other LAB, is often included in the LAB group because of similarities in its biochemical, physiological and ecological properties (Gasser 1994; Aguirre 1993; Adams 1999). Strains of these genera are frequently used on a large scale as starter cultures in food industries or as probiotics. Therefore, *Bifidobacterium* will also be discussed in this chapter on antimicrobial resistance.

In general, LAB are organisms without imposing a health risk for the consumers or the environment. However, there are several studies that have documented the presence and expression of antibiotic resistance genes in food-associated LAB (Borriello 2003; Danielsen 2003; Donohue 1996; Gevers 2003a, b; Saarela 2000; Salminen 1998). When antibiotic resistance is located on mobile genetic elements such as plasmids or transposons, these traits can be transferred to the human or animal commensal microbiota and to pathogenic bacteria temporarily residing in the hosts. The dissemination of antibiotic-resistant strains or of their resistance genes can also reduce the therapeutic possibilities in infectious diseases. Therefore it is very important to look for the presence of transferable antibiotic resistances among LAB strains that are used as probiotics or starter cultures in products for human or animal nutrition.

In PROSAFE (Biosafety Evaluation of Lactic Acid Bacteria used for

Human Consumption, QLRT-2001-01273, funded by the European Commission's 5th Framework Programme) we contacted all companies involved in the worldwide production and/or distribution of probiotics and asked them to submit strains to the PROSAFE project. Eventually, descriptive and other relevant information on more than 200 strains was collected from 30 depositors via a questionnaire (Vankerckhoven 2004). We found that the large majority of commercial probiotic products contain one or multiple strains of lactic acid bacteria (LAB) primarily belonging to the genera *Lactobacillus*, *Bifidobacterium*, *Propionibacterium* spp., *Enterococcus*, *Lactococcus*, and *Pediococcus*. When only considering the cultures of the probiotic category of which the original species identification was confirmed in PROSAFE, our results indicate that *Lb. paracasei*, *Lb. plantarum*, *Lb. acidophilus* and *Lb. rhamnosus* are amongst the most frequently used species in human and/or animal applications (Huys 2006).

In this chapter I will focus on the LAB most widely used as probiotics, based on the PROSAFE inventory, i.e. the genera *Lactobacillus*, *Bifidobacterium*, *Propionibacterium* spp., *Lactococcus*, and *Pediococcus*. *Enterococcus* will not be discussed because extensive reviews on antimicrobial resistance of this genus have been published.

20.2 The problem of antibiotic resistance

20.2.1 Phenotypic mechanisms of resistance

To date, at least four distinctive phenotypic mechanisms of antibiotic resistance have been described in bacteria. These are:

Impermeability

In the case of Gram-negative bacteria, the first line of resistance against an anti-infective agent is the outer membrane, situated outside the peptidoglycan cell wall. The outer portion of this lipid bilayer is made of lipopolysaccharides, which in turn are composed of tightly bound hydrocarbon molecules that prevent the entry of hydrophobic molecules like penicillin and erythromycin. Thus, Gram-negative bacteria are naturally or intrinsically resistant to certain antibiotics. However, the passage of hydrophilic molecules is facilitated by the presence of porins, proteins that are arranged to form water-filled diffusion channels, through which hydrophilic antibiotics, like imipenem pass easily. Bacteria usually produce a large number of porins (10^5 per each *Escherichia coli* cell), but in pathogenic strains mutations can lead to lack of production of specific porins, which results in resistance to antibiotics belonging to different families.

Alteration of the target

The following targets may be altered:

- **The cell-wall:** With this mechanism, resistance occurs mainly by the expression of genes encoding proteins that reprogram cell-wall biosynthesis and thus evade the action of antibiotics. For instance, glycopeptides like vancomycin and teicoplanin interfere with peptidoglycan growth and assemble mainly by binding to D-ala-D-ala at the termini of peptidoglycan precursors. If bacteria are able to alter this D-ala-D-ala, they will become resistant to glycopeptides if this alteration has no effect on normal peptidoglycan biosynthesis.
- **Ribosomes:** This resistance mechanism focuses on mutating or camouflaging of the drug target rather than on its removal or destruction. For instance, resistance to aminoglycosides is acquired by mutations of a part of the binding site, the S12 protein of the 30S subunit, and is a significant cause of resistance among enterobacterial isolates. In contrast to the direct mutations in the binding sites, camouflaging of the binding sites is demonstrated by bacteria against the macrolides-lincosamides-streptogramin A (MLS) group. In MLS resistance, bacteria mono- or dimethylate a specific adenine residue A2058 in the peptidyl transferase loop of the 23S rRNA of the 50S subunit of the bacterial ribosome causing a 10,000 decrease in antibiotic binding.
- **Enzymes:** Here the enzymes targeted by the antibiotics are mutated or changed to prevent either binding or inactivation of the enzyme. β -lactams inhibit bacterial growth by binding covalently to penicillin binding proteins (PBPs) in the cytoplasmic membrane. Alterations of PBPs can lead to β -lactam resistance. Therefore, in Gram-positive bacteria, β -lactam resistance can be due to decreased affinity of the PBPs for the antibiotic or to a change in the amount of PBP produced by the bacterium. However, some penicillin-resistant strains of *Streptococcus pneumoniae* show several changes in their PBPs, i.e., decreased affinity of some PBPs, loss of others, and the appearance of mutant PBPs not present in susceptible cells. The genes encoding these PBPs are mosaics made of segments from susceptible pneumococci and segments from resistant commensal streptococci.

Active efflux

For antibiotics to be effective, they must reach their specific bacterial targets and accumulate at reasonable and effective concentrations. Furthermore, antibiotics that have to reach the bacterial cytoplasm to block one of the essential steps, have to first pass through the barrier membranes (outer and inner for gram negative and only inner for gram positive) and then accumulate to a high enough concentration to exert its action. Bacteria alter their wild-type, energy-dependent membrane pumps that are originally used to transport lipophilic or amphipathic molecules in and out of the cells. These alterations enable these membrane pumps to 'pump out' the antibiotic from the bacterial cell. As a result, there is a decreased accumulation of the antibiotic inside the cell, and hence a lowered killing power. Some transporters such as tetracycline

efflux proteins are dedicated systems that mediate the extrusion of a given drug or class of drug. In contrast to this, specific drug transporters the so-called multidrug transporters can extrude a wide variety of structurally unrelated compounds.

Enzymatic inhibition

Another resistance strategy focuses on enzymatic inhibition. This is an important mechanism of resistance as a vast majority of treatment failures occur as a result of bacterial production of enzymes that modify or inactivate the drug molecule before it has a chance to exert its effects. Resistance to β -lactams is mainly due to production of enzymes called β -lactamases that inactivate these antibiotics by splitting the amide bond of the β -lactam ring via the serine ester mechanism. β -lactamases can be chromosomal or plasmid-mediated, for instance, staphylococcal β -lactamases are usually plasmid mediated. Chromosomal β -lactamases are almost ubiquitous in enterobacteria, however, their contribution to resistance depends upon the amount produced, mode of production and whether their expression is inducible, or, constitutive. Plasmid-mediated β -lactamases, of which over 100 have been identified in enterobacteria alone act against narrow-spectrum cephalosporins, but have minimal activity against the newer, broad-spectrum cephalosporins. However, the past ten years have seen the emergence of 'Extended-spectrum β -lactamases' or ESBLs, which have been the cause of outbreaks in many hospitals. These are essentially mutants of the plasmid-mediated β -lactamases. They differ from the parent enzymes by one to four amino-acid substitutions, which are enough to modify the active site of the enzyme to bind and destroy the extended-spectrum cephalosporins in addition to the narrow-spectrum cephalosporins and monobactams.

20.2.2 Genotypic mechanisms of resistance

In case of antibiotic resistances, genotypically one has to distinguish between the natural (or intrinsic) type and the acquired type of resistance. Natural antibiotic resistance is non-transferable and present in the wild type population of a given taxonomic group. It is a species- or genus-specific property of all (or nearly all) members of the corresponding taxonomic group. These members possess minimal inhibitory concentrations (MICs) to the corresponding antibiotic which are located in the resistant concentration range (see Section 20.2.3 Breakpoint). Natural or intrinsic resistance can be due to inaccessibility of the target (i.e., impermeability resistance due to the absence of an adequate transporter, e.g. aminoglycoside resistance in strict anaerobes), multidrug efflux systems (e.g., *acrE* in *E. coli*; *mexB* in *Pseudomonas aeruginosa*), or drug inactivation (e.g., *ampC* cephalosporinase in *Klebsiella*).

In contrast, bacterial strains with acquired antibiotic resistances are characterized by MICs that are higher than the normal range of the MIC distribution of the wild type population of a given taxonomic group (see

Section 20.2.3 Breakpoint). Acquired resistance can be due to spontaneous mutations in endogenous genes or acquisition of exogenous genes. Spontaneous mutations can occur in structural or regulatory genes. Examples of mutations in structural genes are: expanded spectrum of enzymatic activity (ESBL), target site modification (e.g., streptomycin resistance due to mutations in rDNA genes, *rpsL*; β -lactam resistance due to change in PBPs, penicillin binding proteins; resistance to FQ), or reduced permeability or uptake. Examples of mutations in regulatory genes are: derepression with increased expression (e.g., multidrug efflux systems; overproduction of ampC enzyme); or trimethoprim resistance due to overproduction of DHF, dihydrofolate, reductase). Exogenous acquired genes usually encode for drug inactivating enzymes (e.g., aminoglycoside-modifying enzymes; β -lactamases; chloramphenicol acetyltransferase), enzymes modifying the targets (e.g., methylation in the 23S component of the 50S ribosomal subunit due to Erm methylases); or efflux systems (e.g., tetracyclines), or metabolic by-pass (e.g., trimethoprim resistance: resistant DHF reductase).

These antibiotic resistance properties can be horizontally transferred by: conjugation (cell-cell contact), transformation (uptake of DNA in solution), or transduction (transfer of DNA in bacteriophages). Acquisition of foreign DNA, can be either on a plasmid (as in the case of *Haemophilus influenzae* β -lactamases), on a conjugative transposon (as in the case of some forms of macrolide and tetracycline resistance in streptococci), or by transformation with DNA encoding novel alleles of existing genes (as in the case of penicillin and cephalosporin resistance in streptococci) (Maiden 1998). This requirement for foreign DNA means that the appearance of a novel resistant strain in an individual colonized by drug-susceptible bacteria (emergence of resistance) occurs very rarely. However, fluoroquinolone resistant mutants can be present in infected persons, and, not surprisingly, emergence of resistance during treatment has been documented.

20.2.3 Breakpoint definition

Categorization, the classification of bacterial strains into susceptible, intermediate and resistant categories with regard to an antibiotic is based on the critical values determined for MICs or for inhibition zone diameters. The antibiotic susceptibility (or resistance) of a strain cannot be measured directly but must be deduced from the *in vitro* activity of an antibiotic. Among the various methods available, MIC determination is the most widely used to assess *in vitro* activity for categorization of strains.

The definition of resistance can be based on bacteriological (i.e., epidemiological) or clinical criteria. EUCAST (European Committee on Antimicrobial Susceptibility Testing) has agreed on the following definitions of antimicrobial susceptibility and resistance in relation to 'clinical breakpoints' and 'epidemiological cut-off values'.

Clinical resistance and clinical breakpoints

Clinically susceptible (S): a micro-organism is defined as susceptible by a level of antimicrobial activity associated with a high likelihood of therapeutic success. A micro-organism is categorized as susceptible (S) by applying the appropriate breakpoint in a defined phenotypic test system; this breakpoint may be altered with legitimate changes in circumstances.

Clinically intermediate (I): a micro-organism is defined as intermediate by a level of antimicrobial activity associated with indeterminate therapeutic effect. A micro-organism is categorized as intermediate (I) by applying the appropriate breakpoints in a defined phenotypic test system. These breakpoints may be altered with legitimate changes in circumstances.

Clinically resistant (R): a micro-organism is defined as resistant by a level of antimicrobial activity associated with a high likelihood of therapeutic failure. A micro-organism is categorized as resistant (R) by applying the appropriate breakpoint in a defined phenotypic test system. This breakpoint may be altered with legitimate changes in circumstances.

The clinical breakpoints are presented as $S \leq x \text{ mg/L}$; $I > x, \leq y \text{ mg/L}$; $R > y \text{ mg/L}$.

Microbiological resistance and epidemiological cut-off values

Wild type (WT): a micro-organism is defined as wild type (WT) for a species by the absence of acquired and mutational resistance mechanisms to the drug in question. A micro-organism is categorized as wild type (WT) for a species by applying the appropriate cut-off value in a defined phenotypic test system. This cut-off value will not be altered by changing circumstances. Wild type micro-organisms may or may not respond clinically to antimicrobial treatment.

Microbiological resistance – non-wild type (NWT): a micro-organism is defined as non-wild type (NWT) for a species by the presence of acquired and mutational resistance mechanisms to the drug in question. A micro-organism is categorized as non-wild type (NWT) for a species by applying the appropriate cut-off value in a defined phenotypic test system. This cut-off value will not be altered by changing circumstances. Non-wild type micro-organisms may or may not respond clinically to antimicrobial treatment. The wild type is presented at $WT \leq z \text{ mg/L}$ and non-wild type as $NWT > z \text{ mg/L}$.

The determination of epidemiological MIC breakpoints seems to be more relevant than the definition of clinical breakpoints to recognize acquired and potentially transferable antibiotic resistances in LAB species. Therefore, in PROSAFE, the MICs of all strains were classified within the corresponding species by ‘epidemiological cut-off values’ into wild type (WT) cut-off values (defined as $\leq x \text{ mg/L}$) and non-wild type (non-WT) cut-off values (in analogy with ‘resistance’ defined as $> x \text{ mg/L}$). It was performed by setting the correct MIC cut-offs according to the MIC distributions of an antibiotic within every bacterial species tested, as recommended by EUCAST (Kahlmeter

2003). However, WT and non-WT micro-organisms may or may not respond clinically to antimicrobial treatment (Kahlmeter 2003).

In the simplest case, there is a bimodal distribution of the MICs for bacterial strains belonging to the same species that facilitates the characterization of two populations: one with low MICs evenly distributed and with a range of three to five MIC values; another with much higher MICs corresponding to the strains possessing an acquired resistance mechanism. Between these two populations there are no or few strains. However, distribution can be multimodal because of the multiplicity of resistance mechanisms, or broad because a bell shape distribution of the wild type population is not present. Hence, determination of cut-off values is always a compromise, and subsequent revision of these values may occur, particularly if only a low number of strains of a given species are available (arbitrarily it is agreed to only set cut-off values if at least ten strains have been tested in three institutions for a given species).

From a pragmatic point of view, epidemiological cut-offs can be harmonized for several species belonging to the same genus, if the cut-offs are within one to two-fold dilutions.

20.3 Methods for antimicrobial susceptibility testing of probiotics

A variety of methods and protocols for the determination of antibiotic susceptibilities of non-enterococcal LAB have been described including agar disk diffusion and agar overlay disk diffusion (Charteris 1998a,b; Felten 1999; Huys 2002; Orberg 1985; Ruoff 1988; Tankovic 1993; Temmerman 2003; Yazid 2000), E-test (Charteris 2001; Croco 1994; Danielsen 2004; Eliopoulos 1994; Felten 1999; Frei 2001; Herra 1995; Katla 2001), broth dilution (Bayer 1978; Dubreuil 1999; Elliott 1996; Green 1990; Lim 1993; Matteuzzi 1983; Nagaraja 1987; Parada 1986; Ruoff 1988; Sidhu 2001; Swenson 1990; Yamane 1991), and agar dilution (Brumfitt 1992; Chow 1988; de la Maza 1989; Goldstein 2003; Herra 1995; King 2001). In general, dilution methods are preferred over diffusion-based tests as the former techniques allow to determine MIC values which provide a more reliable indication of the intrinsic or acquired nature of a given resistance phenotype. However, owing to the fact that many non-enterococcal LAB require special growth conditions in terms of medium acidity and carbohydrate supplementation, conventional media such as Mueller-Hinton (MH) and Iso-Sensitest agar or broth are not uniformly suitable for susceptibility testing of lactobacilli, pediococci, lactococci, and bifidobacteria.

In PROSAFE we have tested several broth media for their ability to support the growth of non-enterococcal LAB strains (Klara 2005). Although genus-, species-, and sometimes also strain-dependent effects could be noticed,

the best overall growth support of the examined *Lactobacillus*, *Pediococcus*, and *Lactococcus* strains were obtained with mixed formulation of the antibiotic susceptibility test medium recommended by the British Society for Antimicrobial Chemotherapy, the Iso-Sensitest (IST) broth (90%), with MRS (Man, Rogosa, Sharpe) broth (10%). This broth mixture, referred to as the LAB susceptibility test medium (LSM), was found to be the most optimal medium yielding sufficient to strong growth for all *Lactobacillus*, *Pediococcus*, and *Lactococcus* strains tested. We observed only very minimal differences in growth if lactobacilli, pediococci or lactococci were incubated in LSM broth aerobically or in a 5% CO₂ atmosphere for which reason we recommend to incubate susceptibility tests for these LAB genera aerobically. In further investigations, growth of *Bifidobacterium* species in the mixture of 90% IST broth + 10% MRS broth (supplemented with 0.3 g/L of the reducing agent L-cysteine hydrochloride) after 48h incubation at 37°C under anaerobic conditions was found to be sufficient to test antimicrobial susceptibility of these organisms. Thus, we recommend to perform MIC determinations of bifidobacteria in LSM + 0.3 g/L L-cysteine under anaerobic conditions.

In conclusion, using the two variants of LSM in broth microdilution methods described in PROSAFE, it is possible to determine the antibiotic susceptibilities of lactobacilli, pediococci, lactococci, and bifidobacteria. The methods are easily to perform, and relatively cheap. Additionally, both standardized variants of the microdilution test using the new LSM broth with or without L-cysteine, respectively, eliminate previously reported problems of poorly grown non-enterococcal LAB or unknown antagonistic effects between some tested antimicrobial agents and growth medium components.

20.4 Antibiotic resistance of non-enterococcal LAB

20.4.1 *Lactobacillus*

Lactobacilli possess a wide range of naturally antibiotic resistances (Charteris 1998b; Danielsen 2003). For instance *L. plantarum*, *L. rhamnosus*, *L. fermentum*, *L. casei* or *L. paracasei* are naturally resistant to vancomycin and teicoplanin. However, species of the *L. acidophilus* taxonomic group (*L. acidophilus*, *L. gasseri*, *L. crispatus*) or *L. delbrueckii* are susceptible to the glycopeptides (Danielsen 2003; Felten 1999; Illot-Klein 1994; Nicas 1989; Swenson 1990). Charteris *et al.* (1998b) found that *L. acidophilus* was resistant to vancomycin, which could reflect methodological problems (antibiotic susceptibility testing was done by agar disc diffusion, which is not recommended for glycopeptides).

Several studies showed good activities against penicillins, ampicillin, carbapenems, (imipenem) aminoglycosides (gentamicin, tobramycin, streptomycin), phenicols (chloramphenicol, thiamphenicol), tetracyclines (doxycycline, minocycline), macrolides (erythromycin, roxythromycin, josamycin, clarithromycin), ketolides (telithromycin) lincosamides

(clindamycin), and streptogramins (quinupristin/dalfopristin) (Bayer 1978; Charteris 2001; Chow 1988; De la Maza 1989; Mändar 2001; Swenson 1990; Zarazaga 1999).

Among the fluoroquinolones, excellent activity has been reported for trovafloxacin and moxifloxacin, but ciprofloxacin is less active (Herra 1995; Luh 2000; Mändar 2001). Conflicting results were published for sulfamethoxazole, trimethoprim, and their combination co-trimoxazole. Katla *et al.* (2001) reported that lactobacilli are resistant against sulphadiazine, and trimethoprim. Huovinen *et al.* (1987) mentioned the existence of a trimethoprim-insensitive dihydrofolate reductase in lactobacilli. Danielsen *et al.* (2004) tested lactobacilli for trimethoprim susceptibility on folic acid casei medium and showed that these bacteria do not have intrinsic resistance to this antibiotic. Differences could be related to the used susceptibility testing method and the interaction with components present in the medium.

Rare acquired antibiotic resistances for tetracyclines, macrolides, chloramphenicol, and fluoroquinolones have been described (Felten 1999; Mändar 2001; Vescovo 1982; Zarazaga 1999).

Finally, transfer of antibiotic resistance genes between phylogenetically distant bacteria (by mobile elements that carry the resistance genes) has been reported (Courvalin 1994). Gevers *et al.* (2003b) demonstrated the transferability of the tetracycline resistance gene *tet*(M) from lactobacilli (isolated from fermented dry sausages) to *E. faecalis* (transfer frequencies 10^{-4} to 10^{-6} transconjugants per recipient) and also to *Lc. lactis* subsp. *Lactis*.

20.4.2 *Bifidobacterium*

Bifidobacteria are susceptible to penicillin, macrolides (erythromycin), lincosamides (lincomycin), phenicols (chloramphenicol), bacitracin, and the glycopeptides (vancomycin) (Chow 1988; Edlund 1997; Lim 1993; Miller 1967). Bifidobacteria possess intrinsic resistances to aminoglycosides, nalidixic acid, and polymyxins (Charteris 1998a; Lim 1993; Matteuzzi 1983; Miller 1967). Charteris *et al.* (1998a) found that bifidobacteria were resistant or moderately susceptible to sulfamethoxazole and co-trimoxazole, but this could be related to the presence and interaction with certain components in the nutrient medium. Occasionally resistance is reported to vancomycin, but this is most probably due to incorrect identification (Matteuzzi 1983; Miller 1967; Temmerman 2003). Acquired antibiotic resistance has been reported for macrolides, lincosamides (lincomycin) and tetracycline.

20.4.3 *Pediococcus*

Pediococci are susceptible to penicillins (penicillin G, amoxicillin, ampicillin, piperacillin), carbapenems (imipenem), macrolides (erythromycin), lincosamides (lincomycin and clindamycin), phenicols (chloramphenicol), linezolid, daptomycin, and ramoplanin (Biavasco 1997; Illot-Klein 1994; de

La Maza 1989; Riebel 1990; Swenson 1990; Tankovic 1993; Yamane 1991). *Pediococci* are highly susceptible to the aminoglycosides gentamicin and netilmicin, but they appear to be less sensitive or even resistant to tobramycin, amikacin, and streptomycin. *Pediococci* possess natural high-level resistances to the glycopeptides, vancomycin and teicoplanin (with MICs of $>1024 \mu\text{g/ml}$), tetracycline and fluoroquinolones (ciprofloxacin) (Biavasco 1997; de La Maza 1989; Riebel 1990; Tankovic 1993; Yamane 1991). However, *pediococi* were reported susceptible to trovafloxacin and moxifloxacin (Luh 2000). Although strains appear to be also resistant to co-trimoxazole, this could also be a question of use of the correct test medium. Similarly, Luh *et al.* (2000) reported susceptibility to vancomycin and teicoplanin, but probably some of the strains were not correctly identified because of the known natural resistance of *pediococci* for vancomycin and teicoplanin.

Acquired antibiotic resistance has been reported for erythromycin, lincomycin, and clindamycin, chloramphenicol (Illot-Klein *et al.* 1994; Riebel 1990; Tankovic 1993; Temmerman 2003). Tankovic *et al.* (1993) described MLS resistance carried on a non-conjugative plasmid; the resistance determinant was homologous to *ermAM*, which is widespread in streptococci and enterococci (Leclercq 1991).

20.4.4 *Lactococcus*

Lactococci are susceptible to penicillins (penicillin, ampicillin, amoxicillin/clavulanic acid), cephalosporins (cephalothin, cefuroxime), bacitracin, macrolides (erythromycin), streptogramins (quinupristin/dalfopristin), phenicols (chloramphenicol), aminoglycosides (gentamicin), glycopeptides (vancomycin), and co-trimoxazole (Elliott 1996; Temmerman 2003). Susceptibility to tetracycline, streptomycin, norfloxacin, and ciprofloxacin is variable and may depend on the species. With regards to clindamycin, a clear difference in the MICs of clindamycin for *Lc. lactis* ($\leq 0.12 \mu\text{g/ml}$) vs. *Lc. garvieae* ($\geq$ eight $\mu\text{g/ml}$) can be observed, which could be used to differentiate both species.

Katla *et al.* (2001) found all tested strains resistant to sulphadiazine and trimethoprim; this could, however, be due to the inhibition of these drugs by certain compounds present in the MRS agar used by these investigators.

20.5 Safety testing of probiotics with regard to antimicrobial susceptibility testing

For assessing the safety of probiotics with regard to the presence of acquired antimicrobial resistance properties, strains should be investigated by means of standardized antimicrobial susceptibility testing methods. However, no standards have been proposed for non-enterococcal LAB by the Clinical Laboratory Standard Institute (CLSI), EUCAST or other committees. A variety

of methods and protocols for the determination of antibiotic susceptibilities of non-enterococcal LAB have been reported in the literature, including agar disc diffusion and agar overlay disc diffusion, E-test, broth dilution and agar dilution. However, conventional media such as Mueller-Hinton and Iso-Sensitest agar or broth are not uniformly suitable for susceptibility testing of lactobacilli, pediococci, lactococci, and bifidobacteria. Moreover, interference of media has been reported for these organisms and specific antimicrobials. The E-test allows to more exactly determine antibiotic susceptibilities since it results in quantitative data (MICs) of the strains to the corresponding antibiotics.

The LSM microbroth dilution method as described in the PROSAFE project is a 'tentative reference method' for antibiotic susceptibility testing of probiotic non-enterococcal LAB (Klara 2005). Any other method than the PROSAFE 'tentative reference method' should be validated against the reference method. For trimethoprim, sulfamethoxazole and trimethoprim/sulfamethoxazole, other methods need to be employed.

The determination of epidemiological MIC breakpoints seems to be more relevant than the definition of clinical breakpoints to recognize acquired and potentially transferable antibiotic resistances in non-enterococcal LAB species. Epidemiological cut-offs should be defined at the species level (not genus level). However, there are no published breakpoints for non-enterococcal LAB, and several authors recommend to take the MIC breakpoints of streptococci other than *S. pneumoniae* according to the CLSI documents. The PROSAFE project proposes tentative epidemiological cut-off values.

The minimum set of antibiotics to be tested are: penicillin G, ampicillin, ampicillin/sulbactam, gentamicin, streptomycin, vancomycin, teicoplanin, erythromycin, clindamycin, quinupristin/dalfopristin, oxytetracycline, chloramphenicol, trimethoprim, and sulfamethoxazole/trimethoprim.

Natural (intrinsic or inherent) antibiotic resistance is (probably) non-transferable and present in the wild type population of a given taxonomic group. It is a species- or genus-specific property of all (or nearly all) members of the corresponding taxonomic group. In contrast, bacterial strains with acquired antibiotic resistances are characterized by MICs that are higher than the normal range of the MIC distribution of the wild type population of a given taxonomic group. The notion that an ingested micro-organism could cause an infection in need of antibiotic treatment, speaks in favour of being able to say that only strains without resistance should be used. Acquired resistances result from mutations or accumulation of mutations in the 'own' DNA that finally leads to resistance against the corresponding antibiotic, or from acquisition of transferable resistance genes from donors. Antibiotic resistances due to the acquisition of exogenous DNA can potentially be transferred by plasmids or (conjugative) transposons to other bacterial species or genera. Acquired antibiotic resistance due to mutations of housekeeping chromosomal genes most probably represent a low risk of horizontal dissemination.

The dissemination of antibiotic-resistant genes can reduce the therapeutic possibilities in infectious diseases. Therefore it is very important to look for presence of transferable antibiotic resistances in probiotics that are or could be used for human consumption, but also as starter cultures in products for human or animal nutrition. However, when epidemiological cut-off values are used very few strains will be classified as resistant ('acquired or mutational'). Transfer of acquired resistance genes may occur under proper experimental conditions, and failure to demonstrate *in-vitro* horizontal gene transfer does not exclude the risk of dissemination of genes. Moreover, there are no standardized methods to demonstrate transfer of resistance genes, and there are substantial inter- and intralaboratory variations.

20.6 Future trends

It can be expected that more investigators will report on antimicrobial susceptibility data based on the PROSAFE 'tentative reference method', and that they will validate their or another (commercial) method against the PROSAFE method. These studies will enable revision and amending the method developed by PROSAFE so that eventually a reliable, validated and reproducible antimicrobial susceptibility method is available for the international community. Such studies will also result in more information on MIC distributions of non-enterococcal LAB, which in turn will allow adoption of final epidemiological cut-offs. With internationally agreed cut-offs, the nature of the acquired resistance (intrinsic or acquired) can be characterized. Provided that agreement is reached on methods to study transferability of resistance genes, moveable elements in micro-organisms added to the food chain will be characterized. Eventually, this will allow the risk assessment and establish risk assessment criteria for LAB used as probiotics or starter cultures with acquired antibiotic resistance.

Correct taxonomic identification is essential because intrinsic resistance is inherent of the genus or species. Therefore, all companies, independent of their expertise in bacterial identification, should contact at least one independent taxonomic expert lab for species identification of probiotic or nutritional cultures.

The scientific community should be able to verify the antimicrobial susceptibility profile of a probiotic strain. Therefore, commercial probiotic strains should be deposited in public collections, under conditions of restricted distribution (e.g., these strains should only be made available to researchers through an agreement strictly outlining the terms of use).

In PROSAFE, a large database was constructed which contains data on identification, genotyping, antimicrobial susceptibility, virulence, etc., of nearly all probiotic strains commercially available. An anonymous version could be made available to independent organizations such as the European Food Safety Authority (EFSA, <http://www.efsa.europa.eu>).

20.7 Sources of further information and advice

Guidelines for the Evaluation of Probiotics in Food. Report of a joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food. London Ontario, Canada, 30 April and 1 May, 2002.

Opinion of the Scientific Panel on Additives and Products or Substances used in Animal Feed on the updating of the criteria used in the assessment of bacteria for resistance to antibiotics or human or veterinary importance. *EFSA Journal* (2005), 223, 1–12.

20.8 References

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Development of dairy based functional foods enriched in conjugated linoleic acid with special reference to rumenic acid

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21.1 Introduction

Conjugated linoleic acid (CLA) is the term used to describe the positional and geometric isomers of linoleic acid (LA) with either one or both of the double bonds in the *cis* (*c*) or *trans* (*t*) conformation and separated by simple carbon-carbon linkage. CLA isomers are naturally found in dairy and meat products of ruminant origin such as cows, sheep and goats and in recent years have attracted considerable interest because of the many health-promoting activities observed using both animal and cell culture models of human diseases. It was first purported that CLA might be a potential anti-carcinogen by Pariza *et al.* (1979) when their investigations into mutagenic components of grilled hamburgers provided evidence of a mutagenesis modulator, which was later identified as CLA and shown to be an effective inhibitor of 7, 12-dimethylbenz[a]anthracene induced epidermal carcinogenesis in mice (Ha *et al.*, 1987). In addition to being anti-carcinogenic, CLA manifests activities that confer a reduced risk of atherogenesis, adipogenesis, diabetogenesis, inflammation, bone density loss and immune dysfunction (for reviews see Bhattacharya *et al.* (2006), Pariza (2004) Roche *et al.* (2001), Wahle *et al.* (2004)). Such activities have been primarily attributed to the two major CLA isomers, rumenic acid (*c*9, *t*11 (C18:2)) and the *t*10, *c*12 CLA isomer, and stem from the ability of these isomers to positively influence transcriptional and/or translational control of cytokines, immunoglobulins, lipids including eicosanoids, as well as cell signalling machinery components.

Given the range of health-promoting activities attributed to rumenic acid, ensuring an adequate supply of the isomer would appear desirable. However, in light of the negative perception of milkfat by consumers and prevalence of low-fat nutrition, the concentration of these fatty acids in the diet has fallen in recent years (Lawson *et al.*, 2001) with estimated intakes of 212 and 151 mg d⁻¹ for men and women, respectively, in North America (Ritzenthaler *et al.*, 2001) and 440 and 360 mg d⁻¹ for men and women, respectively, in Germany (Sieber *et al.*, 2004). These levels are lower than the dietary CLA intake of 3.0 g d⁻¹ which Ip *et al.* (1994) estimated to be required, and as such has spurred interest in the development of CLA and in particular rumenic acid enriched foods, especially those of dairy origin. Dietary intake of CLA can be increased through the use of rumenic acid enriched dairy products, derived from the milk of ruminants fed a diet designed to elevate milkfat rumenic acid. For example, a meal containing a serving of high rumenic acid whole milk (460 mg CLA), a sandwich with high rumenic acid butter (365 mg CLA) and high rumenic acid cheddar cheese (721 mg CLA) (Donovan *et al.*, 2000) could be used to provide 1.546 g of CLA, more than half the dose recommended by Ip *et al.* (1994) and supplied in a manner which would not require large adjustments to human dietary habits. Beyond the potential health benefits of CLA and rumenic acid for the consumer, the potential for the dairy industry itself should not be underestimated. Maynard and Franklin (2003) conducted a study into the marketing potential of CLA enriched products in the functional food industry. The willingness-to-pay responses of an experimental group for a range of dairy products naturally enriched in rumenic acid were assessed, and on average, respondents were willing to spend \$0.41 more per gallon for rumenic acid enriched-milk, \$0.38 more per pound of rumenic acid enriched-butter, and \$0.15 more per eight ounce cup of rumenic acid enriched-yoghurt. These data indicate the potential of rumenic acid and CLA isomers in general to add value to dairy products even in the absence of aggressive promotion. The potential of rumenic acid has seen much recent attention directed towards identifying strategies for the enrichment of milk with CLA and the development of CLA enriched dairy products. Strategies for naturally enhancing CLA in milk have included manipulation of the diet of lactating ruminants to favour increased rumenic acid production in the milk, and research into the potential offered by CLA producing starter cultures.

21.2 Health benefits of CLA

The majority of the *in vitro* and *in vivo* studies which have reported health-promoting activities of CLA have employed a mixture of CLA isomers, containing approximately equal parts of rumenic acid and the *t*10, *c*12 CLA isomer (for reviews see Pariza (2004) Roche *et al.* (2001), Wahle *et al.* (2004)). However, the CLA content of milk and dairy products is typically

comprised of ~85–90% rumenic acid (Lock and Bauman, 2004). Some studies do however focus on rumenic acid primarily, and in this regard rumenic acid has been associated with improving cardiovascular health, anti-carcinogenic properties and improved immune function. Studies in hamsters showed that dietary rumenic acid improved the ratio of non-high density lipoprotein (HDL) cholesterol to HDL cholesterol (Valeille *et al.*, 2005), improved HDL cholesterol and the ratio of HDL cholesterol to LDL cholesterol (Valeille *et al.*, 2004) and significantly reduced plasma triglyceride concentrations (Wilson *et al.*, 2006). The anti-atherogenic effects of the ingestion of high CLA milkfat (2.59% rumenic acid, which also contained a low concentration of saturated fat (67%)) versus a low CLA milkfat (0.39% rumenic acid, which contained a high concentration of saturated fat (72%)) were compared in hamsters (Valeille *et al.*, 2006). It was found that hamsters on the high CLA milkfat diet had 25% less aortic cholesteryl-ester deposition, accompanied by a lower LDL cholesterol concentration and ratio of LDL cholesterol to HDL cholesterol, a lower local inflammatory status, and a lower aortic gene expression of vascular cell adhesion molecule-1. In addition, the further supplementation of hamsters on the high CLA milkfat with 9g kg⁻¹ rumenic acid further amplified these anti-atherogenic effects (Valeille *et al.*, 2006). It has been purposed that many of the anti-atherogenic properties attributed to rumenic acid may stem from its influence on peroxisome proliferator-activated receptor α (PPAR α) and sterol regulatory element-binding protein 1c via their influence on stearyl-CoA desaturase, cyclooxygenase (COX), and fatty acid synthase expression and activity (for review see Bhattacharya *et al.* (2006)).

Much of the data surrounding the anti-carcinogenic activity of rumenic acid have been derived from a number of *in vitro* studies which have assessed the impact of the isomer on a diverse range of cancers including prostate cancer (Ochoa *et al.*, 2004; Palombo *et al.*, 2002; Song *et al.*, 2006), colon cancer (Lampen *et al.*, 2005; Miller *et al.*, 2002; Palombo *et al.*, 2002), breast cancer (Chujo *et al.*, 2003; Hubbard *et al.*, 2003; Kim *et al.*, 2005), gastric cancer (Liu *et al.*, 2004; Liu *et al.*, 2002a, 2002b) and leukaemia (Agatha *et al.*, 2004). It has been proposed that the mechanisms for this anti-proliferative activity include the increased uptake of phospholipid hydroperoxides in cancer cells which increase the cells susceptibility to lipid peroxidation; reduced accumulation of arachidonic acid in the cell, resulting in the reduced production of eicosanoids which stimulate the growth of cancer cells; and through PPAR regulated expression of key genes associated with the growth and spread of cancer such as the COX and lipoxygenase enzymes. O'Shea *et al.* (2000) reported the inhibitory effect of milkfat containing an elevated concentration of rumenic acid on the growth of MCF-7 mammary cancer cells, reporting up to a 90% reduction in cancer cell numbers and a 15-fold increase in lipid peroxidation following eight days exposure to CLA rich milkfat.

It has been reported that rumenic acid exhibits anti-inflammatory properties

in inflammatory airway disease (Jaudszus *et al.*, 2005). This anti-inflammatory activity is mediated through reduced production of the inflammatory cytokine interleukin-8 via the nuclear receptor PPAR γ which has been previously shown to regulate inflammatory response (Yu *et al.*, 2002).

Also of interest to this review are the increasing number of reports regarding the anti-carcinogenic activity of the microbially produced *t9, t11* CLA isomer (Beppu *et al.*, 2006; Coakley *et al.*, 2006). Beppu *et al.* (2006) reported the inhibitory effects of this isomer against the Caco-2, HT-29 and DLD-1 colon cancer lines, indicating that exposure of Caco-2 cells to the *t9, t11* isomer resulted in apoptosis due to the uptake of the isomer into the cell which resulted in increased lipid peroxidation. Coakley *et al.* (2006) also reported the anti-proliferative activity of the *t9, t11* isomer on SW480 and HT-29 colon cancer cell with a 55% and 94% reduction in the growth of the SW480 cell line following four days incubation in the presence of 10 $\mu\text{g ml}^{-1}$ and 20 $\mu\text{g ml}^{-1}$ of *t9, t11* CLA, respectively.

21.3 Mechanisms of CLA production in lactating ruminants and starter bacteria

21.3.1 Ruminant CLA production

The presence of CLA in the milk of lactating ruminants is a direct result of the action of the ruminal microbiota on dietary linoleic and linolenic acids. These fatty acids are primarily delivered in the form of glycolipids, phospholipids, and triglycerides from forages and seed oils (Bauman *et al.*, 1999), which are released by indigenous and endogenously produced lipases following ingestion (Bauman *et al.*, 1999; Dawson *et al.*, 1977; Dawson and Kemp, 1970; Keeney, 1970) and subsequently undergo microbial biohydrogenation in the rumen by various ruminant bacteria of which *Butyrivibrio fibrisolvens* is the foremost (Fujimoto *et al.*, 1993; Harfoot and Hazlewood, 1988; Kepler *et al.*, 1966). The biohydrogenation process, which results in the conversion of linoleic and linolenic acids to stearic acid, consists of several steps (see Fig. 21.1). Rumenic acid is formed as the first intermediary in the biohydrogenation of linoleic acid via the activity of the microbial enzyme, linoleic acid isomerase, (Kepler *et al.*, 1966) which catalyses the isomerisation of the *cis* 12 double bond of linoleic acid (Bauman *et al.*, 1999). A portion of the ruminally produced rumenic acid is absorbed in the intestine while the majority is further reduced to vaccenic acid and ultimately to stearic acid. As the hydrogenation of vaccenic acid to stearic acid occurs at a slower rate than the previous step, an accumulation of vaccenic acid in the rumen occurs. Much of this accumulated vaccenic acid is absorbed in the intestine passing into the circulatory system (Harfoot *et al.*, 1973; Kellens *et al.*, 1986; Singh and Hawke, 1979; Tanaka and Shigeno, 1976) and transported to the mammary gland, where it may be converted to rumenic acid via the

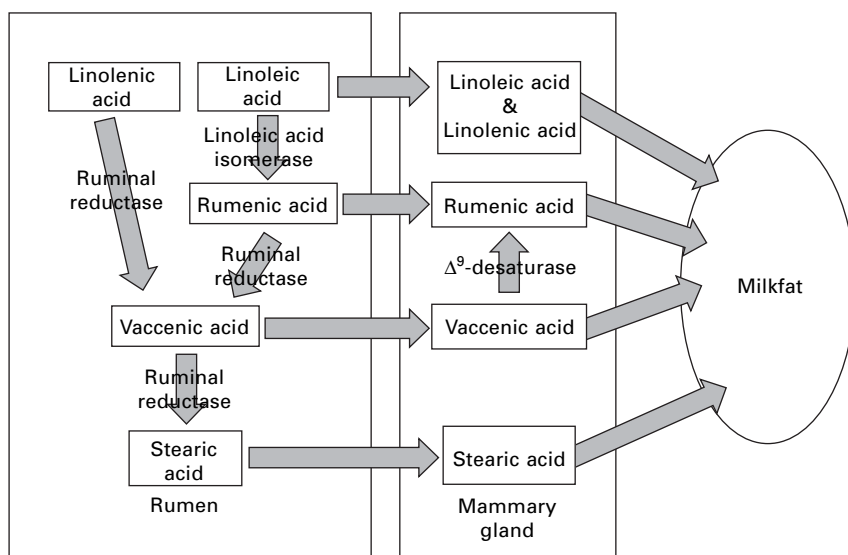


Fig. 21.1 Formation of rumenic acid by ruminants.

action of the enzyme Δ^9 -desaturase (see Fig. 21.1) (Corl *et al.*, 2001; Griinari *et al.*, 2000; Mahfouz *et al.*, 1980; Pollard *et al.*, 1980). This endogenous synthesis of rumenic acid is estimated to account for as much as 75–90% of the total rumenic acid in ruminant milkfat (Griinari *et al.*, 2000; Kay *et al.*, 2004; Lock and Garnsworthy, 2002; Piperova *et al.*, 2002). In addition to rumenic acid, milkfat typically contains a number of other CLA isomers including the *t7*, *c9* CLA which may account for up to 3–16% of total milkfat CLA (Yurawecz *et al.*, 1998), and to a lesser extent the *t10*, *c12* CLA isomer which also has reported biogenic activities. This ruminal and endogenous production of the various CLA isomers gives rise to total milkfat CLA concentrations of 0.2–3.7% in bovine milk (Sebedio *et al.*, 1997), 0.58–1.05% in goats milk and 0.7–2.97% in ovine milk (Parodi, 2002). Variation occurs with animals, lactation number, region, season, stage of lactation, breed and, in particular, diet. It is therefore through manipulation of these factors that marked increases in milkfat CLA can be achieved (see below).

21.3.2 Starter bacteria

Gnotobiotic rats are reported to contain less CLA in their tissue than normal rats when fed free linoleic acid (Chin *et al.*, 1994), leading to speculation that some non-ruminant bacteria may convert linoleic acid to CLA. Since then a number of non-ruminant cultures capable of microbial CLA synthesis have been identified, including strains of *Lactobacillus*, *Propionibacterium*, *Pediococcus*, *Enterococcus*, *Streptococcus*, *Bifidobacterium*, and *Lactococcus*

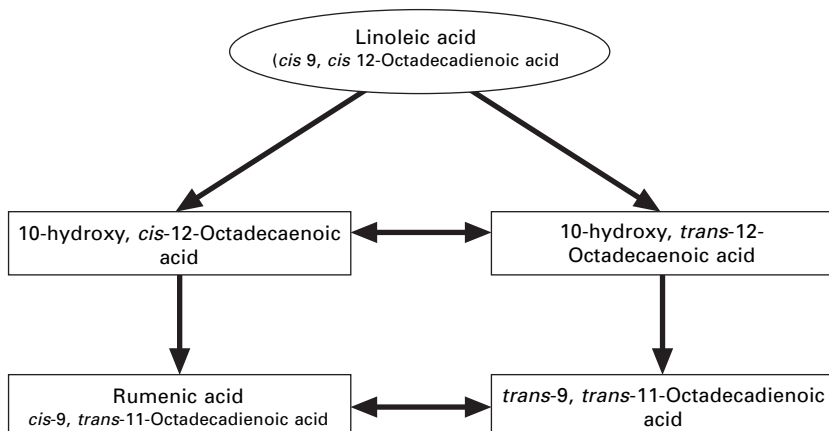


Fig. 21.2 Formation of rumenic acid and *t*9, *t*11 CLA by *Lactobacillus acidophilus* AKU 1137.

(see below). These bacteria convert free linoleic acid to rumenic acid in a similar manner to ruminant bacteria via the action of the enzyme linoleic acid isomerase which results in the conjugation of the *cis* 12 double bond and the formation of rumenic acid (Lin *et al.*, 2002). This rumenic acid is not reduced to vaccenic acid or absorbed, and as such the *cis* 9 double bond may undergo further isomerisation giving rise to the production of the *t*9, *t*11 CLA isomer (Coakley *et al.*, 2003; Kishino *et al.*, 2002) which itself has reported health promoting properties (Beppu *et al.*, 2006; Coakley *et al.*, 2006).

The mechanism of microbial production of rumenic acid and the *t*9, *t*11 CLA isomer was characterised using washed cells of the strain *Lb. acidophilus* AKU 1137 (Ogawa *et al.*, 2001), and involves the production of hydroxy fatty acids as precursors to formation of both CLA isomers. When isolated and introduced to the washed cells these hydroxy fatty acids were rapidly transformed to their respective CLA isomers. Thus, CLA formation by *Lb. acidophilus* was found to consist of two distinct steps, *step one*: the hydration of linoleic acid to 10-hydroxy-18:1 and *step two*: the subsequent dehydration and isomerisation of these hydroxy fatty acids to rumenic acid and the *t*9, *t*11 CLA isomer (see Fig. 21.2). The discovery that strains of bacteria commonly used as dairy starters or probiotics can produce rumenic acid has opened a number of avenues for their use. These include the potential for the enrichment of dairy products with rumenic acid as a result of microbial fermentation, or the possibility of incorporation of such strains into dairy products in numbers where they could colonise the gastrointestinal tract and produce rumenic acid from dietary linoleic acid *in situ*.

21.4 Enrichment of milk with CLA through animal feeding and management strategies

Booth *et al.* (1935) first reported the presence of CLA in milkfat and with the subsequent discovery of its health promoting activity, identification of strategies for the enrichment of milk with this fatty acid have received substantial attention. The rumenic acid content of milkfat is directly affected by a number of factors including species, breed, lactation number, stage of lactation, season, location, farm management strategies and most importantly animal dietary regime (see below). Dietary manipulation has been recognised for decades as being the most successful strategy for elevation of the CLA content in milkfat (Booth *et al.*, 1935), particularly through supplementation of the ruminant diet with fish oils, animal fats, plant oils, ionophores, synthetic CLA supplements and forage (see below).

21.4.1 Plant oils and seeds

A number of different plant oils and seeds derived from a range of different sources such as cottonseed, rapeseed, soybean, corn, sunflower, peanut, safflower, canola and linseed have been fed to ruminants in attempts to elevate CLA production (Tables 21.1 and 21.2). These oils are rich in linoleic acid (cottonseed, soybean, sunflower, safflower, corn) and linolenic acid (linseed), key precursors in the formation of CLA and in particular rumenic acid. The mechanisms by which these oils effect an increase in milkfat CLA have been elucidated and are attributed not only to the increased supply of substrate in the form of linoleic and linolenic acids, which increase the ruminal production of rumenic acid and vaccenic acid, but also as a direct result of inhibition of the reductase enzymes in the rumen responsible for the conversion of vaccenic to stearic acid. This results in the increased accumulation and absorption of vaccenic acid by the animal which can be endogenously

Table 21.1 The principal fatty acids found in some of the most common plant oils

Plant oil seed	14:0	16:0	16:1 (g 100 g ⁻¹)	18:0	18:1	18:2	18:3
Cottonseed	0.8	25.3		2.8	17.1	53.2	0.1
Rapeseed		4.3	0.3	1.7	59.1	22.8	8.2
Soybean		10.7	0.3	3.9	22.8	50.8	6.8
Sunflower	0.2	5.5		3.6	21.7	68.5	0.1
Peanut	0.1	11.5		3.0	53.0	26.0	
Safflower		8.0		3.0	13.5	75.0	0.5
Olive		13.0	1.0	2.5	74.0	9.0	
Canola		4.8		1.9	58.5	23.0	7.7
Linseed		6.4		3.1	20.1	18.2	51.4
Corn				17.0	24.0	59.0	

References: Chouinard *et al.* (2001), Palmquist (1988), Radcliffe *et al.* (2004)

Table 21.2 Effect of animal feeding strategies on milkfat CLA concentrations

	% dDM	CLA content of control diet (% of milkfat)	CLA content of trial diet (% of milkfat)	Ref*
Plant seed oils				
Soybean oil	5.3	N.S.	2.44	1
Soybean oil	2	0.35	1.03	3
Soybean oil	0.5–4	0.5	0.75–2.08	2
Soybean oil	3	0.3	0.7	4
Soybean oil	3.6	0.39	2.1	2
Linseed oil	5.3	N.S.	1.67	1
Linseed oil	1	0.5	0.73	2
Linseed oil	2.2–4.4	0.39	1.58–1.63	2
Linseed oil & vitamin-E	6	0.68	2.8	6
Safflower oil	6	0.45	3.36	6
Safflower oil	2.5	N.S.	7 g d ⁻¹	5
Peanut oil	5.3	N.S.	1.33	1
Cottonseed oil	2	0.35	0.6	3
Corn oil	2	0.35	0.7	3
Canola oil	3	0.3	0.5	4
Canola oil	3.3	0.5	1.1	8
Sunflower oil	2.5	N.S.	5.2 g d ⁻¹	5
PR infusion with safflower oil	150 g d ⁻¹	0.59	0.58	7
Processed plant seed oils				
Raw cracked soybean	18	0.39	0.37	2
Raw cracked roasted soybean	18	0.39	0.77	2
Raw ground soybean	17.5	N.S.	0.31	9
Roasted soybean	17.5	N.S.	0.66	9
Microionized soybean	17.5	N.S.	0.7	9
Extruded soybean	17.5	N.S.	0.89	9
Raw linseed	12.6	0.9	1.4	10
Extruded linseed	12.6	0.9	1.9	10
Cotton seed hull	14	N.S.	0.94	11
Whole cottonseed	14	N.S.	0.97	11
Small cottonseed pellets	14	N.S.	1.47	11
High full fat rapeseed diet	825 kg d ⁻¹	3.94	7.89	12
Low full fat rapeseed diet	1200 kg d ⁻¹	3.94	5.23	12
Protected plant seed oils				
Calcium salt of canola oil	4	0.35	1.32	9
Calcium salt of soybean oil	4	0.35	2.25	9
Calcium salt of linseed oil	4	0.35	1.95	9
Canolamide	3.3	0.5	0.7	8
Marine oils				
Fish oil	1.6	0.16	1.55	13
Fish oil	1	0.71	1.71	14
Fish oil	2	0.71	2.53	14
Fish oil	3	0.71	2.12	14
Fish oil	160 g d ⁻¹	2.25	3.23	15
Fish oil	320 g d ⁻¹	2.25	3.64	15
Soymeal replaced by fish meal	0%	0.53	0.53	16
Soymeal replaced by fish meal	25%	0.53	0.63	16

Table 21.2 Continued

	% dDM	CLA content of control diet (% of milkfat)	CLA content of trial diet (% of milkfat)	Ref*
Soymeal replaced by fish meal	50%	0.53	0.66	16
Soymeal replaced by fish meal	100%	0.53	1	16
Palm and fish oil	2.7	0.61	1.27	17
Marine algae protected	4	0.37	2.31	18
Marine algae unprotected	4	0.37	2.62	18
Animal fats				
Tallow (Jersey)	1.1	N.S.	0.7–1.18	19
Tallow (Holstein)	1.1	N.S.	0.83–0.84	19
Tallow	3	N.S.	1.1	20
PR infusion with tallow	150 g d ⁻¹	0.59	0.61	7
Fresh and conserved forage				
Grass maize silage Day 2	ad libitum	2.43	1.03	21
Grass maize silage Day 6	ad libitum	2.43	0.48	21
Grass maize silage Day 14	ad libitum	2.43	0.44	21
Grass silage early heading	ad libitum	none	1.14	22
Grass silage flowering	ad libitum	none	0.48	22
Grass silage second cutting	ad libitum	none	0.81	22
Pasture	ad libitum	0.44	2.43	21
Pasture	16 Kg d ⁻¹	none	0.39	12
Pasture	20 Kg d ⁻¹	none	0.55–0.57	12
Pasture	24 Kg d ⁻¹	none	0.59–0.68	12
Pasture	50	none	1.57	23
Pasture	65	none	1.61	23
Pasture	80	none	1.9	23
Pasture	ad libitum	0.4	1.1	28
Splega ryegrass	ad libitum	none	1.54	24
Portstewart ryegrass	ad libitum	none	1.71	24
Napoleon ryegrass	ad libitum	none	1.35	24
Millenium ryegrass	ad libitum	none	1.72	24
Annual ryegrass	ad libitum	none	1.2–1.43	25
Sulla	ad libitum	none	1.12–1.25	25
Burr medic	ad libitum	none	1.65–2.3	25
Daisy forb	ad libitum	none	2.33–2.35	25
Red clover	ad libitum	0.4	1.3	28
Miscellaneous strategies				
Monensin	24 ppm	0.48	0.56	6
Monensin	380 mg d ⁻¹	0.8	1.3	26
6% Safflower oil & vitamin-E	150 IU kg ⁻¹	4.16	3.54	6
Mixed ration & vitamin-E	10000 IU d ⁻¹	0.71	0.72	27

***References:** Kelly *et al.* (1998a) =1, Dhiman *et al.* (2000) =2, Zheng *et al.* (2005) =3, Loor and Herbain (2003a) =4, Loor and Herbain (2003b) =5, Bell *et al.* (2006) =6, Bell and Kennelly (2003) =7, Loor *et al.* (2002) =8, Chouinard *et al.* (2001) =9, Gonthier *et al.* (2005) =10, Reveneau *et al.* (2005) =11, Stanton *et al.* (1997) =12, Offer *et al.* (1999) =13, Donovan *et al.* (2000) =14, Rego *et al.* (2005) =15, Abu-Ghazaleh *et al.* (2001) =16, Allred *et al.* (2006) =17, Franklin *et al.* (1999) =18, Morales *et al.* (2000) =19, Jones *et al.* (2000) =20, Elgersma *et al.* (2004) =21, Chouinard *et al.* (1998) =22, Ward *et al.* (2003) =23, Loyola *et al.* (2002) =24, Addis *et al.* (2005) =25, Sauer *et al.* (1998) =26, Kay *et al.* (2005a) =27 and Benchaar *et al.* (2002) =28

N.S. = not stated.

converted to rumenic acid in the mammary gland via the Δ^9 -desaturase enzyme (Griinari and Bauman, 1999). A large number of studies have addressed the effect of dietary plant oils on milkfat CLA and are of general consensus that the supplementation of a basal diet of concentrates and conserved forage with plant-derived oils can substantially increase total milkfat CLA concentrations through elevated rumenic acid production (see Table 21.2). Large variations between the effects of the different oils on the extent of the increase in milkfat CLA have been reported. The differences between oils rich in linoleic and linolenic acids were assessed, by comparing the effect of supplementation of the basal diet with a linoleic acid rich (1% (dDM) soybean oil) or linolenic acid rich (1% (dDM) linseed oil) oil for five weeks. Dietary supplementation increased the CLA content of the milk from 0.50% in the control to 1.45% with soybean oil, and 0.73% with linseed oil (Dhiman *et al.*, 2000). Similarly, Loor and Herbain, (2003a) demonstrated that supplementation of the bovine diet with a high linoleic acid plant oil supplement, i.e. soybean oil (3% (dDM)) led to a greater increase in the milkfat rumenic acid concentrations (0.7%) compared to an oil containing a high oleic acid content (0.5%). Based on these observations it was concluded that plant oils rich in linoleic acid are most effective in increasing milkfat rumenic acid (Dhiman *et al.*, 2000; Loor and Herbein, 2003a).

Several methods for the inclusion of plant oils in the ruminant diet have been used, including the use of free oils, protected oils, whole oilseeds, or processed oilseeds (crushed, extruded, ground, roasted, or microionised). The method by which these plant oils are delivered in the diet can substantially influence the extent of their impact on total milkfat CLA (Chouinard *et al.*, 1997a, 1997b, 2001; Dhiman *et al.*, 1999b, 2000; Loor *et al.*, 2002; Stanton *et al.*, 1997). The variability in effect stems from differences in the availability of the fatty acids from the plant oil for microbial biohydrogenation in the rumen, which is critical to the production of CLA and its endogenous precursor vaccenic acid. A comparison between the effect of diets supplemented with raw linseed or extruded linseed revealed that feeding processed linseed (by means of extrusion) resulted in higher concentrations of milkfat CLA compared to raw linseed (1.90% and 1.40%, respectively) (Gonthier *et al.*, 2005). When the supplementation of the ruminant diet with 14% (dDM) cottonseed hull (control), 14% (dDM) whole cottonseeds or 14% (dDM) small cottonseed pellets was compared, it was found that the processed cottonseed pellets resulted in the greatest increase in milkfat CLA (Reveneau *et al.*, 2005). Clearly, processing results in the release of the oils held within the seeds allowing the rumen microbiota greater access to the fatty acids and hence increased production of rumenic acid and vaccenic acid in milk. Physical processes such as grinding and crushing break the seed, releasing free oils and hence increasing the surface area of the seed exposed to the microbial population. Processes that involve heating can result in partial hydrolysis of bound fatty acid making them more available to the ruminal microbiota. To assess the effect of different processing strategies, Chouinard *et al.* (2001)

fed cows 17.5% (dDM) full fat soybeans treated by grinding, extrusion, microionisation and roasting. Supplementation of these processed oils led to milkfat CLA concentrations of 0.31%, 0.89%, 0.70%, and 0.66%, with the extruded soybeans found to be the most effective in enhancing milkfat CLA. In another study, cows were fed 18% raw cracked soybeans, 18% roasted cracked soybeans or 3.60% soybean oil (dDM) to assess the effect of processing, resulting in total milkfat CLA contents of 0.37%, 0.77% and 2.1%, respectively, compared to the control (0.39%) (Dhiman *et al.*, 2000).

A number of studies have also assessed the effect of dietary supplementation with ruminally protected plant oils, either by formation of calcium salts, fatty acyl amides, a formaldehyde-protein protection matrix, or lipid encapsulation (Chouinard *et al.*, 2001; Loor *et al.*, 2002). These methods of fatty acid protection not only serve to reduce the negative effects that processing and gastric transit can have on the fatty acid composition of the oil, but they also protect the oils from ruminal biohydrogenation and as such their impact on the production of vaccenic acid and CLA in the rumen would be anticipated to be minor. Investigations into the effect that feeding cows calcium salts of canola oil, soybean oil and linseed oil (4% (dDM) for four weeks) had on CLA content of milkfat have also been performed. Milkfat CLA concentrations of 1.32%, 2.25%, and 1.95% were reported for the respective fatty acids compared to 0.35% in the control (Chouinard *et al.*, 2001). Based on these results it is evident that calcium salts of plant oils offer little protection from ruminal biohydrogenation. A similar experiment compared the effect of canolamide, a formaldehyde protected form of canola oil, with unprotected canola oil (3.3% (dDM) for three weeks). As expected, the free oil resulted in a substantial increase in the concentration of milkfat rumenic acid (1.1%) compared to the control (0.5%), however, the formaldehyde protected oil proved less effective (0.7%) (Loor *et al.*, 2002). From these studies it is evident that the manner in which oils are ruminally protected has a large bearing on the success of the oil at increasing the CLA concentration of the milkfat; and in general ruminally protecting oils has a negative effect on CLA production compared to free oils.

21.4.2 Marine oils

Dietary supplementation of lactating ruminants with fish oils and oils derived from marine sources have been shown to result in an elevation of milkfat CLA (Allred *et al.*, 2006; Franklin *et al.*, 1999; Jones *et al.*, 1998; Offer *et al.*, 1999; Rego *et al.*, 2005). These oils are rich in long chain PUFA, such as eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), which inhibit the reductase enzymes that catalyse the conversion of vaccenic acid to stearic acid in the rumen, and results in changes in the microbial population of the rumen (Chow *et al.*, 2004; Griinari and Bauman, 1999; Loor *et al.*, 2005). This leads to an accumulation of vaccenic acid in the rumen, and its subsequent absorption and bioconversion to rumenic acid in the mammary

gland, a process which leads to increased total milkfat CLA and vaccenic acid concentrations. This increase in milkfat CLA through supplementation with fish oils has been reported in a number of studies including Donovan *et al.* (2000) and Rego *et al.* (2005) (see Table 21.2). Fish oil is not the only marine derived oil which has been assessed for its ability to increase milkfat CLA. Cows fed diets supplemented with 4% (dDM) marine algae in both protected and unprotected form have also been shown to produce milk containing elevated concentrations of CLA. In this study milkfat rumenic acid concentrations were increased from 0.37% in the control to 2.31% and 2.62% for the respective diets (Franklin *et al.*, 1999). Thus, it would appear that supplementation with fish oils and marine algae can be an effective strategy for increasing the rumenic acid content of milkfat. The use of processed oils in the form of protected fish oils and fish meal has also been assessed. Allred *et al.* (2006) reported that feeding cows a diet containing 2.7% (dDM) calcium salts of palm and fish oil for six weeks produced a two-fold increase in milkfat CLA concentrations. Abu-Ghazaleh *et al.* (2001) assessed the effect of replacing soy meal in the bovine diet with 25, 50 and 100% fish meal for three weeks reporting milkfat rumenic acid concentrations of 0.44, 0.66 and 0.72%, respectively, compared to 100% soy meal which resulted in a milkfat CLA concentration of 0.39%.

It has been reported that the fatty acid DHA, found in fish oil may play a substantial role in the elevation of CLA in milkfat via increases in the ruminal output of vaccenic acid. A mixed ruminal culture (*in vitro*) supplemented with DHA (5 mg) for 24 hrs produced 1.3-fold more vaccenic acid than a culture supplemented with soybean oil (30 mg) and 2.5-fold more vaccenic acid than the control (AbuGhazaleh and Jenkins, 2004). As the fatty acid composition of fish oils and marine algae vary with species, season, diet, and location, with DHA and EPA concentrations ranging from 2–25% and 4–32%, respectively, it is likely that fish and marine oils derived from different sources differ substantially in their effect on ruminal biohydrogenation and the resulting milkfat CLA and vaccenic acid concentrations.

21.4.3 Animal fats

A number of studies have assessed the effect of supplementation of the ruminant diet with animal fats such as tallow and grease. These animal-derived fats are generally rich in saturated fatty acids and usually contain a low concentration of PUFA. Despite their composition, these sources of animal fat have been shown to elevate milkfat CLA concentrations. One such study supplemented the diet of copper-deficient Holstein and Jersey cows with 1.1% (dDM) tallow, which led to milkfat CLA concentrations of 0.7–1.18% (Morales *et al.*, 2000). In another study, Jones *et al.* (2000) supplemented the bovine diet with 3% (dDM) tallow leading to milkfat CLA concentrations of 1.1%, while Pantoja *et al.* (1996) found that supplementation with 5% (dDM) tallow elevated vaccenic acid production, a key rumenic

acid precursor, from 0.29% to 1.53% of the total milkfat. Onetti *et al.* (2001) on the other hand reported that supplementation of the bovine diet with 0, 2 and 4% tallow (dDM) did not substantially increase milkfat CLA. Feeding animal fats to ruminants is not permitted in the European Union.

21.4.4 Forage

A two- to three-fold increase in the conjugated diene content of cows milk was observed, when cows were turned out to pasture (Riel, 1963). A number of subsequent studies have confirmed this, reporting a dramatic and abrupt increase in the CLA content of milk over the first five days following the transfer of cows from indoor winter feeding to fresh pasture (Dhiman *et al.*, 1999a; Kelly *et al.*, 1998b; Precht and Molkentin, 1997; Stanton *et al.*, 1997; Timmen and Patton, 1988). Furthermore, Elgersma *et al.* (2004) reported that this increase in milkfat rumenic acid is rapidly reversed on a return to indoor feeding. In this study, the rumenic acid content of milkfat fell from 2.30% on day zero when cows were at pasture, to 0.95%, 0.43%, and 0.37%, respectively, after 2, 6 and 14 days on mixed grass-maize silage diet. In temperate countries, the dry matter of fresh grass is composed of about 1–3% fatty acids of which 48–65% is linolenic acid (Bauchart *et al.*, 1984; Chilliard *et al.*, 2001). Studies have suggested that it is the increased supply of substrate along with the improved growth of the ruminal microbiota (due to higher concentrations of fermentable sugar and soluble fibre) which are responsible for the increased rumenic acid production in animals at pasture (Dhiman *et al.*, 2005; Griinari and Bauman, 1999; Stanton *et al.*, 2003).

Comparing the milkfat CLA content of typical US milk (where animals are fed indoors on a diet of fresh and conserved forage, along with concentrates year round) and temperate countries such as Ireland, Australia and New Zealand (where animals receive indoor feeding of conserved forage and concentrate, and fresh pasture on a seasonal basis) highlights that the average milkfat CLA content of US milk is approximately 0.55% while milkfat produced in a temperate climate such as Ireland typically contains approximately 1.6% CLA during access to fresh pasture (Stanton *et al.*, 2003). The factors which effect the impact that fresh pasture has on the CLA content of milkfat have been elucidated and include pasture allowance, forage maturity, forage type, season and supplementation of pasture. In relation to pasture allowance, we found that cows on a high (24 Kg d⁻¹) or medium (20 Kg d⁻¹) pasture allowance had higher milkfat CLA than those on the low pasture allowance (16 Kg d⁻¹) (Stanton *et al.*, 1997). Others have reported that increasing pasture allowance resulted in a linear increase in the total CLA content of the milk in particular the rumenic acid content (Couvreur *et al.*, 2006; Ward *et al.*, 2003). It has been demonstrated that animals fed grass silage which had been cut at three stages of growth, early heading, flowering and second cutting, showed substantial differences in their impact on milkfat CLA content which were recorded at 1.14, 0.48 and 0.81%, respectively (Chouinard *et al.*,

1998). The higher CLA in milk produced by cows grazing early forage is most likely a result of the higher linolenic acid content of the young grass. Investigations into the effect of maturity on the fatty acid composition of a range of traditional and novel forages over three week periods found that in almost all instances the concentration of the key CLA precursors, linoleic and linolenic acids decreased with stage of growth (Clapham *et al.*, 2005) substantiating the findings of Chouinard *et al.* (1998). However, in a similar study Griinari *et al.* (1998) found forage maturity did not substantially affect milkfat CLA or rumenic acid.

The composition of forage supplied to animals may be quite variable and include a range of plant types. The fatty acid composition of these plants may differ substantially and as such their effect on the concentration of CLA in milkfat would be expected to differ. Loyola *et al.* (2002) and Addis *et al.* (2005) both assessed the effect of feeding different plants types or cultivars on milkfat CLA concentrations, investigating differences between the ryegrass cultivars Splega, Portstewart, Napoleon, and Millennium; and between annual ryegrass, sulla, burr medic and daisy forb, respectively. The results highlight substantial differences in the impact that different plant types and cultivars have on milkfat CLA (see Table 21.2).

The effect of season on milkfat rumenic acid and CLA is directly related to the PUFA content of the forage (Bauchart *et al.*, 1984). The total CLA content in milk is found to peak in early spring and autumn and fall in summer in parallel with the linolenic acid concentration of the dietary forage (Chouinard *et al.*, 1998; Mackle *et al.*, 1999; Nudda *et al.*, 2005; Precht and Molkentin, 2000; Thorsdottir *et al.*, 2004). A number of studies have also assessed the effect of supplementation of a forage diet in part with concentrates and grain, a practice which mostly sees the depression of milkfat CLA as a result of the reduced intake of linolenic and linoleic acids (Bargo *et al.*, 2006; Dhiman *et al.*, 1999a; Ward *et al.*, 2003). However, in a study by Chouinard *et al.* (1998), a low forage high concentrate diet was found to increase the concentrations of milkfat CLA in comparison to a high forage low concentrate diet.

Hay and silage (grass and maize) make up a considerable portion of the ruminant feeding strategy and as such, play an important role in milkfat rumenic acid concentrations particularly during indoor feeding. Preserving forage as hay results in a substantial reduction in the concentration of fatty acids and in particular linolenic acid. This effect is seen to a lesser extent with high quality silage but may occur if forage is wilted before ensiling, or under undesirable fermentation conditions (Doreau and Poncet, 2000; Lough and Anderson, 1973). Data on the effects of dietary hay and grass silage on the concentrations of milkfat rumenic acid are scant. However, as a result of the lower concentration of linolenic acid in these feeds, the effect on milkfat CLA would be expected to be less profound than animals receiving fresh pasture. Interestingly, Ward *et al.* (2003) showed that feeding cows fresh forage or hay supplemented with an equivalent concentration of tallow resulted

in milkfat CLA contents of 1.07% and 0.93%, respectively. Assuming that the impact of the tallow was the same in both diets it would appear that fresh forage and hay differed only slightly in terms of their effects on milkfat CLA. Chilliard *et al.* (2001) reported that cows fed a diet consisting of over 60% maize silage had a milkfat CLA content of between 0.4 and 0.6%, considerably less than would be expected from fresh forage.

21.4.5 Miscellaneous feeding strategies

A number of studies have assessed the effect of the addition of ionophores to the ruminant diet. These compounds inhibit the growth of Gram-positive bacteria and as a result directly impact on ruminal biohydrogenation. Using *in vitro* studies the effect of the ionophores nigericin, monensin and tetronasin on the production of CLA by a mixed ruminal population was investigated. The addition of these ionophores resulted in a two-fold increase in the production of rumenic acid through inhibition of the complete biohydrogenation of linoleic acid (Fellner *et al.*, 1997). Furthermore, in a subsequent study it was shown that the use of monensin and soybean oil in combination in continuous cultures of mixed ruminal microorganisms resulted in increased production of $\tau 10$ C_{18:1} a CLA precursor to a greater extent than either additive alone but only when supplemented with barley grains (Jenkins *et al.*, 2003). The effect of supplementation of the ruminant diet with these compounds has also been investigated. On supplementation of the bovine diet with 380 mg monensin it was found that the concentration of milkfat CLA increased from 0.8% in the control group to 1.3% in the monensin supplemented group (Sauer *et al.*, 1998). However, this effect has proved less substantial in other studies (Chouinard *et al.*, 1998; Dhiman *et al.*, 1999a). Bell *et al.* (2006) evaluated the effect of safflower oil in combination with monensin on the concentration of rumenic acid in bovine milk. Cows fed a diet supplemented with 24 ppm of monensin, 6% (dDM) safflower oil, or 6% (dDM) safflower oil and 24 ppm of monensin for 15 days yielded milkfat rumenic acid concentrations of 0.52%, 3.36%, and 5.15% compared to a control value of 0.45%. These data demonstrate that while monensin alone only initiates a small increase in milkfat rumenic acid, its use in combination with plant oil such as safflower was extremely effective in increasing milkfat rumenic acid. The effect of feeding ruminally protected synthetic CLA or post ruminal infusion with synthetically produced CLA on milkfat CLA concentrations has been investigated. Administration of CLA in this manner causes a relatively minor increase in milkfat CLA but also dramatically reduces milk yield and fat (Bell and Kennelly, 2003; Bernal-Santos *et al.*, 2003; Chouinard *et al.*, 1999; Giesy *et al.*, 2002; Mackle *et al.*, 2003; Perfield *et al.*, 2004).

Bell *et al.* (2006) compared the effects of the dietary consumption of 6% (dDM) safflower oil supplemented with vitamin-E (150 IU kg⁻¹ of dDM), or 6% (dDM) safflower oil alone, on the concentration of milkfat CLA. Following

eight weeks treatment it was found that animals fed safflower oil alone produced a higher milkfat CLA (4.16%) than animals fed safflower oil supplemented with vitamin-E (3.54%). In addition, vitamin-E supplementation was also found to reduce milkfat vaccenic acid concentrations. This suggests that vitamin-E may reduce milkfat CLA by negatively affecting ruminal biohydrogenation. In an attempt to determine if vitamin-E was the component of fresh pasture responsible for elevated milkfat CLA concentrations compared to conserved forage or grains Kay *et al.* (2005a) fed cows either fresh pasture, total mixed ration or total mixed ration with vitamin-E supplementation (10,000 IU d⁻¹) for three weeks. These diets yielded milkfat CLA concentrations of 1.84%, 0.71% and 0.72%, respectively, suggesting that vitamin-E does not play a substantial role in the elevation of milkfat CLA which is seen with fresh forage.

21.4.6 Combination diets

A number of studies have assessed the effect of diets containing combinations of fish oils, plant oils, animal fats and forage on the rumenic acid and total CLA content of ruminant milkfat. Following three weeks supplementation with tallow or choice white grease (CWG) at 2% and 4% (dDM) in combination with a corn silage based diet, milkfat CLA concentrations from cows fed the animal fat supplemented diet were lower than the control suggesting the use of conserved forage alone is superior to that supplemented with animal fats (Onetti *et al.*, 2001). Variable effects on milkfat rumenic acid concentrations were reported when plant and fish oils were used in combination. In a large number of these studies, it was found that fish oils and fish meals were superior to combinations of fish and plant oils at increasing milkfat rumenic acid and total CLA (Abu-Ghazaleh *et al.*, 2001; Ramaswamy *et al.*, 2001; Whitlock *et al.*, 2002). Abu-Ghazaleh *et al.* (2001) investigated the effect of replacing soy meal with three diets containing increasing amounts of fish meal on an isonitrogenous basis (100% soy meal, 50% soy meal and 50% fish meal or 100% fish meal), yielding milkfat CLA contents of 0.53%, 0.66% and 1.0%, respectively. Similarly, feeding cows a diet supplemented with 2% fish oil, 2% extruded soybeans, or a combination of 1% fish oil and 1% extruded soybeans yielded milkfat CLA of 2.07%, 1.18% and 1.86%, (Whitlock *et al.*, 2002) and of 2.3%, 1.24% and 2.17% (Ramaswamy *et al.*, 2001), respectively. More recent studies have produced contrasting results, with both Abu-Ghazaleh *et al.* (2002b, 2003) and Allred *et al.* (2006) showing consistently that combinations of fish oil and plant oils were more effective at increasing total milkfat CLA and rumenic acid than either alone.

Supplementation of the bovine diet with 0.5% fish oil in the form of fish meal, 2.5% soybean oil in the form of extruded soybeans, and a combination of both, for a period of four weeks resulted in milkfat CLA concentrations of 0.56%, 0.91%, and 1.59%, respectively (Abu-Ghazaleh *et al.*, 2002b). In a more recent study, the effect of consumption of 2.7% (dDM) calcium salts of

palm and fish oil, a combination diet of 2.7% (dDM) calcium salts of palm and fish oil and 5% (dDM) full fat extruded soybeans, or 2.7% (dDM) calcium salts of palm and fish oil and 0.75% soybean oil, on milkfat CLA concentrations was investigated over six weeks, where milkfat CLA concentrations of 1.27%, 1.44%, and 1.82%, respectively, were obtained (Allred *et al.*, 2006). Abu-Ghazaleh *et al.* (2003) assessed the effect of fish oil in combination with different plant oils on total milkfat CLA concentrations, by feeding a diet containing 1% (dDM) fish oil supplemented with 2.0% (dDM) high oleic acid sunflower seeds, 2% (dDM) high linoleic acid sunflower seeds, or 2% (dDM) linseed (high linolenic acid), (all of which were cracked with rollers) to investigate which plant oil had the greatest synergistic effect on milkfat CLA when used in combination with fish oil over a four week trial period. Total milkfat CLA concentrations of 1.21%, 1.94%, and 1.21%, respectively were achieved, indicating combinations of high linoleic acid oils with fish oil yield the greatest increases in total milkfat CLA.

21.4.7 Management strategies, lactation number, breed, and stage of lactation

The effect of ruminant feeding strategies such as indoor feeding of concentrates and conserved forage year round versus seasonal pasture feeding, restricted versus unrestricted dietary intake, and the effect of supplementation of the ruminant diet with lipid supplements on the total CLA and rumenic acid concentration in ruminant milk have been investigated. In addition, studies have addressed a number of other factors which could potentially have a bearing on the total CLA and rumenic acid content of milkfat such as altitude, farm management strategy, animal breed, lactation number and stage of lactation. Differences in milkfat CLA concentrations in the milk of cows grazing at different altitudes have been observed. In one such study milkfat CLA concentrations of 0.85%, 1.58%, and 2.34% were reported from the milk of cows grazing in the lowlands (600–650 m above sea level), mountains (900–1210 m above sea level), and highlands (1275–2120 m above sea level) of Switzerland, respectively (Collomb *et al.*, 2002). These differences in total milkfat CLA were attributed to variations in the plant species between these regions, although differences in the fatty acid metabolism of the animals at the different locations cannot be ruled out (Collomb *et al.*, 2001).

In a study on the impact of farm management on the rumenic acid content of ruminant milk, Jahreis *et al.* (1997) compared three different farm management strategies: indoor feeding of conserved forage which resulted in a low concentrations of rumenic acid (0.34%); indoors and pasture feeding on a seasonal basis which resulted in a milkfat rumenic acid content (0.61%); and ecological farming practice which resulted in the highest milkfat rumenic acid content (0.8%). This was attributed to differences in the forage type and fatty acid composition of the different strategies (herd size and elevation differed between the management strategies). Ellis *et al.* (2006) collected

bulk-tank milk derived each month from 17 organic and 19 conventional dairy farms in the UK over a 12-month period, to assess differences in the respective CLA (rumenic acid) content of the milk. The study showed that organically produced milk contained 12% more CLA than milk from conventionally managed farms, which was attributed to differences in animal management and nutrition between the two systems.

The effect of breed on total milkfat CLA has been investigated in a number of studies and included investigations into the CLA content of milk derived from Holstein-Friesian, Brown Swiss, Jersey, Normande, Montbeliarde, Ayrshire and Guernsey cows (Capps *et al.*, 1999; Dhiman *et al.*, 2002; Kelsey *et al.*, 2003; Lawless *et al.*, 1999; Medrano *et al.*, 1999; Morales *et al.*, 2000; Ramaswamy *et al.*, 2001; White *et al.*, 2001; Whitlock *et al.*, 2002). The data indicates that at pasture Montbeliardes produce the highest concentrations of milkfat CLA (1.85%), followed by Normandes (1.64%), Holstein-Friesians (0.72–1.66%), Brown Swiss (1.22%), Jersey (0.59–0.77%), Ayrshire (0.57%), and Guernsey (0.36%) (Data from Table 21.2, Dhiman *et al.*, 2005). The difference between the milkfat concentrations of CLA produced by different breeds of cows has been attributed to differences in the activity of the mammary Δ^9 -desaturase (Medrano *et al.*, 1999; Stanton *et al.*, 2003). The effect of lactation number on total milkfat CLA and rumenic acid has been reported (Lal and Narayanan, 1984; Stanton *et al.*, 1997). We compared the fatty acid composition of milkfat from cows with a lactation number of five with those with a lactation number of between two and four. Following eight weeks of grazing supplemented with grass nuts, milkfat CLA concentrations of 0.59% and 0.41%, respectively, were obtained (Stanton *et al.*, 1997), substantiating previous data indicating that milk of cows of higher lactation number yielded a higher milkfat CLA concentration than low lactation number cows (Lal and Narayanan, 1984). The factors responsible for the higher milkfat CLA levels in higher lactation number cows has not been fully elucidated, but may be associated with changes in the microbial population of the rumen and fatty acid metabolism of the animal (Dhiman *et al.*, 2005). In a recent study, milk fatty acid composition was recorded over sixteen weeks postpartum in both a low merit and high merit bovine genetic line (in terms of milk yield). It was found that milkfat CLA content increased from a low of 0.31% on week one, to 0.46% on week eight to a high of 0.54% on week sixteen. During this period, the activity of the enzyme Δ^9 -desaturase remained relatively constant with the increase in milkfat CLA attributed to an increased supply of vaccenic acid (Kay *et al.*, 2005b). In another study by Lock *et al.* (2005a), changes in milkfat CLA concentrations and Δ^9 -desaturase activity from winter to summer were investigated. It was reported that the rumenic acid content of the milk varied from 0.1% to 3.2% over the sampling period, concluding that under normal conditions, stage of lactation had no bearing on milkfat CLA concentrations and that again desaturase activity remained relatively constant. Furthermore, it was observed that milk yield, fat content and fat

yield did not affect either the CLA content of the milkfat or desaturase activity, substantiating the earlier observations of Kelsey *et al.* (2003).

21.5 CLA producing cultures of dairy significance

In recent years strains of a number of dairy starter and probiotic cultures have been identified as possessing the ability to biosynthesise CLA including strains of *Lactococcus*, *Streptococcus*, *Enterococcus*, *Lactobacillus*, *Bifidobacterium*, and *Propionibacterium*. These cultures are of extreme importance to the dairy industry and are essential for the production of a range of traditional and novel dairy products.

21.5.1 Lactococci, streptococci, and enterococci

Lactococci, streptococci, and enterococci are some of the most important lactic acid bacteria (LAB) involved in the dairy industry and play a critical role in the manufacture of fermented dairy products such as buttermilk, lactic butter, ripened cream, yoghurt and cheese. In recent years, these cultures have received substantial attention as a result of reported probiotic activity and through their use in alleviation of a number of gastrointestinal conditions (Benyacoub *et al.*, 2005; Cremonini *et al.*, 2002; Marteau *et al.*, 2001; Steidler *et al.*, 2000). In addition to these properties, a number of recent investigations have indicated their ability to produce CLA and in particular rumenic acid, from free linoleic acid (Kishino *et al.*, 2002; Lin *et al.*, 1999) (see Table 21.3). Lin *et al.* (1999) assessed the CLA producing abilities of *Lc. lactis* subsp. *cremoris* CCRC12586, and *Lc. lactis* subsp. *lactis* CCRC10791 and the streptococcal strain *S. salivarius* subsp. *thermophilus* CCRC12257 in a culture media containing 12% skim milk powder (w/v) and 0.1 or 0.5 mg ml⁻¹ linoleic acid after 24 hrs incubation. The fermentation resulted in the production of 0.0415 mg ml⁻¹, 0.0575 mg ml⁻¹ and 0.0485 mg ml⁻¹ of CLA, respectively, at a linoleic acid concentration of 0.1 mg ml⁻¹, and 0.0440 mg ml⁻¹, 0.0525 mg ml⁻¹, and 0.0645 mg ml⁻¹ of CLA, respectively, at a linoleic acid concentration of 0.5 mg ml⁻¹. Kishino *et al.* (2002) assessed the CLA producing abilities of a number of LAB including the strain *E. faecium* AKU 1021. Strains were screened for CLA production at 28°C under O₂ limited conditions for 24–72 hrs shaking in the presence of 0.6 mg ml⁻¹ linoleic acid, the products recovered were 0.04 mg ml⁻¹ rumenic acid and 0.06 mg ml⁻¹ of the *Δ*⁹, *Δ*¹¹ CLA isomer. Lin *et al.* (1999) and Kishino *et al.* (2002) reported the ability of strains of lactococci, streptococci, and enterococci to produce CLA, however, many other studies have found no such bioconversion. We investigated the potential of a number of food cultures including *Lactococcus* to produce CLA in MRS medium containing 0.55 mg ml⁻¹ free linoleic acid, and found that all lactococcal strains assayed were negative for CLA production (Coakley *et al.*, 2003). Similar findings were observed in

Table 21.3 CLA production by strains of *Lactococcus*, *Streptococcus* and *Enterococcus*

Culture	Culture Type	Medium	Linoleic acid mg ml ⁻¹	Incubation time	Total CLA mg ml ⁻¹	Ref
<i>Lc. lactis</i> subsp. <i>lactis</i> NCFB 176	Growing culture	MRS	0.025	72 hrs	N.D.	Jiang <i>et al.</i> (1999)
<i>Lc. lactis</i> subsp. <i>lactis</i> ATCC 19435	Growing culture	MRS	0.025	72 hrs	N.D.	Jiang <i>et al.</i> (1999)
<i>Lc. lactis</i> subsp. <i>cremoris</i> ATCC 19257	Growing culture	MRS	0.025	72 hrs	N.D.	Jiang <i>et al.</i> (1999)
<i>Lc. lactis</i> subsp. <i>cremoris</i> NCFB 924	Growing culture	MRS	0.025	72 hrs	N.D.	Jiang <i>et al.</i> (1999)
<i>Lc. lactis</i> subsp. <i>lactis</i> DPC3147	Growing culture	MRS	0.55	24 hrs	N.D.	Coakley <i>et al.</i> (2003)
<i>Lc. lactis</i> subsp. <i>lactis</i> DPC 436	Growing culture	MRS	0.55	24 hrs	N.D.	Coakley <i>et al.</i> (2003)
<i>Lc. Lactis</i> subsp. <i>cremoris</i> CCRC12586	Growing culture	12 % RSM	0.5	24 hrs	0.044	Lin <i>et al.</i> (1999)
<i>Lc. lactis</i> subsp. <i>lactis</i> CCRC10791	Growing culture	12 % RSM	0.1	24 hrs	0.0575	Lin <i>et al.</i> (1999)
<i>S. salivarius</i> subsp. <i>thermophilus</i>	Growing culture	MRS	0.025	25 hrs	N.D.	Jiang <i>et al.</i> (1999)
<i>S. salivarius</i> subsp. <i>thermophilus</i> ATCC 19258	Growing culture	MRS	0.025	25 hrs	N.D.	Jiang <i>et al.</i> (1999)
<i>S. salivarius</i> subsp. <i>thermophilus</i> CCRC12257	Growing culture	12 % RSM	0.5	24 hrs	0.0645	Lin <i>et al.</i> (1999)
<i>E. faecium</i> AKU 1021	Growing culture	MRS	0.6	24–72 hrs	0.1	Kishino <i>et al.</i> (2002)

N.D. = not detected.

the work of Jiang *et al.* (1998) who assessed the potential for the production of CLA from the lactococcal strains *Lc. lactis* subsp. *lactis* NCFB 176, *Lc. lactis* subsp. *lactis* ATCC 19435, *Lc. lactis* subsp. *cremoris* ATCC 19257, and *Lc. lactis* subsp. *cremoris* NCFB 924, and the streptococcal strains *S. salivarius* subsp. *thermophilus*, and *S. salivarius* subsp. *thermophilus* ATCC 19258 when grown in MRS containing 0.025 mg ml⁻¹ free linoleic acid.

21.5.2 Propionibacteria

Dairy propionibacteria are commonly found in Swiss type cheeses where they produce acetate, propionate, and carbon dioxide which contribute to flavour and characteristic eyes of the cheese, however, they have also been isolated from soil, silage, brines for olive fermentation and rum distilleries (Cummins and Johnson, 1986). In addition to their role in the manufacture of dairy products, *Propionibacterium* have been reported to produce B-vitamins (Quesada-Chanto *et al.*, 1994; Roessner *et al.*, 2002), bacteriocins (Brede *et al.*, 2004; Lyon *et al.*, 1993; Van der Merwe *et al.*, 2004), and bifidogenic compounds (Kaneko *et al.*, 1994) spurring increased interest in these cultures. The ability of propionibacteria to produce rumenic acid and other CLA isomers from linoleic acid has been confirmed and resulted in the identification of a large number of rumenic acid and *t*9, *t*11 CLA producing strains (Jiang *et al.*, 1998; Kishino *et al.*, 2002; Rainio *et al.*, 2001, 2002; Verhulst *et al.*, 1987) (see Table 21.4). Verhulst *et al.* (1987) examined 36 strains of *Propionibacterium* for the production of CLA from 0.02 mg ml⁻¹ linoleic acid in modified BHI (mBHI) medium resulting in the identification of a large number of CLA producing strains of *P. freudenreichii* subsp. *freudenreichii*, *P. freudenreichii* subsp. *shermanii*, *P. acidi-propionici*, and *P. technicum* with between 50% and 80% of the CLA produced in the form of rumenic acid. Similarly, Kishino *et al.* (2002) reported *P. shermanii* AKU 1254 exhibited linoleic acid isomerase activity when grown in MRS containing 0.6 mg ml⁻¹ linoleic acid converting 15% and 3.33% of the linoleic acid to rumenic acid and the *t*9, *t*11 isomer, respectively. Jiang *et al.* (1998) assayed a number of dairy cultures for CLA production including six strains of propionibacteria using MRS containing 0.025 mg ml⁻¹ linoleic acid. It was found that strains of *P. freudenreichii* subsp. *freudenreichii* and *P. freudenreichii* subsp. *shermanii* were capable of producing rumenic acid (see Table 21.4). Using these strains the authors assessed the effect of incrementally increasing the linoleic acid from 0 to 1.5 mg ml⁻¹ on rumenic acid production showing that each strain had an optimum linoleic acid concentration and that at concentrations above this, both growth and CLA production were inhibited.

Most of the studies on the production of CLA by strains of propionibacteria have used synthetic media or milk containing free linoleic acid emulsified using detergents such as Tween 80 or through the formation of complexes with proteins such as BSA. Vahvaselkä *et al.* (2004) used an alternative approach where a species of oats (*Avena sativa* L.) with a high linoleic acid

Table 21.4 CLA production by strains of *Propionibacterium*

Culture	Culture Type	Medium	Linoleic acid mg ml ⁻¹	Incubation time	Total CLA mg ml ⁻¹	Ref
<i>P. freudenreichii</i> subsp. <i>freudenreichii</i> NCIB 8896	Growing culture	mBHI	0.02	24 hrs	Detected	Verhulst <i>et al.</i> (1987)
<i>P. freudenreichii</i> subsp. <i>freudenreichii</i> NCIB 5959	Growing culture	mBHI	0.02	24 hrs	Detected	Verhulst <i>et al.</i> (1987)
<i>P. freudenreichii</i> subsp. <i>shermanii</i> NCIB 10585	Growing culture	mBHI	0.02	24 hrs	Detected	Verhulst <i>et al.</i> (1987)
<i>P. freudenreichii</i> subsp. <i>shermanii</i> NCIB 5964	Growing culture	mBHI	0.02	24 hrs	Detected	Verhulst <i>et al.</i> (1987)
<i>P. freudenreichii</i> subsp. <i>shermanii</i> NCIB 8099	Growing culture	mBHI	0.02	24 hrs	Detected	Verhulst <i>et al.</i> (1987)
<i>P. acidi-propionici</i> NCIB 8070	Growing culture	mBHI	0.02	24 hrs	Detected	Verhulst <i>et al.</i> (1987)
<i>P. acidi-propionici</i> NCIB 5959	Growing culture	mBHI	0.02	24 hrs	Detected	Verhulst <i>et al.</i> (1987)
<i>P. technicum</i> NCIB 5965	Growing culture	mBHI	0.02	24 hrs	Detected	Verhulst <i>et al.</i> (1987)
<i>P. acnes</i> ATCC 6919	Growing culture	mBHI	0.02	24 hrs	Detected	Verhulst <i>et al.</i> (1987)
<i>P. acnes</i> ATCC 6921	Growing culture	mBHI	0.02	24 hrs	Detected	Verhulst <i>et al.</i> (1987)
<i>P. acnes</i> no 27	Growing culture	mBHI	0.02	24 hrs	Detected	Verhulst <i>et al.</i> (1987)
<i>P. acnes</i> VPI 163	Growing culture	mBHI	0.02	24 hrs	Detected	Verhulst <i>et al.</i> (1987)
<i>P. acnes</i> VPI 164	Growing culture	mBHI	0.02	24 hrs	Detected	Verhulst <i>et al.</i> (1987)
<i>P. acnes</i> VPI 199	Growing culture	mBHI	0.02	24 hrs	Detected	Verhulst <i>et al.</i> (1987)
<i>P. acnes</i> VPI 186	Growing culture	mBHI	0.02	24 hrs	Detected	Verhulst <i>et al.</i> (1987)
<i>P. acnes</i> VPI 174	Growing culture	mBHI	0.02	24 hrs	Detected	Verhulst <i>et al.</i> (1987)
<i>P. acnes</i> VPI 170	Growing culture	mBHI	0.02	24 hrs	Detected	Verhulst <i>et al.</i> (1987)
<i>P. avidum</i> , (VPI 575, VPI 576, VPI 598, VPI 668, VPI 671, ATCC 25557, CN 6976, CN 5888, and CN 6278)	Growing culture	mBHI	0.02	24 hrs	N.D.	Verhulst <i>et al.</i> (1987)
<i>P. jensenii</i> (NCIB 5960, NCIB 5967, and NCIB 5962)	Growing culture	mBHI	0.02	24 hrs	N.D.	Verhulst <i>et al.</i> (1987)
<i>P. thoenii</i> (NCIB 8072, and NCIB 5966)	Growing culture	mBHI	0.02	24 hrs	N.D.	Verhulst <i>et al.</i> (1987)
<i>P. lymphophilum</i> CN 5936	Growing culture	mBHI	0.02	24 hrs	N.D.	Verhulst <i>et al.</i> (1987)
<i>P. freudenreichii</i> subsp. <i>freudenreichii</i> ATCC 6027	Growing culture	MRS	0.1	72 hrs	0.023	Jiang <i>et al.</i> (1999)
<i>P. freudenreichii</i> subsp. <i>freudenreichii</i> Propioni-6	Growing culture	MRS	0.75	73 hrs	0.265	Jiang <i>et al.</i> (1999)

Table 21.4 Continued

Culture	Culture Type	Medium	Linoleic acid mg ml ⁻¹	Incubation time	Total CLA mg ml ⁻¹	Ref
<i>P. freudenreichii</i> subsp. <i>shermanii</i> 9093	Growing culture	MRS	0.5	74 hrs	0.112	Jiang <i>et al.</i> (1999)
<i>P. shermanii</i> AKU 1254	Growing culture	MRS	0.6	24–72 hrs	0.11	Kishino <i>et al.</i> (2002)
<i>P. freudenreichii</i> subsp. <i>shermanii</i> JS	Growing culture	WPM	2	N.S.	1.6	Rainio <i>et al.</i> (2002)
<i>P. freudenreichii</i> subsp. <i>shermanii</i> 56	Growing culture	Yoghurt	5	N.S.	Detected	Xu <i>et al.</i> (2005)
<i>P. freudenreichii</i> subsp. <i>shermanii</i> 51	Growing culture	Yoghurt	5	N.S.	Detected	Xu <i>et al.</i> (2005)
<i>P. freudenreichii</i> subsp. <i>freudenreichii</i> 23	Growing culture	Yoghurt	5	N.S.	Detected	Xu <i>et al.</i> (2005)
<i>P. freudenreichii</i> subsp. <i>shermanii</i> strain JS	Growing culture	hydrolysed oat flour slurry	12.6 mg g ⁻¹ (DM)	30 hrs	10.1 mg g ⁻¹ (DM)	Vahvaselkä <i>et al.</i> (2004)
<i>P. freudenreichii</i> subsp. <i>shermanii</i> DSM 20270	Growing culture	hydrolysed oat flour slurry	30 mg g ⁻¹ (DM)	20 hrs	11.5 mg g ⁻¹ (DM)	Vahvaselkä <i>et al.</i> (2006)

N.D. = not detected; N.S. = not stated.

content and endogenous lipolytic activity was used to prepare a linoleic acid enriched slurry (5% (w/v)) as the substrate for CLA production by *P. freudenreichii* subsp. *shermanii* JS under optimised conditions. Once optimised, such slurries were capable of yielding CLA concentrations of up to 0.44 mg ml⁻¹ which was further increased to 0.85 mg ml⁻¹ by increasing the flour content of the slurry up to 15% (w/v). The CLA produced via this fermentation was concentrated into the solid phase by acidification and was easily removed from this solid material by centrifugation or filtration. Vahvaselkä *et al.* (2006) repeated the fermentation using the strain *P. freudenreichii* subsp. *shermanii* DSM 2027 reporting the production of 116 mg g⁻¹ fat of CLA from the oat flour slurry following 20 hrs fermentation.

21.5.3 Lactobacilli

Lactobacilli are one of the most common dairy starter cultures used in the production of a diverse range of products including acidophilus buttermilk, yoghurt, kefir, cheese and koumiss, where they contribute to acid production and flavour through the production of lactic acid, acetic acid or ethanol. In addition to their use as starter bacteria, lactobacilli have been frequently used as probiotics and have been associated with the alleviation of a number of gastro-intestinal disorders (Bergonzelli *et al.*, 2005; Cremonini *et al.*, 2002; Gosselink *et al.*, 2004; O'Mahony *et al.*, 2005; Orrhage *et al.*, 2000; Sartor, 2005; Schultz and Sartor, 2000). Added to these benefits a large number of studies have also reported that lactobacilli possess the ability to conjugate linoleic acid and produce CLA (see Table 21.5). Kim and Liu (2002) assayed strains of *Lb. acidophilus*, *Lb. bulgaricus*, *Lb. helveticus*, *Lb. johnsonii*, and *Lb. plantarum* for CLA production in both MRS and in whole milk at a linoleic acid concentration of 0.1 mg ml⁻¹. The study identified four strains of CLA producing lactobacilli (*Lb. acidophilus* 96, and *Lb. plantarum* 4191, *Lb. acidophilus* 56, and *Lb. acidophilus* 43121) whose ability to produce the isomer differed substantially with the type of medium used. When grown in MRS only the strains *Lb. acidophilus* 96, and *Lb. plantarum* 4191 proved positive for CLA production while growth in whole milk resulted in additional CLA production by the strains *Lb. acidophilus* 56, and *Lb. acidophilus* 43121 and improved CLA production by the strain *Lb. plantarum* 4191.

In a similar study, Alonso *et al.* (2003) reported that two strains of *Lb. acidophilus* (L1, and O16) and two strains of *Lb. casei* (E5, and E10) exhibited CLA producing capabilities in MRS and milk containing 0.2 mg ml⁻¹ linoleic acid. Production of CLA in milk was also assessed by Lin *et al.* (1999) using the strains *Lb. acidophilus* CCRC 14079, *Lb. delbrueckii* subsp. *bulgaricus* CCRC 14009, and *Lb. delbrueckii* subsp. *lactis* CCRC 14078 in 12% (w/v) reconstituted skim milk containing 1.0 mg ml⁻¹ linoleic acid with all four strains proving positive for CLA production. Kishino *et al.* (2002) identified 15 strains of CLA producing lactobacilli using MRS containing 0.6 mg

Table 21.5 CLA production by strains of *Lactobacillus*

Culture	Culture type	Medium	Linoleic acid mg ml ⁻¹	Incubation time	Total CLA mg ml ⁻¹	Ref
<i>Lb. acidophilus</i> ATCC 4356	Growing culture	MRS	0.025	24 hrs	N.D.	Jiang <i>et al.</i> (1999)
<i>Lb. bulgaricus</i>	Growing culture	MRS	0.025	24 hrs	N.D.	Jiang <i>et al.</i> (1999)
<i>Lb. casei</i>	Growing culture	MRS	0.025	24 hrs	N.D.	Jiang <i>et al.</i> (1999)
<i>Lb. casei</i> F-19	Growing culture	MRS	0.025	24 hrs	N.D.	Jiang <i>et al.</i> (1999)
<i>Lb. fermentum</i>	Growing culture	MRS	0.025	24 hrs	N.D.	Jiang <i>et al.</i> (1999)
<i>Lb. helveticus</i> ATCC 15009	Growing culture	MRS	0.025	24 hrs	N.D.	Jiang <i>et al.</i> (1999)
<i>Lb. reuteri</i> ATCC 23272	Growing culture	MRS	0.025	24 hrs	N.D.	Jiang <i>et al.</i> (1999)
<i>Lb. lactis</i> subsp. <i>lactis</i> NCFB 176	Growing culture	MRS	0.025	24 hrs	N.D.	Jiang <i>et al.</i> (1999)
<i>Lb. lactis</i> subsp. <i>lactis</i> ATCC 19435	Growing culture	MRS	0.025	24 hrs	N.D.	Jiang <i>et al.</i> (1999)
<i>Lb. lactis</i> subsp. <i>cremoris</i> ATCC 19257	Growing culture	MRS	0.025	24 hrs	N.D.	Jiang <i>et al.</i> (1999)
<i>Lb. lactis</i> subsp. <i>cremoris</i> ATCC NCFB 924	Growing culture	MRS	0.025	24 hrs	N.D.	Jiang <i>et al.</i> (1999)
<i>Lb. Reuteri</i> NCIMB 11951	Growing culture	MRS	0.55	24 hrs	N.D.	Coakley <i>et al.</i> (2003)
<i>Lb. Reuteri</i> NCIMB 701359	Growing culture	MRS	0.55	24 hrs	N.D.	Coakley <i>et al.</i> (2003)
<i>Lb. Reuteri</i> NCIMB 701089	Growing culture	MRS	0.55	24 hrs	N.D.	Coakley <i>et al.</i> (2003)
<i>Lb. Reuteri</i> NCIMB 702655	Growing culture	MRS	0.55	24 hrs	N.D.	Coakley <i>et al.</i> (2003)
<i>Lb. Reuteri</i> NCIMB 702656	Growing culture	MRS	0.55	24 hrs	N.D.	Coakley <i>et al.</i> (2003)
<i>Lb. heleveticus</i> NCIMB 700257	Growing culture	MRS	0.55	24 hrs	N.D.	Coakley <i>et al.</i> (2003)
<i>Lb. heleveticus</i> ATCC 15009	Growing culture	MRS	0.55	24 hrs	N.D.	Coakley <i>et al.</i> (2003)
<i>Lb. heleveticus</i> NCIMB 701244	Growing culture	MRS	0.55	24 hrs	N.D.	Coakley <i>et al.</i> (2003)
<i>Lb. paracasei</i> UCC 43338	Growing culture	MRS	0.55	24 hrs	N.D.	Coakley <i>et al.</i> (2003)
<i>Lb. paracasei</i> UCC 43364	Growing culture	MRS	0.55	24 hrs	N.D.	Coakley <i>et al.</i> (2003)
<i>Lb. paracasei</i> UCC 42319	Growing culture	MRS	0.55	24 hrs	N.D.	Coakley <i>et al.</i> (2003)
<i>Lb. paracasei</i> UCC 43348	Growing culture	MRS	0.55	24 hrs	N.D.	Coakley <i>et al.</i> (2003)
<i>Lb. paracasei</i> DPC 5336	Growing culture	MRS	0.55	24 hrs	N.D.	Coakley <i>et al.</i> (2003)
<i>Lb. delbreuckii</i> subsp. <i>lactis</i> NCIMB 8117	Growing culture	MRS	0.55	24 hrs	N.D.	Coakley <i>et al.</i> (2003)

Table 21.5 Continued

Culture	Culture type	Medium	Linoleic acid mg ml ⁻¹	Incubation time	Total CLA mg ml ⁻¹	Ref
<i>Lb. delbreuckii</i> subsp. <i>lactis</i> NCIMB 8118	Growing culture	MRS	0.55	24 hrs	N.D.	Coakley <i>et al.</i> (2003)
<i>Lb. salivarius</i> UCC 43310	Growing culture	MRS	0.55	24 hrs	N.D.	Coakley <i>et al.</i> (2003)
<i>Lb. fermentum</i>	Growing culture	MRS	5	24 hrs	Detected	Ham <i>et al.</i> (2002)
<i>Lb. acidophilus</i> 96	Growing culture	MRS	0.1	24 hrs	< 2 mg g fat	Kim and Liu (2002)
<i>Lb. acidophilus</i> 56	Growing culture	MRS	0.1	24 hrs	N.D.	Kim and Liu (2002)
<i>Lb. acidophilus</i> ATCC 4356	Growing culture	MRS	0.1	24 hrs	N.D.	Kim and Liu (2002)
<i>Lb. acidophilus</i> ATCC 43121	Growing culture	MRS	0.1	24 hrs	N.D.	Kim and Liu (2002)
<i>Lb. bulgaricus</i>	Growing culture	MRS	0.1	24 hrs	N.D.	Kim and Liu (2002)
<i>Lb. helveticus</i> ATCC 15009	Growing culture	MRS	0.1	24 hrs	N.D.	Kim and Liu (2002)
<i>Lb. johnsonii</i> 88	Growing culture	MRS	0.1	24 hrs	N.D.	Kim and Liu (2002)
<i>Lb. plantarum</i> 4191	Growing culture	MRS	0.1	24 hrs	< 2 mg g fat	Kim and Liu (2002)
<i>Lb. acidophilus</i> 96	Growing culture	Whole milk	0.1	24 hrs	< 2 mg g fat	Kim and Liu (2002)
<i>Lb. acidophilus</i> 56	Growing culture	Whole milk	0.1	24 hrs	< 2 mg g fat	Kim and Liu (2002)
<i>Lb. acidophilus</i> ATCC 4356	Growing culture	Whole milk	0.1	24 hrs	N.D.	Kim and Liu (2002)
<i>Lb. acidophilus</i> ATCC 43121	Growing culture	Whole milk	0.1	24 hrs	< 2 mg g fat	Kim and Liu (2002)
<i>Lb. bulgaricus</i>	Growing culture	Whole milk	0.1	24 hrs	N.D.	Kim and Liu (2002)
<i>Lb. helveticus</i> ATCC 15009	Growing culture	Whole milk	0.1	24 hrs	N.D.	Kim and Liu (2002)
<i>Lb. johnsonii</i> 88	Growing culture	Whole milk	0.1	24 hrs	N.D.	Kim and Liu (2002)
<i>Lb. plantarum</i> 4191	Growing culture	Whole milk	0.1	24 hrs	2–4 mg g fat	Kim and Liu (2002)
<i>Lb. acidophilus</i> L1	Growing culture	MRS	0.2	24 hrs	0.132	Alonso <i>et al.</i> (2003)
<i>Lb. acidophilus</i> O16	Growing culture	MRS	0.2	24 hrs	0.069	Alonso <i>et al.</i> (2003)
<i>Lb. casei</i> E5	Growing culture	MRS	0.2	24 hrs	0.111	Alonso <i>et al.</i> (2003)
<i>Lb. casei</i> E10	Growing culture	MRS	0.2	24 hrs	0.08	Alonso <i>et al.</i> (2003)
<i>Lb. acidophilus</i> L1	Growing culture	10% RSM	0.2	24 hrs	0.116	Alonso <i>et al.</i> (2003)
<i>Lb. acidophilus</i> O16	Growing culture	10% RSM	0.2	24 hrs	0.054	Alonso <i>et al.</i> (2003)
<i>Lb. casei</i> E5	Growing culture	10% RSM	0.2	24 hrs	0.01	Alonso <i>et al.</i> (2003)

Table 21.5 Continued

Culture	Culture type	Medium	Linoleic acid mg ml ⁻¹	Incubation time	Total CLA mg ml ⁻¹	Ref
<i>Lb. casei</i> E10	Growing culture	10% RSM	0.2	24 hrs	0.071	Alonso <i>et al.</i> (2003)
<i>Lb. acidophilus</i> CCRC 14079	Growing culture	12% RSM	0.1	24 hrs	0.105	Lin <i>et al.</i> (1999)
<i>Lb. delbrueckii</i> subsp. <i>bulgaricus</i> CCRC 14009	Growing culture	12% RSM	0.1	24 hrs	0.087	Lin <i>et al.</i> (1999)
<i>Lb. delbrueckii</i> subsp. <i>lactis</i> CCRC 14078	Growing culture	12% RSM	0.1	24 hrs	0.078	Lin <i>et al.</i> (1999)
<i>Lb. acidophilus</i> AKU 1137	Growing culture	MRS	0.6	24–72 hrs	1.5	Kishino <i>et al.</i> (2002)
<i>Lb. acidophilus</i> IAM10074	Growing culture	MRS	0.6	24–72 hrs	0.6	Kishino <i>et al.</i> (2002)
<i>Lb. acidophilus</i> AKU 1122	Growing culture	MRS	0.6	24–72 hrs	0.12	Kishino <i>et al.</i> (2002)
<i>Lb. brevis</i> IAM 1082	Growing culture	MRS	0.6	24–72 hrs	0.55	Kishino <i>et al.</i> (2002)
<i>Lb. paracasei</i> subsp. <i>paracasei</i> IFO 12004	Growing culture	MRS	0.6	24–72 hrs	0.2	Kishino <i>et al.</i> (2002)
<i>Lb. paracasei</i> subsp. <i>paracasei</i> JCM 1109	Growing culture	MRS	0.6	24–72 hrs	0.07	Kishino <i>et al.</i> (2002)
<i>Lb. paracasei</i> subsp. <i>paracasei</i> AKU 1142	Growing culture	MRS	0.6	24–72 hrs	0.07	Kishino <i>et al.</i> (2002)
<i>Lb. paracasei</i> subsp. <i>paracasei</i> IFO 3533	Growing culture	MRS	0.6	24–72 hrs	0.09	Kishino <i>et al.</i> (2002)
<i>Lb. pentosus</i> AKU 1148	Growing culture	MRS	0.6	24–72 hrs	0.08	Kishino <i>et al.</i> (2002)
<i>Lb. pentosus</i> IFO 12011	Growing culture	MRS	0.6	24–72 hrs	0.13	Kishino <i>et al.</i> (2002)
<i>Lb. plantarum</i> AKU 1138	Growing culture	MRS	0.6	24–72 hrs	0.45	Kishino <i>et al.</i> (2002)
<i>Lb. plantarum</i> AKU 1009a	Growing culture	MRS	0.6	24–72 hrs	3.41	Kishino <i>et al.</i> (2002)
<i>Lb. plantarum</i> JCM 8341	Growing culture	MRS	0.6	24–72 hrs	0.19	Kishino <i>et al.</i> (2002)
<i>Lb. plantarum</i> JCM 1551	Growing culture	MRS	0.6	24–72 hrs	1.02	Kishino <i>et al.</i> (2002)
<i>Lb. rhamnosus</i> AKU 1124	Growing culture	MRS	0.6	24–72 hrs	1.41	Kishino <i>et al.</i> (2002)
<i>Lb. acidophilus</i> AKU 1137	Washed cells	KPB	5	96 hrs	4.9	Ogawa <i>et al.</i> (2001)

Table 21.5 Continued

Culture	Culture type	Medium	Linoleic acid mg ml ⁻¹	Incubation time	Total CLA mg ml ⁻¹	Ref
<i>Lb. plantarum</i> AKU 1009a	Washed cells	KPB	20	48 hrs	8.9	Kishino <i>et al.</i> (2002)
<i>Lb. Reuteri</i> ATCC 55739	Immobilised cells	Silica gel	0.5	1 hr	0.175	Lee <i>et al.</i> (2003)
<i>Lb. delbrueckii</i> subsp. <i>bulgaricus</i> CCRC 14009	Immobilised cells	Polyacrylamide	3	24 hrs	2.211	Lin <i>et al.</i> (2005)
<i>Lb. delbrueckii</i> subsp. <i>bulgaricus</i> CCRC 14009	Immobilised cells	Chitosan	3	24 hrs	0.283	Lin <i>et al.</i> (2005)
<i>Lb. delbrueckii</i> subsp. <i>bulgaricus</i> CCRC 14009	Free cells	KPB	3	24 hrs	0.01	Lin <i>et al.</i> (2005)
<i>Lb. acidophilus</i> CCRC 14079	Immobilised cells	Polyacrylamide	3	24 hrs	0.218	Lin <i>et al.</i> (2005)
<i>Lb. acidophilus</i> CCRC 14079	Immobilised cells	Chitosan	3	24 hrs	0.055	Lin <i>et al.</i> (2005)
<i>Lb. acidophilus</i> CCRC 14079	Free cells	KPB	3	24 hrs	0.022	Lin <i>et al.</i> (2005)
<i>Lb. rhamnosus</i>	Growing culture	yoghurt	5	N.S.	Detected	Xu <i>et al.</i> (2005)

N.D. = not detected; N.S. = not stated.

ml⁻¹ linoleic acid (see Table 21.5). Of these strains *Lb. acidophilus* AKU 1137 produced the highest concentration of rumenic acid (0.85 mg ml⁻¹), while the strain *Lb. plantarum* AKU 1009a produced the highest total CLA (3.41 mg ml⁻¹) primarily found in the form of the *t*9, *t*11 isomer. Studies into the production of CLA by lactobacilli have generally found the majority of the CLA to be intimately associated with the cells or located within. Storage of CLA in this manner by the cells increases the potential for the use of lactobacilli as probiotic vectors for delivery of CLA and in particular rumenic acid to the human gastrointestinal (GI) tract. However, not all studies have demonstrated the CLA producing abilities of lactobacilli including those of Jiang *et al.* (1998), Coakley *et al.* (2003) and Ham *et al.* (2002).

The use of growing cells of lactobacilli as a means of rumenic acid and CLA production has proved a very successful strategy, it is not, however, the only avenue being explored. Ogawa *et al.* (2001) used washed cells of *Lb. acidophilus* AKU 1137 which when exposed to 5 mg ml⁻¹ free linoleic acid complexed to BSA produced 4.9 mg ml⁻¹ of CLA of which almost all was in or associated intimately with the cells. Kishino *et al.* (2002) assessed the CLA forming abilities of washed cells of *Lb. plantarum* AKU 1009a, varying reaction conditions such as pH, temperature, form of linoleic acid supplied, linoleic acid concentration, oxygen exposure and the ratio of BSA to linoleic acid. Optimum CLA production was achieved by maintaining the pH and temperature at the optimum for the strain's linoleic acid isomerase, while only the free form of linoleic acid was converted to CLA. The optimum ratio of BSA to linoleic acid was deemed to be 1:5 or 2.5:5 (weight ratio), and when exposed to 120 mg ml⁻¹ free linoleic acid, these washed cells produced 40 mg ml⁻¹ of CLA after 108 hrs, while reduction of this concentration to 26 mg ml⁻¹ resulted in a 50% reduction in CLA production. Based on the observations of Ogawa *et al.* (2001) and Kishino *et al.* (2002) the use of washed cells would appear to be an efficient and effective method for the natural production of CLA and in particular rumenic acid on an industrial scale.

The use of immobilised cells in the production of rumenic acid and other CLA isomers was reported by Lee *et al.* (2003). Immobilised cells of *Lb. reuteri* ATCC 55739 in a silica gel matrix were reported to produce 0.175 mg ml⁻¹ CLA from 0.5 mg ml⁻¹ linoleic acid following incubation for one hour under optimised conditions in the presence of 1.0 mM Cu²⁺. The strategy proved extremely successful when compared to the production of CLA by washed cells of the same strain which produced only 0.032 mg ml⁻¹ CLA under optimised conditions. Furthermore, these immobilised cells could be reused up to five times resulting in the production of over 0.344 mg ml⁻¹ CLA from 0.5 mg ml⁻¹ linoleic acid.

More recently Lin *et al.* (2005) investigated the use of immobilised cells of *Lb. delbrueckii* subsp. *bulgaricus* CCRC 14009, and *Lb. acidophilus* CCRC 14079 in two different gel matrices (chitosan, and polyacrylamide) for the

production of CLA following incubation in the presence of 3 mg ml⁻¹ linoleic acid for 24 hrs. Using this approach *Lb. delbrueckii* subsp. *bulgaricus* CCRC 14009 produced 1.23 mg ml⁻¹, and 0.052 mg ml⁻¹ of rumenic acid in a polyacrylamide and chitosan gel matrix, respectively, compared to washed cells of the same strain which produced only 0.03 mg ml⁻¹ of rumenic acid. Similar results were also obtained with *Lb. acidophilus* CCRC 14079 which produced substantially more CLA in polyacrylamide and chitosan gel matrices compared to washed cells.

Lin *et al.* (2003) and Lin (2006) used crude enzyme extracts which harbour the enzyme linoleic acid isomerase extracted from lactobacilli as the catalyst for CLA production. Partially purified enzyme extract derived from the strain *Lb. acidophilus* CCRC 14079 was used for the production of CLA from 50 and 75 mg of linoleic acid using increasing concentrations of enzyme extract (25–75 mg) (Lin *et al.*, 2003). The production of CLA increased in parallel with the concentration of enzyme extract supplied resulting in the production of 0.305 mg and 0.439 mg of CLA at linoleic acid concentrations of 50 and 75 mg, respectively. Lin (2006) investigated the effect of the exposure of linoleic, linolenic, and oleic acids to an enzyme extract derived from the strain *Lb. delbrueckii* subsp. *bulgaricus* CCRC 14009. In the assay, 25 mg of each fatty acid was mixed with 50 mg of the enzyme extract and incubated at 37°C for 108 hrs, yielding 0.0085 mg, 0.0035 mg and 0.0047 mg of CLA from linoleic, linolenic and oleic acid, respectively. The use of crude enzyme extracts in the production of CLA resulted in the formation of a diverse range of CLA isomers. In the study by Lin *et al.* (2003), eight different CLA isomers were detected, while Lin (2006) reported the production of six CLA isomers.

21.5.4 Bifidobacteria

Bifidobacteria have been used for centuries in the production of bifidus milks and, more recently, in the production of functional dairy products. As natural inhabitants of the human gastrointestinal tract bifidobacteria have been associated with a large number of probiotic properties and with the prevention or alleviation of a number of human gastrointestinal conditions (Cremonini *et al.*, 2002; Gionchetti *et al.*, 2000a, 2000b; O'Mahony *et al.*, 2005; Orrhage *et al.*, 2000; Saavedra, 2000; Sartor, 2004). In addition, a number of recent studies have reported the production of CLA (primarily rumenic acid and the *Δ*9, *Δ*11 isomer) from linoleic acid by bifidobacteria (see Table 21.6).

The production of CLA by these cultures was first reported by Coakley *et al.* (2003) following the screening of strains of *B. adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. dentium*, *B. infantis*, *B. lactis*, and *B. pseudocatenulatum*, for the ability to bioconvert 0.55 mg ml⁻¹ linoleic acid to CLA (see Table 21.6). *B. breve*, *B. dentium*, and *B. pseudocatenulatum* were found to produce the highest concentration of CLA with the majority as

Table 21.6 CLA production by strains of *Bifidobacterium*

Culture	Culture Type	Medium	Linoleic acid mg ml ⁻¹	Incubation time	Total CLA mg ml ⁻¹	Ref
<i>B. acodelescentis</i> NCFB 2230	Did not grow	cys-MRS	0.55	48 hrs	N.D.	Coakley <i>et al.</i> (2003)
<i>B. acodelescentis</i> NCFB 2204	Growing culture	cys-MRS	0.55	48 hrs	0.0035	Coakley <i>et al.</i> (2003)
<i>B. acodelescentis</i> NCFB 2231	Growing culture	cys-MRS	0.55	48 hrs	0.0028	Coakley <i>et al.</i> (2003)
<i>B. angulatum</i> NCFB 2236	Growing culture	cys-MRS	0.55	48 hrs	0.0012	Coakley <i>et al.</i> (2003)
<i>B. bifidum</i> NCFB 795	Growing culture	cys-MRS	0.55	48 hrs	0.001	Coakley <i>et al.</i> (2003)
<i>B. breve</i> NCFB 2257	Growing culture	cys-MRS	0.46	48 hrs	0.2311	Coakley <i>et al.</i> (2003)
<i>B. breve</i> NCFB 2258	Growing culture	cys-MRS	0.55	48 hrs	0.3982	Coakley <i>et al.</i> (2003)
<i>B. breve</i> NCTC 11815	Growing culture	cys-MRS	0.55	48 hrs	0.2151	Coakley <i>et al.</i> (2003)
<i>B. breve</i> NCIMB 8815	Growing culture	cys-MRS	0.55	48 hrs	0.2281	Coakley <i>et al.</i> (2003)
<i>B. breve</i> NCIMB 8807	Growing culture	cys-MRS	0.46	48 hrs	0.1279	Coakley <i>et al.</i> (2003)
<i>B. dentium</i> NCFB 2243	Growing culture	cys-MRS	0.55	48 hrs	0.1598	Coakley <i>et al.</i> (2003)
<i>B. infantis</i> NCFB 2205	Growing culture	cys-MRS	0.55	48 hrs	0.0036	Coakley <i>et al.</i> (2003)
<i>B. infantis</i> NCFB 2256	Growing culture	cys-MRS	0.55	48 hrs	0.0246	Coakley <i>et al.</i> (2003)
<i>B. lactis</i> Bb12	Growing culture	cys-MRS	0.55	48 hrs	0.281	Coakley <i>et al.</i> (2003)
<i>B. pseudocatenulatum</i> NCIMB 8811	Growing culture	cys-MRS	0.55	48 hrs	0.0233	Coakley <i>et al.</i> (2003)
<i>B. breve</i> NCFB 2258	Growing culture	cys-MRS	0.5	72 hrs	36.7 %	Rosberg-cody <i>et al.</i> (2004)
<i>B. breve</i> (PFGE pattern B)	Growing culture	cys-MRS	0.5	72 hrs	29.0 %	Rosberg-cody <i>et al.</i> (2004)
<i>B. breve</i> (PFGE pattern F2)	Growing culture	cys-MRS	0.5	72 hrs	27.4 %	Rosberg-cody <i>et al.</i> (2004)
<i>B. bifidum</i> (PFGE pattern A1)	Growing culture	cys-MRS	0.5	72 hrs	17.9 %	Rosberg-cody <i>et al.</i> (2004)
<i>B. breve</i> KCTC 10462	Growing culture	cys-MRS	0.5	48 hrs	0.16	Oh <i>et al.</i> (2003)
<i>B. pseudocatenulatum</i> KCTC 10208	Growing culture	cys-MRS	0.5	48 hrs	0.135	Oh <i>et al.</i> (2003)
<i>B. breve</i> KCTC 3461	Growing culture	cys-MRS	4	40 hrs	0.69	Song <i>et al.</i> (2005)

rumenic acid. Oh *et al.* (2003) identified two further strains of CLA producing bifidobacteria (*B. breve* KCTC 10462 and *B. pseudocatenulatum* KCTC 10208) following the screening of faecal samples derived from breast fed infants. Both Coakley *et al.* (2003) and Oh *et al.* (2003) observed that the CLA produced by bifidobacteria was found almost exclusively in the supernatant.

Bifidobacteria are commonly isolated from the intestine and faeces of adults and infants, which represents a large reservoir for the isolation of rumenic acid producing strains. Rosberg-Cody *et al.* (2004) reported the isolation of novel strains of bifidobacteria from infant faecal material, and the identification of two strains with efficient CLA producing capabilities belonging to the species *B. breve* and *B. bifidum*. This study along with that of Oh *et al.* (2003) demonstrate that populations of bifidobacteria with ability to produce CLA and in particular rumenic acid develop in the neonate shortly after birth and as such it may be assumed they play an important role in the health of neonates.

The anti-carcinogenic activity of CLA (rumenic acid and *tt*10, *cc*12 CLA isomer) naturally produced by the probiotic mix VSL3 (mixture of CLA producing strains of *Lb. acidophilus*, *Lb. bulgaricus*, *Lb. casei*, *Lb. plantarum*, *B. breve*, *B. infantis*, *B. longum* and *S. thermophilus*) from 0.5 mg ml⁻¹ linoleic acid on HT-29 and Caco-2 cell lines was investigated (Ewaschuk *et al.*, 2006) and reduced viability and increased apoptosis reported in both cell lines. Furthermore, in an *ex vivo* assay it was shown that following administration of the probiotic VSL3, murine faeces supplemented with linoleic acid produced 100-fold more CLA than faeces collected prior to VSL3 feeding. These observations suggest the ability of CLA producing probiotic bacteria to produce CLA isomers *in vivo*. We have recently demonstrated the anti-proliferative effect of the two main CLA isomers formed by *B. breve*, *i.e.* rumenic acid and *tt*9, *tt*11 CLA using SW480 and HT-29 human colon cancer cells, which were cultured in the presence of the CLA isomers (Coakley *et al.*, 2006). This study demonstrated that the *tt*9, *tt*11 CLA had a more potent anti-proliferative effect than rumenic acid and supports the earlier observations of Beppu *et al.* (2006) who also reported the higher anti-proliferative activity of the *tt*9, *tt*11 CLA isomer compared to rumenic acid and *tt*10, *cc*12 CLA isomer.

21.6 Production of rumenic acid enriched dairy products

Our ability to successfully manipulate the CLA content of ruminant milkfat and the identification of CLA producing bacteria have opened up avenues for the development of CLA enriched dairy products (see Table 21.7). Using this knowledge a number of studies have proceeded to produce a range of CLA enriched dairy products, including, UHT milk, butter, yoghurt and in particular cheeses.

Table 21.7 CLA content of a range of fermented and non-fermented dairy products

Product	Total fat g 100 g ⁻¹	Rumenic acid mg g ⁻¹ fat	Total CLA mg g ⁻¹ fat	Ref.*
Milk and milk powder				
2% fat milk	N.S.	4.14	N.S	1
Evaporated milk 1	N.S.	3.38	N.S	1
Evaporated milk 2	N.S.	6.39	N.S	1
Whole milk	N.S.	4.49	N.S	1
Skim milk powder	0.1	N.S	1.8	2
Whole milk	3.2	N.S	3.4	2
1% milk	1	N.S	4.3	2
2% milk	2.1	N.S	5	2
3% fat milk	3	5.88	N.S	5
1.5% fat milk	1.5	5.83	N.S	5
Cheese				
Blue cheese 1	N.S.	4.87	N.S	1
Blue cheese 2	N.S.	7.96	N.S	1
Brie	N.S.	4.75	N.S	1
Medium cheddar	N.S.	4.02	N.S	1
Sharp cheddar	N.S.	4.59	N.S	1
Cougar Gold cheese	N.S.	3.72	N.S	1
Cream cheese	N.S.	4.3	N.S	1
Cottage cheese	N.S.	4.8	N.S	1
Edam cheese	N.S.	5.38	N.S	1
Monteray Jack cheese	N.S.	4.8	N.S	1
Mozzarella cheese	N.S.	4.31	N.S	1
Processed American cheese	N.S.	3.64	N.S	1
Processed cheese spread 1	N.S.	4.26	N.S	1
Processed cheese spread 2	N.S.	4.02	N.S	1
Parmesan cheese	N.S.	4	N.S	1
Swiss cheese	N.S.	5.45	N.S	1
Viking cheese	N.S.	3.59	N.S	1
Buttermilk	N.S.	4.66	N.S	1
Sour cream	N.S.	4.14	N.S	1
Yoghurt	N.S.	3.82	N.S	1
Goat cheese	28.5	N.S	2.7	2
Brie cheese	27.9	N.S	3.8	2
Italian parmesan cheese	28.3	N.S	4.2	2
Mozzarella cheese	24.9	N.S	4.6	2
Cheddar cheese	34.6	N.S	4.2	2
Imperial cheddar cheese	33	N.S	4.7	2
Farmer cheese	28.9	N.S	4.7	2
Cream cheese	33.8	N.S	2.7	2
Yoghurt	5.4	N.S	4.4	2
Butter	91.1	N.S	4.7	2
Cheese Whiz	19.1	N.S	4.9	2
Sour cream	12.6	N.S	5	2
Processed Parmesan cheese	28.5	N.S	5.3	2
Cottage cheese	3.1	N.S	5.9	2
Processed cheese	24.3	N.S	6.2	2
Turkish processed cheese 1	26.5	3.63	N.S	3
Turkish processed cheese 2	20	1.5	N.S	3

Table 21.7 Continued

Product	Total fat g 100g ⁻¹	Rumenic acid mg g ⁻¹ fat	Total CLA mg g ⁻¹ fat	Ref.*
Turkish processed cheese 3	31	2.36	N.S	3
Turkish processed cheese 4	18.5	2.1	N.S	3
Turkish processed cheese 5	26	2.72	N.S	3
Turkish processed cheese 6	27	1.98	N.S	3
Mozzarella cheese	11.5	4.15	4.96	4
Gouda cheese	30	5.18	5.96	4
Cheddar cheese	32	3.94	5.02	4
Blue cheese	39	6.2	N.S	5
Cheddar cheese	35	5.86	N.S	5
Pratost cheese	31	5.01	N.S	5
Herragardsost cheese	28	5.45	N.S	5
Vasterbottenost cheese	31	6.02	N.S	5
Greve cheese	28	7.06	N.S	5
Butter				
Turkish butter 1	83	4.41	N.S	3
Turkish butter 2	82.5	4.67	N.S	3
Turkish butter 3	82	4.49	N.S	3
Turkish butter 4	83	3.87	N.S	3
Turkish butter 5	82	3.82	N.S	3
Turkish butter 6	82.5	3.88	N.S	3
Turkish butter 7	82.25	4.62	N.S	3
Turkish butter 8	82	2.85	N.S	3
Butter (salted)	80	6.42	8.11	4
Butter (unsalted)	80	6.11	7.82	4
Butter	80	6.19	N.S	5
Cream				
Whipping cream	N.S.	4.24	N.S	1
Half/half cream	12.2	N.S	5.5	2
Turkish cream 1	35	7.94	N.S	3
Turkish cream 2	35	5.74	N.S	3
Sour cream	19	5.86	7.49	4
Ice milk	5	2.79	3.8	4
Ice cream	10	3.77	4.95	4
Whipping cream	40	6.18	N.S	5
Sour cream	34	6.22	N.S	5
Dairy blend	80	4.32	N.S	5
Low dairy blend	40	2.31	N.S	5
Fermented milk & yoghurt				
0.05% fat yoghurt	0.05	3.73	5.25	4
1% fat yoghurt	1	7.37	9.01	4
3.25% fat yoghurt	3.25	4.27	5.12	4
Yoghurt 1	3	6.15	N.S	5
Yoghurt 2	0.5	6.22	N.S	5
Fjallfal fermented milk	4.2	6.12	N.S	5
Mellanfil fermented milk	1.5	6.07	N.S	5
Bifilus fermented milk	1.5	4.47	N.S	5
Dofilus fermented milk	0.5	5.16	N.S	5
Halsofil fermented milk	0.5	5.24	N.S	5

Table 21.7 Continued

Product	Total fat g 100g ⁻¹	Rumenic acid mg g ⁻¹ fat	Total CLA mg g ⁻¹ fat	Ref.*
Turkish kaymak				
Turkish kaymak 1	65	6.09	N.S	3
Turkish kaymak 2	65	5.28	N.S	3
Turkish kaymak 3	60	5.83	N.S	3
Turkish kaymak 4	60	4.33	N.S	3

*References: Lin *et al.* (1995) =1, Ma *et al.* (1999) =2, Seckin *et al.* (2005)=3, Shanta *et al.* (1995) =4 and Jiang *et al.* (1997) =5.

N.S. = not stated.

21.6.1 UHT milk

In a recent study, high rumenic acid milk (9.1-fold more rumenic acid than control), produced through the supplementation of the basal diet of Friesian cows with sunflower oil and fish oil, was utilised to produce a UHT milk (Jones *et al.*, 2005). Following UHT treatment, the rumenic acid content of the experimental milk was 9.33-fold greater than the control milk, and while sensory characteristics differed compared to the control, a negative impact on the quality of the milk was not obtained. The results demonstrate the stability of rumenic acid enriched milk to the processing conditions employed in the production of UHT milk.

21.6.2 Butter

A number of studies have investigated the fatty acid composition of a range of different butters from a range of locations (Jiang *et al.*, 1997; Lin *et al.*, 1995; Ma *et al.*, 1999; Seckin *et al.*, 2005; Shantha *et al.*, 1995). These studies have highlighted the differences in the rumenic acid content of butters, which can be attributed to factors such as the animals diet or farm management practices. Butter enriched in rumenic acid has been produced in a number of studies through the use of rumenic acid enriched milk produced by dietary supplementation of ruminants with plant and fish oils (Bauman *et al.*, 2000; Jones *et al.*, 2005; Ramaswamy *et al.*, 2001). The resulting butters contained elevated concentrations of both rumenic acid and its precursor vaccenic acid a direct reflection of the composition of the milk from which they were manufactured. Butter produced from the milk of animals supplemented with plant and fish oils were found to be very similar to the controls in most respects but were less firm than the controls (Baer *et al.*, 2001; Jones *et al.*, 2005).

21.6.3 Fermented milk, and yoghurt

Boylston and Beitz (2002) produced a high CLA yoghurt using milk derived from animals fed a diet supplemented with 5% soybean oil. The CLA content

of this yoghurt was almost identical to the CLA content of the raw milk from which it was manufactured. This result suggests that the CLA content of milk remains stable throughout the fermentation process. In addition, the stability of CLA in the yoghurt was demonstrated over seven days refrigerated storage.

As in the case of butter, the rumenic acid content of yoghurt can differ substantially (Table 21.7) a fact which can in part be attributed to the different dietary and animal management practices employed; however, as yoghurt is a fermented product, the influence of the previously described rumenic acid producing bacteria cannot be overlooked. The ability of strains to produce CLA from free linoleic acid during the fermentation of milk has been shown in a large number of studies including those by Kim and Liu (2002), Jiang *et al.* (1998), Lin *et al.* (1999) and Alonso *et al.* (2003). As a number of these strains are starter bacteria, it is therefore possible that the starters used in the production of fermented milks and yoghurts could convert linoleic acid naturally found in milk to rumenic acid. Evidence for this can be obtained from fermented dairy products, such as Dahi where milk fat CLA concentrations increased approximately 4.8-fold following fermentation (Aneja and Murthy, 1990), 0.05% fat yoghurt with a 1.19-fold increase in CLA following fermentation (Shantha *et al.*, 1995), 3.0% fat yoghurt with a 1.05-fold increase in CLA following fermentation (Jiang *et al.*, 1997), 1.5% fat Mellanfil with a 1.03-fold increase in CLA following fermentation (Jiang *et al.*, 1997), and 5.4% fat yoghurt with a 1.29-fold increase in CLA compared to the raw whole milk (Ma *et al.*, 1999). Lin (2003) assessed the use of the rumenic acid producing strain *Lb. acidophilus* CCRC 14079 in co-culture with traditional starters (*Lb. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*) in the production of non-fat set yoghurt supplemented with 1.0 mg ml⁻¹ linoleic acid. It was found that the starter strains exhibited some linoleic acid isomerase activity, increasing the rumenic acid content of the yoghurt from 1.10 mg g⁻¹ to 1.63 mg g⁻¹, but that when combined with the rumenic acid producing strain *Lb. acidophilus* CCRC 14079 this conversion was substantially increased compared to the control which contained the yoghurt cultures alone (0.93 mg g⁻¹ to 2.95 mg g⁻¹).

In a similar study, Xu *et al.* (2005) investigated the effect of using one of three rumenic acid forming propionibacteria (*P. freudenreichii* subsp. *shermanii* 56, *P. freudenreichii* subsp. *shermanii* 51, and *P. freudenreichii* subsp. *freudenreichii* 23) or a rumenic acid producing *Lb. rhamnosus* strain on the rumenic acid content of a fermented milk when used alone or in co-culture with the traditional yoghurt cultures *Lb. delbrueckii* subsp. *bulgaricus* and *S. salivarius* subsp. *thermophilus* YC-180. In this study, 12% (w/v) skimmed milk was supplemented with hydrolysed soybean oil to give a free linoleic acid concentration of 5.0 mg ml⁻¹, fermented, and stored at 4 °C for 14 days. During storage, it was observed that the co-culture of CLA producing propionibacteria with yoghurt cultures resulted in on average a 10.53% increase in the concentration of CLA compared to yoghurt cultures alone while

co-culture of the starter cultures with the *Lb. rhamnosus* strain resulted in a 70% increase in the CLA concentration. Importantly, in both the studies by Lin (2003) and Xu *et al.* (2005), the product acceptability of the yoghurt prepared through the use of the CLA producing strains in co-culture with traditional yoghurt cultures was unaffected when compared to the control.

21.6.4 Cheese

Milk enriched in rumenic acid produced through animal dietary supplementation has been used for the production of a number of rumenic acid enriched cheeses (Addis *et al.*, 2005; Dhiman *et al.*, 1999b; Jones *et al.*, 2005). The rumenic acid content (reflective of the rumenic acid content of the milk from which they have been produced) and key sensory properties of these cheeses were found to be unaffected by the cheese-making process, but the texture was changed in some cases with the rumenic acid enriched cheese being generally softer than the control.

Studies on the fatty acid compositions of different cheeses have highlighted the often substantial variations in the rumenic acid and CLA content between cheeses. These differences are for the most part a result of the differences in the rumenic acid and CLA contents of the raw milk used in cheese making, but given the existence of CLA producing starter bacteria their potential impact cannot be overlooked (see above). Strains of *Lactobacillus*, *Lactococcus*, *Streptococcus thermophilus*, *Enterococcus faecium*, and *Propionibacterium* have all been identified as producing CLA and are commonly used in the production of commercial and farmhouse varieties of cheese as starter or adjunct cultures. It has been reported that the CLA content of cheese (manufactured with milk from the same season) increased from 16.1 mg g⁻¹ fat after five months ripening to 17.3 mg g⁻¹ fat after one year of ripening (Lavillonniere *et al.*, 1998). In another study, hard cheeses which were aged longer had higher CLA content than hard cheeses with a shorter aging time (Zlatanov *et al.*, 2002). Other studies have shown that CLA content remains unchanged during cheese-making and ripening (Jiang *et al.*, 1997; Luna *et al.*, 2005). Addis *et al.* (2005) found that the fatty acid composition of cheeses produced from the milk of sheep on a diet of Mediterranean forages did not differ after 1 and 60 days ripening, while Shanta *et al.* (1995) reported that the total CLA and rumenic acid concentration of Mozzarella, Gouda, and cheddar cheeses did not change over 32 weeks at 4°C. Both observations suggest inactivity by the culture used in terms of CLA formation.

A number of studies have commented on the influence of the manufacturing conditions employed during the production of cheese on rumenic acid and CLA content. Gnadig *et al.* (2004) reported that neither the type of milk used (raw, or thermised milk) nor cooking had any effects on the CLA content of cheese, but that the use of low and high lipolytic *Propionibacterium* strains did cause a small elevation in the CLA content of the cheese from 9.5 mg g⁻¹ fat in the control to 9.9 mg g⁻¹ fat and 10 mg g⁻¹ fat for the low and high

lipolytic strains, respectively. Previously Garcia-Lopez *et al.* (1994) reported an increase in the total CLA content of cheese following the application of heat during processing. This observation supports an earlier study where it was found that the use of elevated temperatures (80°C) during the manufacture of processed cheese could also increase the concentration of CLA present (Kanner *et al.*, 1987).

While some studies demonstrate the positive influence of processing on CLA and rumenic acid concentrations, a number of studies also suggest this is not the case. The effect of manufacturing on the CLA content of processed cheese was examined at four points of manufacture (raw material, following cooking, following creaming and in the final product) (Luna *et al.*, 2005). Only negligible changes in the CLA and linoleic acid concentration of the cheese throughout manufacture were observed. A similar finding was made by Jiang *et al.* (1997) who investigated the effect of manufacturing conditions on the production of the hard cheeses Grevé and Herragårdssost at various time points during manufacture and ripening. They found that the CLA concentration remained relatively unchanged in both cheeses. These studies suggest that neither the manufacturing nor ripening of cheese influence the CLA content and that in such products the starter or adjunct cultures do not produce substantial amounts of CLA during ripening or storage.

21.7 Assessing the safety of CLA enriched foods on human health

While a plethora of data report the health-promoting activities of rumenic acid in both *in vivo* and *in vitro* studies, reports of a number of negative health effects attributed to the *t*10, *c*12 CLA isomer (Larsen *et al.*, 2003; Pariza, 2004; Wahle *et al.*, 2004) and the technical classification of rumenic acid as a *trans* fatty acid have raised a number of questions as to the safety of consuming rumenic acid enriched foods. Scimeca (1998) assessed the effect of the dietary intake of CLA in rats receiving either a basal diet or a diet supplemented with 1.5% (dDM) of a CLA mix (42.5% rumenic acid, and 43% *t*10, *c*12 CLA) for 36 weeks on Fisher 344 rats, with weekly assessment of food intake, and body weight along with post mortem analysis of 15 organs from 10 random animals from both the test and control group. The study showed that the dietary intake of CLA did not have any toxicological effects during the trial period. The long-term effects of feeding a CLA mix (1.0% dDM of 41.9% rumenic acid, and 43.5% *t*10, *c*12) to Fisher 344 rats was studied by Park *et al.*, (2005). I found that rats fed the CLA mix had a lower food intake but no differences in the percentage fat and tissue weight of the CLA fed animals were reported. Interestingly, CLA feeding did result in significant reductions in blood glucose levels, mean corpuscular volume and cholesterol. During the study, animals from both groups developed chronic renal disease, a condition attributed to the high protein content of the feed

and characterised by increases in urinary protein concentrations. Interestingly it was observed that dietary CLA intake reduced the amount of protein in the animal's urine suggesting that it may reduce the severity of renal failure.

Using Clarinol G80, a product containing a 50:50 mix of the two main CLA isomers (rumenic acid and *t*10, *c*12 CLA), O'Hagan and Menzel (2003) conducted a 90-day toxicological feeding study using Wistar out bred [CrI:(WI)WU BR] rats. In addition, the effects of exposure to Clarinol G80 on bacterial mutation and on chromosome aberration in human peripheral blood lymphocytes were examined *in vitro*. The study showed Clarinol G80 to be non-mutagenic and that at a concentration of 5% did not cause any adverse health effects. However, at the highest dose level (15% w/w) Clarinol G80 was found to initiate hepatocellular hypertrophy, an effect that was reversible upon withdrawal of test material. An increase in plasma insulin concentration was observed but plasma glucose concentrations remained unaffected. Whigham *et al.* (2004) assessed the safety of a dietary intake of 6 g d⁻¹ of Clarinol on obese humans over 12 months. This study found that dietary intake of CLA did not negatively affect serum glucose concentrations, insulin resistance, or alkaline phosphatase activity. The CLA group also reported significantly lower frequencies of skin rash, depression, irritability, hair loss, and infection compared to the control group. Based on these observations, it would appear that the use of CLA in the form of Clarinol in the treatment of obesity for up to one year is safe.

The effect of the dietary intake of a high CLA butter on cholesterol and lipoprotein metabolism compared to a control butter or partially hydrogenated vegetable oil (PHVO) has recently been reported using Golden Syrian Hamsters (Lock *et al.*, 2005b). The high-CLA butter was produced using milk enriched in CLA and vaccenic acid through supplementation of the bovine diet with sunflower oil resulting in a product that contained 15.36 g of vaccenic acid per 100 g of fat and 3.61 g rumenic acid per 100 g fat. The hamsters were fed a basal diet supplemented with 0.2% crystalline cholesterol and either 20% fat derived from control butter, a high-CLA butter or PHVO. It was shown that the group fed the high-CLA butter had a lower VLDL cholesterol, and a reduced ratio of intermediate density lipoprotein (IDL) and low density lipoprotein (LDL) to high density lipoprotein (HDL), compared with the control or PHVO groups.

A recent report assessed the effect in humans of ingesting butter containing elevated levels of vaccenic acid (3.1 g 100 g⁻¹) and rumenic acid (1.3 g 100 g⁻¹), compared to a control butter (vaccenic acid 0.4 g 100 g⁻¹, rumenic acid 0.3 g 100 g⁻¹) (Tholstrup *et al.*, 2006). Subjects consumed 115 g d⁻¹ of the CLA and vaccenic acid enriched butter each day for five weeks. Ingestion of butter with elevated CLA and vaccenic acid did not significantly affect body weight, serum insulin, serum glucose, or inflammatory response, oxidative stress, or haemostatic risk factors. The diet containing elevated CLA and vaccenic acid did, however, result in a lower total and HDL plasma cholesterol level which the authors attributed to the greater concentration of monounsaturated fatty acids and lower concentration of saturated fatty acids in the test butter compared to the control.

A study by Tricon *et al.* (2006) assessed the effects of ingestion of CLA enriched butter, milk and cheese over six weeks on body weight, blood lipid profile, inflammatory response, serum insulin, serum glucose and the ratio of LDL to HDL cholesterol. During the trial 32 healthy male participants consumed a control diet containing 0.151 g d^{-1} CLA and a test diet containing 1.421 g d^{-1} CLA delivered in the form of cheese, butter and milk naturally enriched in CLA. The study demonstrated that the high CLA and vaccenic acid diet did not significantly affect body weight, inflammatory markers, serum glucose and insulin concentrations, triacylglycerols, or total LDL to HDL cholesterol. The diet did, however, result in a minor increase in the ratio of LDL to HDL cholesterol. Overall, the study concluded that the consumption of dairy products naturally enriched in CLA did not have a significant effect on blood lipid profile or pose any increase in cardiovascular disease risk variables.

Raff *et al.* (2006) assessed the effect of the dietary intake of 115 g d^{-1} fat derived from milk containing elevated vaccenic acid ($23.4 \text{ g } 100 \text{ g}^{-1}$ fat) and rumenic acid ($1.3 \text{ g } 100 \text{ g}^{-1}$ fat) on blood pressure and arterial elasticity in healthy young men with a BMI of 22.5 kg m^2 . Following consumption of the CLA and vaccenic acid enriched milk no differences in systolic and diastolic blood pressure, pulse pressure, isobaric arterial compliance, distensibility, or volume could be found, leading the authors to conclude that the dietary intake of CLA or vaccenic acid through consumption of CLA and vaccenic acid enriched milk does not impact on blood pressure or arterial elasticity indices in healthy young men compared with a control diet.

21.8 Conclusion

Research into the health-promoting activity of CLA and rumenic acid is vast and suggests that these fatty acids may positively impact some of the major conditions affecting human health. Selecting appropriate animal feeding strategies by the use of dietary supplementation with plant and/or fish oils in combination with pasture feeding, yield CLA enriched milk, which can be effectively transformed into health-promoting CLA enriched dairy products. In addition, CLA producing dairy cultures may be employed in the production of fermented dairy products enriched in CLA. Furthermore, the presence of CLA producing bacteria as natural inhabitants of the human gastrointestinal tract may impart additional benefits to human health.

21.9 Sources of further information and advice

There are a number of helpful reviews (Banni *et al.*, 2004; Lee *et al.*, 2005; Mir *et al.*, 2004; Tricon and Yaqoob, 2006), papers (AbuGhazaleh *et al.*,

2002a; Gaullier *et al.*, 2005; Kim *et al.*, 2002; Medrano *et al.*, 1999; Toomey *et al.*, 2005), books (*Advances in Conjugated Linoleic Acid Research*, Vol. 1, *Advances in Conjugated Linoleic Acid Research*, Vol. 2; Kritchevsky (2004); Siems *et al.* (2001)) and web-pages (www.curezone.com/foods/fatspercent.asp; www.feedenergy.com/Conjugated_Linoleic_Acid; www.wisc.edu/fri/clarefs.htm) not mentioned in this review which further address the topic of CLA.

21.10 References

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21.11 Abbreviations

CLA	Conjugated linoleic acid
COX	cyclooxygenase
dDM	dietary dry matter
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid

GI	gastrointestinal
HDL	high density lipoprotein
IDL	intermediate density lipoprotein
LDL	low density lipoprotein
PHVO	partially hydrogenated vegetable oil
PPAR	peroxisome proliferator-activated receptor
PUFA	polyunsaturated fatty acids
UHT	ultra high temperature

Developing dairy weight management products – basis for Valio ProFeel[®] products

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22.1 Introduction: the importance of developing dairy weight management products

Obesity is a growing health problem both in Western and developing countries. Obesity and overweight are a problem especially among men in the United States (Flegal, 2002) and women in Eastern Europe (James, 2004). Finland is a leader in the statistics for Europe (James, 2004). Half of Finnish adults are at least slightly overweight and approximately one-fifth can be classified as obese (Aromaa and Koskinen, 2002). Increasing obesity especially among children and young people is of special concern. There has been a clear interest in and a need for low-energy foodstuffs during recent years, demonstrated among consumers and by health professionals. Prevention is the most effective measure in solving the obesity problem and is best realised through a balanced lifestyle in terms of exercise, nutrition and appropriate food choices.

22.2 Reducing the fat and calorie content of milk products

Carbohydrates, proteins and fats are a primary source of energy for humans. An essential aspect in weight loss is to reduce the amount of energy supplied by the diet. In obesity prevention, energy balance is the most critical factor. As fat is the most energy-dense nutrient, its removal results in a remarkable reduction of energy content.

Skimmed milk as such is a suitable product for weight control. It is a challenging task to achieve a remarkable reduction in its already low energy

content and this is best accomplished by reducing the carbohydrate content. Valio Ltd has patented methods for the production of lactose-free milk drinks and they are based on removing the lactose. One third of the total amount of carbohydrates in skimmed milk can be removed from the concentrate by means of chromatography so that the total energy content of the remaining components does not exceed 20 kcal/100 g. Alternatively, carbohydrates can be removed from the raw material by ultrafiltration. Remaining carbohydrates, i.e. mainly lactose, are hydrolysed to introduce sweetness into the milk beverage and thereby improve its taste.

22.3 Adding functional ingredients to milk products

22.3.1 Calcium

The beneficial effect of calcium on fat metabolism at the cellular level and the subsequent beneficial effect on weight control have been stated in a number of scientific publications (DiRienzo, 2003; Parikh and Yanowski, 2003; Zemel and Miller, 2004; Teegarden, 2005). In epidemiological studies, high dairy calcium intake is associated with lower weight (Loos, 2004; Rosell, 2004; Mirmiran, 2005; Marques-Vidal, 2005). High calcium intake from milk products seems both to prevent weight gain and to improve the results of dieting (Zemel, 2004; Zemel, 2005a; Zemel, 2005b). Milk has a stronger effect than calcium and, as a part of a low-energy diet, it reinforces weight loss (Zemel, 2004). A strong reverse correlation has been observed between regular and high consumption of milk products and the development of metabolic syndrome (Pereira 2002; Azadbahkt, 2005) and type 2 diabetes in adults (Choi, 2005). It has also been noticed that an increase in calcium intake from food affects metabolism of a fat cell by reducing lipogenesis and increasing lipolysis (Melanson, 2003; Zemel, 2003; Gunther, 2005; Jacobsen, 2005).

Calcium may be added to the milk beverage or to other dairy products in different forms, for example in the form of Ca lactate gluconate, milk calcium, Ca gluconate, Ca lactate, Ca citrate or in some other soluble form of calcium salt. Vitamin D can also be added to dairy products. It enhances calcium absorption and thereby, through calcium, has an indirect effect on energy balance and may contribute to the beneficial effects of calcium on weight (Zemel, 2003).

22.3.2 Fibre

Fibre has many beneficial effects on health, one of which may be to aid in energy intake control. Fibre increases the feeling of fullness and balances the blood sugar levels. It may have an impact on energy control through a plural number of mechanisms (Burton-Freeman, 2000; Pereira and Ludwig, 2001; Howarth, 2001; Feinle, 2002; Anderson and Woodend, 2003). After a meal,

the rate of glucose absorption influences the secretion of insulin, which is needed to metabolise glucose. This determines the change in blood glucose concentration following the meal. Regulation of postprandial blood glucose concentration is important especially in diabetes but also in healthy individuals it may help to keep hunger away longer. Fibre also reduces the energy density of food. Eating the same weight of a less energy-dense food has been reported to increase satiety and decrease energy intake in short term studies (Howarth, 2001). Fibre may also cause the stomach to expand due to increased salivation and secretion of gastric acid. Soluble fibre slows the evacuation of the stomach by forming a viscous gel matrix that contains nutrients and slows down the release of food from the stomach. This slows down digestion, whereby nutrients are absorbed more uniformly and the feeling of satiety lasts longer. Fibre may act on energy balance also through a hormonal mechanism. Fibre, and soluble fibre in particular, reduces the absorption of fat and protein possibly by reducing the physical contact between nutrients and the villi in the intestines. An increase in the daily intake of fibre reduces energy intake and thereby causes weight loss in the long term (WHO, 2003). Positive effects are achieved both by means of fibre obtained from natural sources and from a fibre supplement (Howarth, 2001). Fibre also improves taste and thereby contributes to the organoleptic properties of a fat-free dairy product. Soluble fibre that may be added to a milk beverage and to other dairy products includes polydextrose, inulin, fructo-oligosaccharides, galacto-oligosaccharides, pectin, β -glucan, guar gum, xanthan gum, carrageen, and carob powder. The addition of insoluble fibres is more challenging but not impossible.

22.4 Role of proteins

The protein content of milk is made up of 80% casein proteins and 20% whey proteins. Protein may play a key role in weight management due to its satiating effects and also its effects on thermogenesis (Westerterp-Platenga, 2003; Anderson and Moore, 2004; Tome, 2004; Westerterp-Platenga and Lejeune, 2005). Compared with caseins and other protein sources, whey protein increases satiety and reduces the amount of food intake during subsequent meals (Anderson and Moore, 2004). Due to its amino acid composition and satiety increasing effect, a whey protein fraction included in foodstuffs has been observed to have an advantageous effect on the metabolic syndrome and weight control (Anderson and Moore, 2004). Therefore protein should be retained in weight management products. Milk, other liquid dairy products, as well as cheese, are excellent sources of protein and different types of protein may also be added to dairy products.

22.5 Role of blood sugar level

Milk products are generally useful for lowering the glycaemic index of the diet (Foster-Powell, 2002). This means that the glucose level of blood after a meal does not rise as high as it would if the meal contained a lot of highly processed carbohydrates, i.e. products of high glycaemic index (GI). A diet rich in products of high glycaemic index has been associated with central obesity, cardiovascular disease and type 2 diabetes (Bell and Sears, 2003). Low GI foods are associated with greater satiety and may promote weight loss (Ludwig, 2000, 2003; Augustin, 2002). Also, several studies have found improvements in glycaemic control with low GI foods (Augustin, 2002). Therefore, it has been suggested that low GI diets may have clinical implications in the prevention and management of chronic Western diseases like obesity and type 2 diabetes (Augustin, 2002).

22.6 Assessing the effect of new dairy weight management products on human health

The scientific background needed for a new dairy weight management product depends on what one wants to claim on the package. It is generally known that low energy and low fat or fat free products are suitable for a weight management diet and there is no need to conduct clinical trials to show the effects. One can also use the scientific evidence that already exists if it is suitable for the nature of the product. For example, the fact that dietary fibre intake helps in weight management is so well established that it is easy to use the information in marketing. If one wants to have a more specific claim on the package, like 'Improves glucose control', 'Decreases body fat' or 'Reduces appetite', the effects have to be shown in well controlled clinical trials. Ideally the effects should be shown at least in two randomised, placebo controlled, independent trials. The endpoints should be clearly defined to measure the specific effects to which one wants to refer in the marketing. Whenever possible, the postulated mechanism of action should also be investigated in experimental trials. For evaluation of long term benefits of the product, the effects should be examined also in a long-term clinical trial.

Useful information on the potential biomarkers for body weight regulation, insulin sensitivity and diabetes risk is provided in the PASSCLAIM (Process for the Assessment of Scientific Support for Health Claims on Foods) (Riccardi, 2004).

22.7 Very low energy milk – a case example

In this chapter, we describe the development process of a dairy weight management product by using the Valio ProFeel® brand, a product family

intended for weight control, as a case example. Valio ProFeel® milk drink was launched at the beginning of September 2005. It was the first low energy milk-like product on the market designed for weight controlling. Its effect is based on a very low energy content (20 kcal/100 g), high fibre content (1.5 g/100 g) and added calcium (180 mg/100 g). The product also contains added vitamin D (0.5 µg/100 g), as well as all nutrients naturally present in milk. Compared with normal fat-free milk, the Valio ProFeel® milk drink has 40% fewer calories, 63% less carbohydrates, 1.5 times more calcium and 3 times more fibre. The low energy content is obtained by removing fat and most of the lactose content. As the residual lactose is hydrolysed, the product becomes lactose free and it is therefore suitable for even the most sensitive of lactose intolerant consumers.

The concept combines an optimal composition of nutrients that have a positive effect on weight control and on dietary treatment of diabetes and metabolic syndrome. The non-fat milk beverage has a low carbohydrate content and thereby an extremely low energy content. Valio ProFeel® milk drink is intended primarily for consumption with meals or snacks. There is a patent pending for this milk drink (Tervala, 2005).

With the same concept it is possible to produce yoghurts, other fermented dairy products, flavoured milks etc., and to create a whole family of very low energy foods. At the moment, the Valio ProFeel® product family consists of the milk drink, yoghurts and cheese spread.

22.7.1 The effect of Valio ProFeel® milk drink on blood glucose levels

The effect of the fibre enriched Valio ProFeel® milk drink and fat-free milk on blood glucose levels was studied in healthy subjects (Kekkonen, 2006). The aim of the study was to compare the effects of water, fat-free milk and a novel fibre enriched Valio ProFeel® milk drink on blood glucose levels after a standardised meal in a randomized, placebo-controlled setting. Twelve healthy volunteers ingested 250 ml of glucose water as a standard meal after an overnight fast. Immediately after this, they drank 200 ml of water, fat-free milk or very low-caloric polydextrose and calcium enriched fat- and lactose-free milk drink (Valio ProFeel®, Valio Ltd, Helsinki, Finland) within 15 minutes on three different study days. Blood glucose levels were measured before ingestion of the study products and after 15, 30, 45, 60, 90 and 120 minutes. The area under curve (AUC) for blood glucose was calculated for each study product and the baseline glucose value was used as a covariable in the analysis. AUC values for blood glucose between the products differed significantly ($p = 0.054$): the very low-caloric fibre enriched fat free milk drink had significantly lower AUC for blood glucose than water or normal fat free milk (0.431 vs. 0.796, $p = 0.019$ and 0.431 vs. 0.732, $p = 0.042$) (see Fig. 22.1). It was concluded that enrichment of milk with polydextrose fibre helps to balance blood glucose levels after a standardised meal and Valio

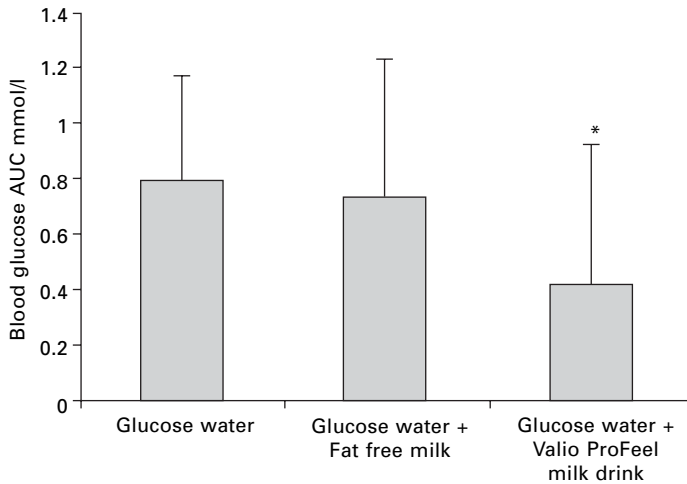


Fig. 22.1 The fibre enriched Valio ProFeel® milk drink helps to balance blood glucose levels after a standardised meal.

ProFeel® milk drink may improve glucose control. In a recent study with 26 healthy volunteers it was shown that Valio ProFeel® milk drink had significantly lower AUC for blood insulin than normal fat free milk (7.4 vs. 10.3, $p = 0.014$) (Lummela, 2007). Milk is known to raise blood insulin more than could be expected by its glucose response and the addition of fibre may help to lower the high insulin response caused by milk.

22.7.2 Marketing and successfully launching dairy weight management products

Health-oriented women are the most receptive consumer group with regard to weight management regimens (Anon, 2004). Women are, for instance, the major customers for weight management groups e.g. Weight Watchers. Therefore the marketing, package decorations, and other means of consumer communications, are designed keeping the main target group in mind.

The trend is changing from negative nutrition (low and light) to positive nutrition (fortified and functional) (Anon, 2005). The concept of Valio ProFeel® products suits both trends, but the main focus in advertising is the positive one. In concept tests carried out among potential consumers before launching the brand, the most frequently mentioned characteristics that best described Valio ProFeel® milk drink were: healthy, modern, functional, and aid in weight control. This also implied that this type of product was expected to work actively in weight reduction. Some consumers commented in the concept tests that the product sounded almost too good to be true. Others were more sceptical: the addition of fibre to milk was considered strange and raised questions about the taste and consistency of the product, as did the term

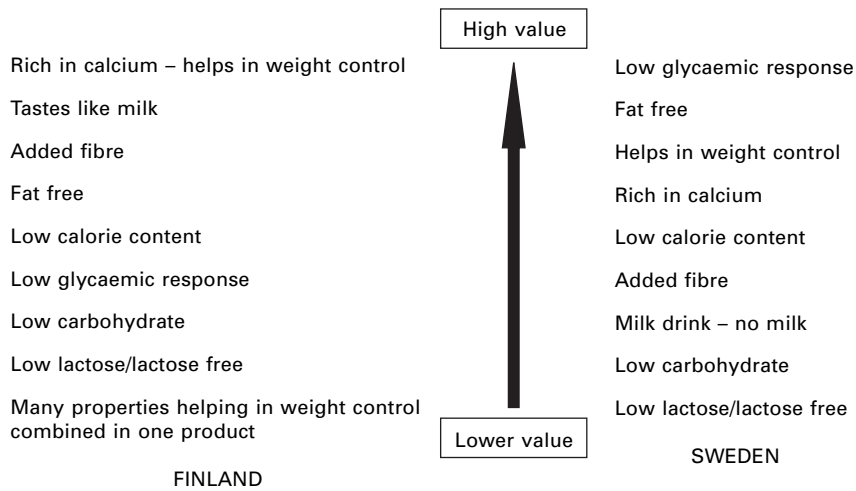


Fig. 22.2 Ranking of added value features for Valio ProFeel® milk drink in concept tests by IRO Research, Finland and Augur, Sweden, 2005 (unpublished).

‘milk drink’. Many consider milk as something very natural and are against any manipulation of it. Finnish and Swedish consumers valued slightly different characteristics of the product in concept tests performed before launching the milk drink, as shown in Fig. 22.2.

Most Finnish consumers watching their weight have enough knowledge about weight control, as there is plenty of information readily available. What they feel they need is support in adapting this knowledge into practice, easy food recipes, as well as convenient and tasty foods. As a response to these requests Valio offers home pages under the theme ‘weight reduction’ at www.valio.fi/painonhallinta. This website offers background support including a nutritional values calculator, a seven-week diet, and information about physical exercise. The website naturally also works as a means of marketing. Other promotional activities for Valio ProFeel® include advertising on TV, radio and in magazines; brochures for both consumers and health professionals; co-operation with Weight Watchers; and seminars and fairs for health professionals. It is always emphasised that products intended for weight management only work together with a balanced healthy diet and appropriate physical exercise.

22.8 Conclusions and future trends

It is obvious that no single food product or even product family can provide a solution to today’s obesity problem. Weight control is a coherent whole that takes in many factors ranging from genes and inherent exercising to learned habits and preferences. The energy content of the food we eat is

nevertheless highly significant. A reduced daily energy intake of just a few dozen kilocalories on a regular basis adds up to a weight loss of several kilos in a year. If weight loss or control is based only on limiting the amount of food eaten, there is a risk of having too low an intake of protective nutrients, i.e. vitamins, minerals and trace elements, and at the same time, of lapses in the regimen caused by hunger. Products which contain very little energy, but supply protective nutrients and components that create a feeling of fullness and improve glucose control, together with calcium which has a beneficial effect on energy metabolism, can offer an active and effective approach in weight control regimens.

The food industry possesses a central role in the solution of the obesity problem. It is obviously not solved entirely by the action of the food industry, but the whole society has to be involved. And still, the individuals themselves make the final choices. Tasty, ordinary foods of high quality that are easy to include in one's diet are probably the most feasible in terms of weight control. The fundamental elements are the very low energy and low fat everyday products which may be further developed in the future to include, for example, ingredients that enhance the feeling of satiety. Products that would decrease the risk of body weight gain or decrease body fat would be welcome but the development would need years of extensive scientific research to show the benefits. Successful weight control requires sensible daily choices of foods and drinks, which can be supported by the expertise of the food industry.

22.9 Sources of further information and advice

Codex Guidelines: CAC/GL 23-1997, Rev. 1-2004

Process for the Assessment of Scientific Support for Health Claims on Foods (PASSCLAIM)

www.valio.com, +358 (0) 10 381 121

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Developing a functional dairy product: from research on *Lactobacillus helveticus* to industrial application of Cardi-04™ in novel antihypertensive drinking yoghurts

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23.1 Introduction

23.1.1 Functional dairy foods: microbial and non-microbial based products

In the last few decades, research into treatments and cures for several diseases, such as diabetes, heart disease and cancer has become particularly intensive, and as supported by the unveiling of the human genome, diet and nutrition may play a critical role in providing health benefits. Today, scientists take new approaches to traditional nutrition and have begun to consider several functional properties of food, such as their potential roles in disease prevention (Milner, 2000). Functional foods have been defined as ‘any food or food ingredient that may provide a health benefit beyond the traditional nutrients that it contains’ (Thomas, 1994). Features of a functional food are defined as:

- (i) conventional or everyday food consumed as part of the normal diet;
- (ii) composed of naturally occurring components, sometimes in increased concentration or present in foods that would not normally supply them;
- (iii) scientifically demonstrated positive effects on target functions beyond basic nutrition;
- (iv) provide enhancement of the state of well-being and health to improve the quality of life and/or reduce of the risk of disease; and
- (v) supported by claims authorized by a relevant authority (Clydesdale, 1997; Bellisle, 1998; Roberfroid, 2000).

Proteins, peptides, lipids and many minor constituents of milk (vitamins, minerals, minor carbohydrates) are being recognized as having potential health benefits and are a part of the total functional food picture. Dairy products with recognized bioactive functions include yoghurt and other fermented dairy foods, whey products, milk protein concentrates, milk and cheese. Current health benefit evidence is based primarily on *in vitro* and *in vivo* animal studies. Other 'good-for-you' ingredients, such as probiotic cultures which are associated with lowering serum cholesterol, aiding in digestion, improving absorption of vitamins and nutrients, and having a beneficial effect against intestinal viral infection, colitis and disease-causing organisms, are being added to an array of dairy products (Gorski, 1996). Some key concepts concerning functional dairy foods include:

- (i) strong evidence, based on a multitude of *in vitro* and *in vivo* animal studies, that dairy foods provide numerous ways to protect health and prevent disease;
- (ii) almost all components of milk and its products possess physiological functionality beyond nutrition;
- (iii) many of the components of milk have more than one physiological function; and
- (iv) dairy foods possess a natural 'cluster' of nutrients that protect health and enhance the immune system. However, to date, only a limited number of human clinical trials have supported the concept that dairy foods do indeed provide a basis for enhancement of human health. There is need for additional human clinical trials to provide final evidence for claims currently in the literature.

Physiological functions and the dairy products and components associated with these functions include the following:

- (i) immunomodulation (modified whey protein concentrate; colostrum; probiotics; lactoferrin; lactoperoxidase; kappa casein glycomacropeptide; conjugated linoleic acid (CLA); calcium);
- (ii) anti-microbial/ anti-viral activity (lactoferrin/lactoferricin; lactoperoxidase; lactalbumin; casein glycomacropeptide);
- (iii) microbial toxin binding; (whey protein concentrate; beta-lactoglobulin; alpha-lactalbumin; casein glycomacropeptide);
- (iv) protection from some cancers (whey protein concentrate; lactoferrin; alpha-lactalbumin; peptides; sphingolipids; CLA; butyric acid);
- (v) anti-inflammatory activity (peptides; colostrum; immune milk);
- (vi) anti-thrombotic activity (lactoferrin; peptides);
- (vii) reduction in cholesterol levels (whey protein concentrates; whey protein isolates; stearic acid);
- (viii) opioid-like activity (peptides);
- (ix) prebiotic effects such as stimulation of growth of Bifidobacteria (casein glycomacropeptide; oligosaccharides; amino sugars);

- (x) maintaining a healthy gut (dairy products containing probiotic microorganisms);
- (xi) anti-oxidative effects (whey protein concentrates/isolates; lactoferrin; bovine serum albumin; vitamin C); and
- (xii) protection from hypertension (immune whey protein concentrate; whey protein isolates, minerals such as Ca, Mg and K) (for recent review, see: Cross, 2000; Shah, 2000; Ha, 2003). Especially the role of fermented dairy products in improving cardiovascular health by regulating blood pressure has been emphasized tremendously during the past few years (Agerholm-Larsen 2000; Hata 1996; Hayakawa 2004; Mizuno 2005; Seppo 2003; Sipola 2002; Takano, 2002; Tuomilehto 2004).

23.1.2 Cardiovascular risks in the current world

Managing heart health is receiving increased focus not only by the medical profession but also by ordinary people. 'Managing health' is the term used for the on-going trend of living healthily and focusing on the positive aspects of eating and living (Hornstra, 1998). Indeed, in the large majority of cases the etiology of hypertension is unknown, but probably multi-factorial. Environmental factors such as the Westernised life style have been associated with development of hypertension (Burszty, 1987). Diet is another factor which could be responsible for the development of hypertension. Especially sodium has been blamed for causing high blood pressure, but other dietary factors such as a high-energy intake, or enhanced fat and alcohol consumption may also contribute to high blood pressure (Chapmann, 1949; Graudal, 2005).

Hypertension is a worldwide problem:

- (i) in many industrial countries over 25% of the adult population suffers from hypertension (nearly 50 million persons in USA); and
- (ii) the number of people diagnosed with hypertension is increasing (Fields, 2004). Often patients with hypertension experience no warning signs or symptoms resulting in poorly controlled or uncontrolled hypertension which can result in stroke, heart disease and kidney disease (Chobanian, 2003). Medication or changes in the patient's lifestyle are used in typical treatment of hypertension, including diminished use of salt and alcohol and weight reduction. Anti-hypertensive drugs on the market belong to four categories: ACE inhibitors; beta-blockers; diuretics; and calcium channel blockers (Whelton, 2003).

Non-drug treatment of hypertension, for example using functional foods, began to attract attention some years ago and has been recommended as first-line treatment of mild hypertension in recent years (Burszty, 1987). Such non-pharmacological treatments should be implemented as the 'first drug of choice' for all degrees of hypertension, primarily because they lack the unwanted side effects of drug treatment.

23.1.3 Non-pharmacological alternatives to medicine treatments of hypertension

A variety of products are available on the market today, both as food supplements and as bacterial-based functional foods. Food supplements are typically a mixture of whey protein hydrolysates (BioZate, Davisco Foods; Minnesota) or single purified peptides originating from *in vitro* digestion of milk α_{s1} -casein by proteolytic enzymes (DSM; The Netherlands). As purified components, these products often have the disadvantage that they result in off-flavours and bitterness especially when used at high concentrations in a product. Two functional food products claiming to reduce blood pressure are available, one on the Japanese market (Ameal S; Calpis, Kanagawa, Japan) and one on the Finnish market (Evolus; Valio, Finland). Both contain bioactive peptides, among them the two well-known and well-documented tripeptides IPP and VPP. Recently, Cardi-04™ (Chr. Hansen, Denmark), a new product made by fermentation of milk by *Lactobacillus helveticus* strain Cardi-04™ that offers a unique concept with respect to its mode of action on the cardiovascular system has been described (Bejder, 2003).

23.2 The functional dairy product Cardi-04™: translating research into industrial applications

23.2.1 Link between dairy products and reduction of blood pressure

The role of the probiotic

In contrast to traditional probiotic products which by definition involve direct bacterial interaction with the host resulting in beneficial effects (e.g., bacteria that adhere to the intestine), the action of lactic acid bacteria (LAB) on the cardiovascular system is via the release of peptides from food proteins during the fermentation process. To date, no data has clearly shown a direct effect of probiotic bacteria on the host cardiovascular system (Tanida, 2005). Although *Lb. helveticus*, the species reported to be responsible for the release of bioactive peptides, does have the capacity to survive in the intestine, it is not considered to be a probiotic species (Malinen, 2001), as it does not fulfil all probiotic criteria (e.g., adhesion to the intestinal mucosa; Reid, 1999).

The role of proteins and peptides

Milk proteins are a good source of anti-hypertensive molecules, with peptides originating from both casein degradation (80% of the total milk proteins) known as casokinins, and peptides originating from degradation of α -lactalbumin and β -lactoglobulin (20% of the total milk proteins) known as lactokinins (Mullally, 1996). Some regions of the primary structure of caseins contain overlapping peptide sequences that exert different biological effects.

These regions have been considered as 'strategic zones' which are partially protected from proteolytic breakdown (Meisel, 1997). These peptides are inactive when present in the amino acid sequence of the precursor proteins, but can be released by proteolysis during intestinal digestion or during food processing (Gobbetti, 2002). Although chemical and physical treatments may have some influence, proteolysis by endogenous milk enzymes, and enzymes from microbial cultures such as lactic acid bacteria (*Lb. helveticus*, *Lactococcus lactis* and *Lb. delbrueckii* subsp. *bulgaricus*) have been shown to generate bioactive anti-hypertensive peptides during dairy fermentations (Gobbetti, 2000; Nakamura, 1995; Yamamoto, 1999). The responsible bacterial enzyme is generally believed to be the cell wall proteinase which is well known for digesting milk caseins into oligopeptides which are used as nutritional sources for rapid bacterial growth (Kunji *et al*, 1996; Yamamoto, 1994). The link between the proteolytic system and the cell wall proteinase specificity and activity of lactic acid bacteria (especially in the well-characterized *L. lactis* proteolytic system) has been reported as being a fundamental parameter in delivery of bioactive peptides (Gobbetti, 2000; Nakamura, 1995; Tossavainen, 2001). Although an increasing amount of effort has been used to understand the proteolytic system of lactobacilli and other thermophilic lactic acid bacteria, less is known for these species and available data are based on reports obtained from enzyme purification and characterization. Despite this lack of data, Chr. Hansen A/S has found and characterized a distinctive strain of *Lb. helveticus* which is able to release specific bioactive compounds that significantly reduce both the blood pressure and the heart rate from the breakdown of milk components.

Research was carried out over several years aiming at screening, isolating and identifying anti-hypertensive peptides from dairy products through *in vitro* and *in vivo* methods, targeting especially the activity of the well-known angiotensin-converting enzyme (ACE). The bioactive peptides affecting the heart rate reported recently as being produced by the activity of the Cardi-04™ bacterium on milk proteins represent a pioneering bioactivity characteristic. Whereas the beneficial effects of blood pressure lowering are well established, the potential beneficial effects of fermented milk peptides resulting in a heart rate reduction have only been addressed recently (Mulder, 2007). Indeed, the increase in left ventricular diastolic filling, the improvement in myocardial perfusion together with the reduction of myocardial oxygen demand are at the origin of the beneficial effects of 'pure' heart rate reducing pharmaceutical agents used in patients with angina and the basis for models of heart failure and myocardial infarction, illustrating that heart rate reduction exerts beneficial effects. While several drugs, such as If current blockers, Ca-antagonists, and β -blockers reduce resting heart rate, ingestion of sour milk has never been shown to reduce heart rate alone or in combination with blood pressure reduction. In research described here we demonstrate the effects of a fermented milk product that results in blood pressure and resting heart rate reduction in experimental hypertension.

23.2.2 Scientific documentation: from *in vitro* to *in vivo* studies

In vitro screening

The research approach of focusing on two screening systems; the enzymatic characteristics of the bacterial cell wall proteinases and the properties of the strains to reduce ACE activity *in vitro* has been very fruitful (Flambard, 2002; 2007). The high cell wall-associated proteinase activity of lactic acid bacteria was reported to be a sufficient screening method for targeting lactic acid bacteria with ACE inhibitors properties (Fulgsang, 2003). However, the data did not illustrate that the specificity of the cell wall proteinase from *Lb. helveticus* is a crucial parameter nor how it influences the anti-hypertensive functional food property. The specificity of the enzyme determines the sequence of the liberated peptides which is a critical parameter for any bioactivity of the peptide. Our research, carried out mostly on the *Lb. helveticus* species, revealed three specific cell wall proteinase patterns that were correlated to ACE inhibition activity *in vitro* (Flambard, 2002; 2007). From the results, *Lb. helveticus* Cardi-04™ was documented as a natural lactic acid bacterium with a unique proteolytic pattern.

The anti-hypertension effect of selected strains, based on the best *in vitro* ACE inhibition values, was then studied *in vivo* by oral administration of fermented products to rats. The correlations between *in vivo* and *in vitro* systems are not apparent. Many of the strains inhibiting ACE activity *in vitro* do not reduce blood pressure in rats. The *in vitro* test mimics only the last interaction of the substrate with the ACE, while the *in vivo* system involves many other steps comprising unknown proteolytic and metabolic parameters. For example, phosphorylation or methylation may occur following ingestion, and gastrointestinal and blood peptidases and proteinases may further breakdown casein-derived oligopeptides.

Two products fermented by *Lb. helveticus* and one by *Lb. acidophilus* significantly reduced the systolic and diastolic blood pressure of spontaneously hypertensive rats (SHR), measured by the tail cuff method. The product fermented by *Lb. helveticus* Cardi-04™ was selected for further study as this product showed the best potential, reducing the blood pressure by 20 mm Hg on spontaneously hypertensive rats (SHR) (still after 24 hours, using the general tail cuff measurement method) and presenting the unique feature of reducing the heart rate. To further document the anti-hypertensive properties of the *Lb. helveticus* Cardi-04™ strain, a sensitive telemetry detection method was employed allowing a visualisation of the kinetic effect of the product over time on SHR (Fig. 23.1 and Fig. 23.2). Safety and effectiveness of the product were monitored accordingly.

Safety and efficacy: importance of the clinical documentation

Choice of the detection system

Anti-hypertension studies have mainly been performed *in vitro*, by simple incubation of ACE with compounds or peptide pools, and by subsequent measurement of the inhibition of the enzyme. The anti-hypertensive effects

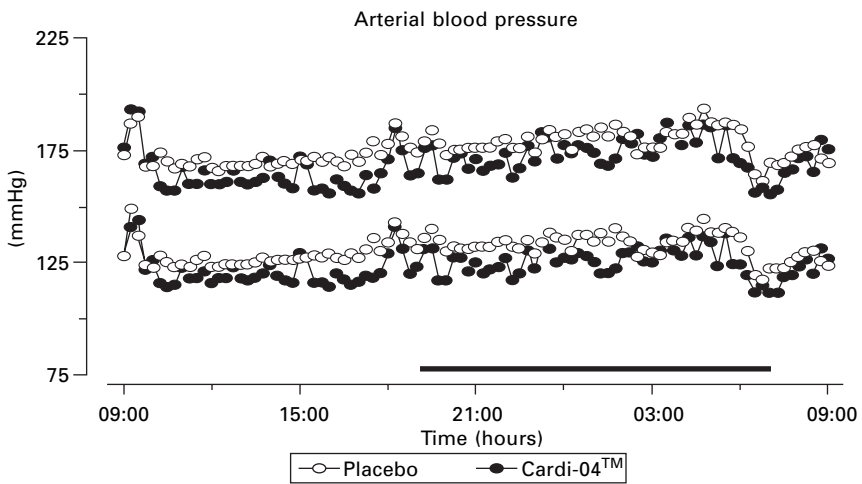


Fig. 23.1 Time-course of systolic blood and diastolic pressures determined in conscious hypertensive (SHR) rats treated with placebo (○) or Cardi-04™ fermented milk (●).

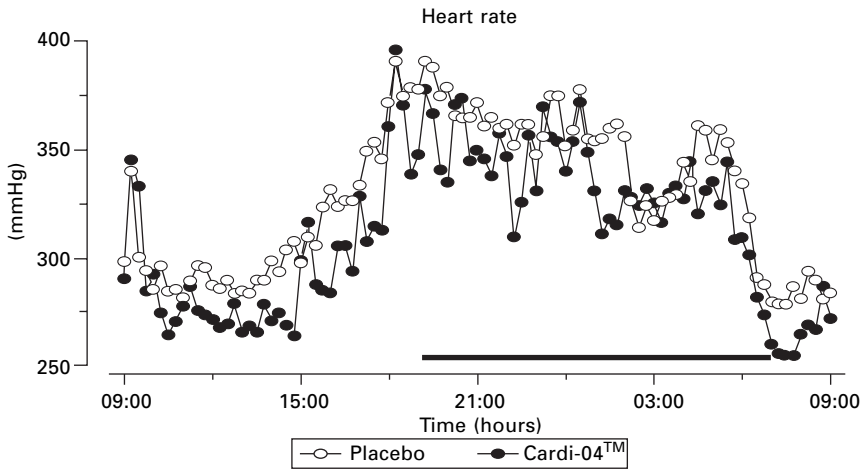


Fig. 23.2 Time-course of heart rate determined in conscious hypertensive (SHR) rats treated with placebo (○) or Cardi-04™ fermented milk (●).

of some purified peptides or fermented milks have also been successfully tested *in vivo* by the oral administration of peptides to rats and humans (Hata, 1996; Nakamura, 1995). For animal studies, two methods are used today to follow the blood pressure and heart rate variations. The tail cuff method is the traditional measurement system for screening procedures whereas the telemetry method is used for accurate data reporting.

Tail cuff method: This method is a traditional indirect method of blood pressure measurement. Rat-tails are passed through a cuff (13 mm long, with a 9 mm diameter) and immobilized by adhesive tape in a V-shaped block between a light source above and a photo resistor below the tail. Blood flow in the tail produces oscillating waveforms that are displayed in real time on a monitor and computer analysed before and during a programmable routine of cuff inflation and deflation.

Advantages: (i) This method gives a fast and clear picture of the effect of the product on blood pressure and heart rate; and (ii) has moderate costs. Disadvantages: (i) No continuous reporting of the data; and (ii) the method generates a lot of stress for the rats due to frequent human interventions.

Telemetry method: This method is a direct method of blood pressure measurement and involves the use of a saline-filled catheter and a radio transmitter operated into the rats under anaesthesia. Once in place, the catheter allows a measurement of blood pressure without the need to handle the rats. Telemetry offers the ability to obtain a high fidelity recording of blood pressure continuously, over relatively long periods of time, in conscious, free-moving animals, without the limitations of restraint or anaesthesia. This method can detect small variations in both systolic and diastolic blood pressures, and heart rate (Fig. 23.1 and Fig. 23.2). This technique reduces the stress factor by limiting the human intervention and continuously monitors the blood pressure parameters.

Advantages: (i) accurate data generation; (ii) kinetic effect of the product on blood pressure and heart rate determined; and (iii) no stress for the animals. Disadvantage: high cost.

Efficacy on blood pressure

Milk fermented by *Lb. helveticus* Cardi-04™ significantly reduces the systolic and diastolic blood pressure of spontaneously hypertensive rats. The results (Fig. 23.1) show that the blood pressure lowering effect of milk fermented by Cardi-04™ occurs during the 24 hours after feeding with a systolic blood pressure which falls from 176 ± 9 to 169 ± 9 mm Hg ($p < 0.05$). The same reduction pattern is observed for the diastolic blood pressure which is reduced from 131 ± 5 to 123 ± 5 mm Hg ($p < 0.05$) (Fig. 23.1). A significant effect is already observed in the period 3–6 hours after feeding. It is maintained through the 12–15 hour period and the 21–24 hour period after feeding. An investigation of the chronic effect of the anti-hypertensive product on SHR over a period of eight days indicates a continuous reduction of blood pressure from 8 mm/Hg at day one to 20 mm Hg at day four and a stabilization which lasts at least until day eight.

In order to satisfy safety concerns and to provide documentation for substantial health claims, the fermented product made with Cardi-04™ was enrolled in a prospective, randomized, placebo-controlled, double blind tolerability study, to record potential adverse effects in normotensives, a

population that is representative of the population majority. Cardi-04™ shows complete safety without any adverse side effects.

Efficacy on heart rate

Simultaneously, an effect of Cardi-04™ on heart rate is observed in SHR (Fig. 23.2). The product not only reduces the oscillation variation of the heartbeat, it also reduces the pulse significantly by 15 beats/min averaged over 24 h, with a peak reduction of 26 beats/min observed 12–15 h after feeding the rats (Fig. 23.2).

These two features, blood pressure and heart rate reduction, associated in one product is a significant novelty of this functional food. None of the food today known to interact with the cardiovascular system shows a heart rate reducing effect.

The clinical data on animals reveals that the product is most effective when the animals are in an active period (black bar in the x-axis; Fig. 23.2). An extrapolation of the results from animals to humans therefore strongly suggests that the product would be especially efficient as a prevention factor when daily triggers that influence cardiac rhythm, such as stress peaks or slight hypertension, occur. Consequently, in addition to benefiting the at-risk population, the described functional food could benefit the group of people with no reported cardiovascular problems or non-drug controlled cardiovascular disease; i.e. stressed people, people suffering from shortness of breath, and slightly hypertensive people. The overall effect of a fermented product made by Cardi-04™ as a prevention factor, would be to bring blood pressure to normal levels or to restore heart rate rhythm to a normal state. The total benefit of having both reduction of hypertension and heart rate in a single product would be a reduction of organ damage over time.

23.2.3 Action mechanisms

Blood pressure reduction

Blood pressure is controlled by various factors, such as heart rate, arterial contraction, blood volume, and the nervous system. The renin angiotensin system (RAS) and the kinin kallikrein system (KKS) in particular, have an important role in the control of blood pressure. Angiotensin-converting enzyme (ACE) is a multi-functional biological agent associated with the regulation of peripheral blood pressure in mammals by converting angiotensin-I into angiotensin-II, a highly potent vasoconstrictor molecule. In the KKS, ACE hydrolyses bradykinin into a vaso-inactive peptide. Inhibition of ACE is known to decrease hypertension by two additive effects; reduction of the vasoconstricting activity of angiotensin-II, and enhancement of the vasodilating activity of bradykinin (Petrillo, 1982).

The mode of action of Cardi-04™ on blood pressure reduction has been studied in spontaneously hypertensive rats by measuring the response to increasing doses of angiotensin-I (Fig. 23.3). As has already been shown

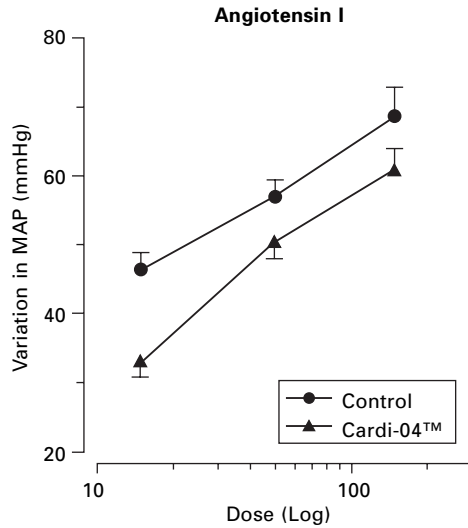


Fig. 23.3 Values (mean $\pm$ SEM) of the variations in mean arterial blood pressure induced by increasing doses of angiotensin-I (15, 50 and 150 $\mu\text{g.kg}^{-1}$) in untreated (black column and black circle), and Cardi-04™ treated animals (hatched column and black triangle). *: $p < 0.05$ vs. untreated.

with peptides generated by enzymatic digestion of casein and whey proteins by other *Lb. helveticus* strains, Cardi-04™ liberates bioactive compounds that interfere with the renin-angiotensin system by inhibiting the well-known angiotensin converting enzyme (ACE). While the antihypertensive activities of these peptides do not compare to those of the commonly used drugs such as Captopril and Bradykinin potentiator B, mixtures of these peptides may exert protective anti-hypertensive effects through daily ingestion of functional food made with Cardi-04™. Milk products containing such peptides are being developed to act as adjunct therapies in maintenance of good health.

Heart rate reduction

To date, the mechanism of action is unknown. From a general point of view, the reduction of heart rate has a very positive impact on general cardiovascular health, especially when considering diseases such as angina pectoris. When one reduces the heart rate, the diastolic time is by definition increased and the systolic time is reduced. This results in a weak heart becoming more efficient in pumping blood and consequently delivering more oxygen enriched-blood to meet the needs of the body's other organs.

All coronary heart diseases benefit indirectly from a reduction of heart rate. This large group of diseases forces the heart to work harder for multiple reasons. When the heart is beating faster, it burns more oxygen instead of distributing it to other organs. Reducing the heart rate helps the heart pump blood more efficiently.

Finally, arrhythmia type of diseases such as tachycardia (fast pulse with regular pace) also benefit from lowering the heart rate. Even though Cardi-04TM is, by definition, not a drug, the results presented here show that Cardi-04TM could have potential in preventing the further weakening of the heart in heart rate dysfunction-related diseases.

23.3 Product development

23.3.1 Consumer acceptance

To support American consumer protection and rights, the Federal Trade Commission (FTC) encourages the US Food and Drug Administration (FDA) to consider allowing truthful, non-misleading health claims for more foods. The FTC gives comments to the (FDA) with the main objective to ‘help consumers make informed dietary choices and promote competition among manufacturers to develop and market healthier food products’. Consequently, FTC does not classify claims by type (functional or structure) as the FDA does, but requires that the adviser must have a reasonable basis for all express and implied product claims based

- (i) on the totality of the evidence,
- (ii) ‘competent and reliable scientific evidence’ based on accepted norms in the relevant field of research, and
- (iii) based on ‘reasonable consumer’ standard.

23.3.2 Regulation and labelling

When bringing a functional food to market, careful consideration needs to be given to the form of the functional food as well as to the claims that will be associated with the product. This is to ensure that the marketing of the product is legal in the markets under consideration and that the consumer is properly protected. The regulation of functional dairy products is discussed in more detail in Chapter 12 (von Wright).

Even though the term ‘functional food’ is not specifically defined by law in the United States, regulation of functional food is the responsibility of the Food and Drug Agency (FDA). One important element of this regulation is the control of claims which may appear on the label of the functional food. Structure and function claims describe the effects of the functional food on the normal functioning body but may not imply that the food can prevent or cure a specific disease. A disease-reduction claim implies a relationship between the functional food and a specific disease or health condition. A disease-reduction claim can only be used following approval from the FDA and requires a thorough review of the scientific evidence for the claim. With the current level of scientific documentation, a structure and function claim could be made on yoghurt made with Cardi-04TM.

The European Union is in the process of harmonizing legislation in the area of health claims. Currently, food producers can only make nutrition claims or general health claims selected from a positive list of permissible claims. Novel health claims, in particular those which claim to reduce the risk of specific disease such as heart disease would require an authorization by the European Food Safety Authority (EFSA).

The situation in Japan is clearer where the concept of Foods for Specified Health Use (FOSHU) has been used to regulate functional foods since 1991. This system is based on a list of approved foods and ingredients, including specific microorganisms which the Minister of Health and Welfare believes to have substantiated health benefits. In order to use the FOSHU label, the food producer simply needs to be able to show the presence of the functional ingredient. Cardi-04TM is not currently on the list of approved ingredients.

23.3.3 Intellectual property rights

Patents exist for the benefit of society; ensuring that useful inventions can be described, further developed and possibly commercialized, without the danger of copy products reaping the benefits of the often-high investment in research and development required to create the invention. The technology surrounding Cardi-04TM is protected by a number of patent applications. These include the use of lactic acid bacteria to prepare anti-hypertensive peptides and functional foods containing these (WO 2003/082019 A3; WO 2004/015125 A1) as well as fermented food products containing bioactive compounds which reduce the heart rate (WO 2004/089097 A1). Additional patent applications have been submitted but these are not yet published.

23.4 Future trends

It is of fundamental importance that professionals with different viewpoints, such as dieticians, the food industry, the health authorities, the scientific community, and the media continue to work together to ensure that the public has accurate information regarding the emerging area of functional food and nutrition science. Currently, the food industry is facing many challenges with consumer acceptance and health authority approval of functional foods. As no regulation properly addresses functional food products, too many foods claiming health benefits without any substantial clinical documentation are on the market. Analyzing the content of physiologically active components in functional foods and evaluating their role in health promotion will be necessary with solid scientific evidence (clinical trial, animal studies, experimental *in vitro* laboratory studies, epidemiologic studies) required to support this emerging area of food and nutrition. Knowledge of the mode of action of active food components (physiological role; level of intake; synergy with other components; safety) from both phytochemicals

and zoochemicals, will need to be accurately evaluated and communicated as each functional food should be evaluated on the basis of scientific evidence to ensure appropriate integration into a varied diet.

23.5 Conclusions

Managing cardiovascular health is part of the on-going trend of living healthy and focusing on the positive aspects of eating and living. Medication or changes in lifestyle are used in typical treatments of hypertension. However, today non-drug treatments of hypertension attract attention especially with regard to prevention of factors which lead to the development of hypertension.

Functional foods based on fermented milk have been shown to reduce the blood pressure through the release of bioactive ACE inhibitory peptides. Today, only *Lb. helveticus* is well documented and reported being capable of releasing the specific peptides from caseins. As for probiotic products, the most important feature of the validation of a potential functional food is the clinical documentation. The product can only be validated if the concept has been proven in human trials with a certain level of pre-clinical documentation on animals.

The potential of other lactic acid bacteria to ferment or release anti-hypertensive peptides from casein or other protein sources should be evaluated and validated to open the way to a number of other food applications and food products. This would support the current increased market for probiotics in general or health benefit products within the cardiovascular domain in particular.

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