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Probiotic lactic acid bacteria in the gastro-intestinal tract: health benefits, safety and mode of action

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Abstract

Lactic acid bacteria (LAB) have received considerable attention as probiotics over the past few years. This concept has grown from traditional dairy products to a profitable market of probiotic health supplements and functional foods. Extensive research is done on novel potential probiotic strains, with specific emphasis on their health benefits and mode of action. Criteria for the selection of probiotic strains have only recently been formulated by the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO). Several *in vitro* techniques have been developed to evaluate the probiotic properties of strains. In many cases, this is followed by *in vivo* tests. Safety studies are also obligatory, as a few cases of bacteremia caused by LAB have been reported. This review focuses on the health benefits and safety of LAB probiotics, the criteria used to select a probiotic, mode of action and the impact these organisms have on natural microbiota in the gastro-intestinal tract.

Keywords: probiotics, lactic acid bacteria

1. Introduction

The concept of probiotics evolved from a theory first proposed by Metchnikoff in 1908, which suggested that the long and healthy life span of Bulgarian peasants could be ascribed to the consumption of fermented milk products. Over the years many definitions for probiotics have been proposed. Fuller (1989) defined probiotics as 'a live microbial feed supplement, which beneficially affects the host by improving its intestinal microbial balance'. Marteau *et al.* (2002) considered probiotics as 'microbial cell preparations or components of microbial cells that have a beneficial effect on health and well-being'. Gorbach (2002) defined probiotics as 'living microorganisms which upon ingestion in certain numbers exert health benefits beyond inherent general nutrition'. In 2002, the FAO/WHO defined a probiotic as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2002).

Many claims relating to probiotic properties have been made, varying from the prevention of infectious diseases (Rolfe, 2000), curing of irritable bowel syndrome, alleviation of allergies, digestion of lactose and lowering of serum cholesterol levels (Andersson *et al.*, 2001) to the prevention of cancer (Gibson and Macfarlane, 1994). The question now arises as to whether any given microorganism that adheres to any one or more of these definitions could be considered a probiotic.

In general, probiotic lactic acid bacteria (LAB) do not cause immunological side effects (Salminen *et al.,* 1998). However, *Lactobacillus casei* has been associated with symptoms of fever, arthritis and hepatobiliary lesions (Schwab, 1993). Symptoms such as these may be caused by cell wall components such as peptidoglycans that elicit cytokines (Miettinen *et al.,* 1996). Immunological side effects are often caused by cells that invade epithelial cells, migrate through mucus (Tang *et al.,* 1993) and degrade mucus (Salminen *et al.,* 1998). In a recent publication (Vogel, 2008), medical practitioners from the University

of Utrecht, the Netherlands, announced that a probiotic supplement of six strains was responsible for the death of 24 patients. It is, however, important to note that the patients suffered from acute pancreatitis and that they were immune-compromised when treated. In another report, *L. casei* and *Lactobacillus rhamnosus* have been associated with bacteremia and endocarditis (Cannon *et al.,* 2005). This is extremely rare (Salminen *et al.,* 1998). Such strains would be able to metabolise glycoproteins and lyse fibrin clots (Oakley *et al.,* 1995) and need to be tested for these properties. A few strains of LAB have been associated with urinary tract infections, wound, tissue and other infections (Vankerckhoven *et al.*, 2008). In immunocompromised patients some lactobacilli have been associated with arthritis and meningitis (Vankerckhoven *et al.*, 2008).

Of specific concern is the safety of *Enterococcus* spp. In a few cases *Enterococcus* infections have been associated with abnormal physiological conditions, underlying disease and immunosuppression (Franz and Holzapfel, 2004). This is rather surprising, as these organisms are closely associated with the human environment and gastro-intestinal tract and one would assume this minimises the chance of causing diseases, at least in healthy individuals. Clinical trials have shown that the probiotic *Enterococcus faecium* SF68 is effective in the prevention and treatment of diarrhoea (Lewenstein *et al.,* 1979) and lowers the cholesterol levels in serum (Agerbaek *et al.,* 1995). *E. faecium* CRL 183 lowered cholesterol levels by 43% in *in vitro* tests (Rossi *et al.,* 1999). *E. faecium* Fargo 688® alleviated the symptoms of irritable bowel syndrome and was successfully used in the production of cheddar cheese (Allen *et al.,* 1996; Gardiner *et al.,* 1999). Despite these findings, controversy still surrounds the use of enterococci as probiotics (Vankerckhoven *et al.*, 2008).

Five years ago the Scientific Committee on Animal Nutrition classified *E. faecium* DSM 7134, NCIMB 10415, CECT 4515, NCIMB 30098, NCIMB 1181, DSM 5464, DSM 3520, NCIMB 10415, DSM 4788, DSM 4789 and *Enterococcus mundtii* CNCM MA 27/4E safe to be used as probiotics in animals. In studies conducted on rats with a probiotic strain of *E. mundtii* (ST4SA), no haematological and histological abnormalities were detected (Botes *et al.*, 2008).

Most studies on probiotic LAB are performed *in vitro* (Lin *et al.*, 2006). Adhesion to mucus, glycoproteins and epithelial cells are studied using human cell lines such as Caco-2, HT-29 and HT29-MTX (Sambuy *et al.*, 2005). *In vitro* models, simulating the gastrointestinal tract in humans, have been developed to represent *in vivo* conditions. Examples of such models are the gastro-intestinal model (GIM) developed by Botes *et al.* (2008), the stomach and duodenum (upper gastro-intestinal tract) model (Mainville *et al.*, 2005), an anaerobic three-vessel continuous-flow culture system (Payne *et al.*, 2003), a three-stage compound continuous culture system simulating the proximal colon (Macfarlane *et al.*, 1998), an upper gastro-intestinal model representing the stomach, duodenum, jejunum and ileum (Minekus *et al.,* 1995) and a similar model mimicking the colon (Minekus *et al.,* 1999), and a simulated human intestinal microbial ecosystem (SHIME) developed by Molly *et al.* (1993). Although *in vitro* studies have to be done and are valuable in the selection of a probiotic, claims regarding the safety of a strain can only be made once *in vivo* trials have been carried out (Mishra and Prasad, 2005).

Genome sequencing has revealed a number of genes encoding specific enzymes that may be considered as favourable probiotic properties, e.g. ornithine decarboxylase and its role in acid tolerance of *Lactobacillus acidophilus* NCFM (Alterman *et al.,* 2005), bile salt hydrolase (BSH) and bile transport by *Lactobacillus johnsonii* NCC 533 (Pridmore *et al.*, 2004). In the case of *Lactobacillus plantarum* WCFS1, gene clusters encode cytoplasmic membrane and cell-wall-associated functions involved in bile tolerance (Bron *et al.*, 2004a,b,c).

2. Health benefits and safety of probiotics

Lactic acid bacteria have a long history in the dairy industry. For more than 70 years, a number of species have been used as probiotics (Salminen *et al.,* 1998). The biosafety of probiotics was addressed at a workshop in 2006 as part of the EU-PROSAFE project (Vankerckhoven *et al.*, 2008). Sixty academics and scientists from the industry took part in the discussions. Methods for testing antimicrobial susceptibilities, recommendations for the grouping of strains into susceptible, intermediate and resistant categories (and the setting of epidemiological cut-offs), and the ability of strains to share antibiotic resistance through horizontal gene transfer have been discussed (Vankerckhoven *et al.,* 2008).

Immune modulation

Microbiota in the intestinal tract regulate the systemic and local immune responsiveness by affecting the development of gut associated lymphoid tissue (GALT) at an early age (Dugas *et al.,* 1999). Microbial colonisation leads to maturation of the humoral immune mechanisms, particularly circulation of the IgA and IgM-secreting cells. The balance of the different T helper (Th) subsets is particularly important in mucosal immunity. After priming, memory B and T cells migrate to effector sites. This is followed by active proliferation, local induction of certain cytokines and production of secretory antibodies (IgA). Upon antigen exposure, immune cells respond with the release of a host of cytokines that then direct the subsequent immune responses. One of the major mechanisms by which the GALT maintains homeostasis is

via local cytokine regulation, particularly TGF-β-associated low-dose tolerance immunity.

A strain of *L. casei* that inhibited the growth of pathogenic strains of *Pseudomonas aeruginosa* and *Listeria monocytogenes* in mice led to an increase in the levels of macrophages (Driessen and de Boer, 1989). In another study, Miettinen *et al.* (1996) observed that LAB could induce the production of proinflammatory cytokines, tumor necrosis factor alpha and interleukin-6 from human peripheral blood mononuclear cells (PBMC), thereby stimulating non-specific immunity. Schiffrin *et al.* (1995) showed that strains of *L. acidophilus* and *Bifidobacterium bifidum* could enhance non-specific immunity and concluded that specific LAB could play a role in specific age groups, specifically neonates or the elderly.

A limited number of studies are being conducted on stimulation of the host's immune system (Isolauri *et al.*, 2001). Malin *et al.* (1996) investigated the effect of oral *L. rhamnosus* GG on the intestinal immunological barrier in a small study of 14 children with Crohn's Disease and seven control patients (hospitalised for investigation of abdominal pain but with no evidence of intestinal disease). Strain GG was administered to patients and controls at 10^{10} colony forming units (cfu), twice daily, mixed in liquid. A significant increase in IgA immune response was recorded for the Crohn's patients, but not for the controls. In another study, on children with mild to moderate stable Crohn's Disease, administration with strain GG improved gut barrier function and clinical status after six months of therapy (Gupta *et al.*, 2000). However, a randomised, double-blind, placebo-controlled trial of 45 post-surgery Crohn's patients given strain GG for one year proved less effective than the placebo in preventing disease recurrence (Prantera *et al.*, 2002).

Anticarcinogenic and antitumour activity

Microbial enzymes such as azoreductase, β-glucuronidase and nitroreductase may convert procarcinogens into carcinogens and cause colon cancer (Fernandes and Shahani, 1990; Goldin, 1990). Goldin and Gorbach (1977) have shown that *L. acidophilus* could decrease nitroreductase, azoreductase and β-glucuronidase activities in carnivorous animals. In a subsequent study, Goldin *et al.* (1992) have shown that *L. rhamnosus* GG could lower bacterial β-glucuronidase activity in the large intestine.

LAB may also retard or prevent the initiation and promotion of tumours. *L. acidophilus* and *Lactobacillus bulgaricus* and/or *L. casei* suppressed Ehrlich ascites tumour or Sarcoma 180 in mice (Goldin *et al.*, 1996). Tumour suppression is associated with intact viable cells, intact dead cells, and cell wall fragments of lactobacilli and bifidobacteria. *Lactobacillus* GG positively affected the initiation or promotion of DMH-induced tumours in rats on a high-fat diet. Orally administered strains of *L. casei* were effective in preventing the recurrence of superficial bladder cancer (Aso *et al.*, 1995).

Nitrites used in food processing are converted to carcinogenic nitrosamines in the GIT. Cellular uptake of nitrites by lactobacilli and bifidobacteria has been shown *in vitro* (Grill *et al.,* 1995). Bile salts have been implicated in the initiation of colon carcinogens (Lewis and Gorbach, 1972). *L. acidophilus* reduced the biotransformation of primary to secondary bile salts, thus reducing the possible initiation of cancer (Fernandes and Shahani, 1990). Modler *et al.* (1990) suggested that the reduction of intestinal pH, through metabolic activities of LAB, could inhibit the growth of putrefactive bacteria and thus prevent large bowel cancer.

Aflatoxins produced by moulds are known to cause cancer. At least 13 aflatoxins, of which B_1, B_2, G_1, G_2, M_1 and M_2 are the best known, have been described for *Aspergillus* spp. (Groopman *et al.*, 2008). Aflatoxin B_1, the best studied of all, causes liver cancer in humans. Gourama and Bullerman (1995) reported the inhibition of mould growth and aflatoxin production by LAB: *L. casei* subsp. *pseudoplantarum* inhibits the biosynthesis of aflatoxins B_1 and G_1 (Gourama and Bullerman, 1997). *L. rhamnosus* GG binds aflatoxin B_1, and to a lesser extent aflatoxins B_2 and G_1 (El-Nezami *et al.,* 1996).

Reduction of cholesterol

Several studies in animals and humans have focused on the effect of fermented milk or milk containing viable LAB on serum cholesterol levels. A strain of *Streptococcus thermophilus* and *L. acidophilus* reduced cholesterol levels in rats (Grunewald, 1982). Milk fermented with LAB and *Streptococcus cerevisiae* led to lower serum cholesterol levels, phospholipids and bile acids in the faeces of mice (Tamai *et al.,* 1996). Similar findings were reported by Fukushima and Nakano (1996) and Tortuero *et al.* (1997). Zacconi *et al.* (1992) showed that serum cholesterol levels were lower in axenic mice colonised with *E. faecium* and *L. acidophilus*. Gilliland and Walker (1990) have shown that the consumption of *L. acidophilus* reduced serum cholesterol levels in pigs that have been fed a high-cholesterol diet. Studies conducted by de Rodas *et al.* (1996) supported these findings. However, the serum lipoprotein levels of 334 individuals remained unchanged when they were treated with *L. acidophilus* and *L. delbrueckii* subsp. *bulgaricus* (8×10^6 cfu/day) (Lin and Chen, 2000). *E. faecium* administered over six weeks to adults resulted in an initial increase in total cholesterol and LDL, followed by a sharp decrease two weeks after termination of treatment (Mikeš *et al.,* 1995). The decrease corresponded with an increase in the reduction of iodonitrotetrazolium and superoxide

production by peripheral neutrophils and an elevated production of IgG.

In all studies conducted thus far, the real factor responsible for a reduction in cholesterol levels remains unknown. Klaver and Van der Meer (1993) suggested that the reduction of cholesterol is not due to assimilation or to a direct interaction between the bacteria and cholesterol, but rather due to the co-precipitation of cholesterol with deconjugated bile salts at pH values below 6.0. This would not explain reduction of cholesterol *in vivo* as the pH of the lower GIT is neutral to alkaline. Marshall and Taylor (1995) also observed co-precipitation of cholesterol with deconjugated bile salts, but also reported cholesterol removal in the absence of bile. They suggested a physical association between cholesterol and the cell surface.

Bile salt deconjugation may also play a role in the reduction of cholesterol. Cholesterol and bile salt metabolism is closely linked, where cholesterol is the precursor for synthesis and bile salts the water-soluble excretory end product. Bile salts are deconjugated during enterohepatic circulation (EHC) by bile salt hydrolase (BSH) (E.C.3.5.1.24). The free bile acids as well as glycine and taurine, are not so easily reabsorbed and are excreted in the faeces (De Smet *et al.,* 1994). The loss in bile salts increases the catabolism of cholesterol to bile acids, resulting in lower cholesterol levels (De Rodas *et al.,* 1996; Driessen and De Boer, 1989). BSH activity has been shown in *Lactobacillus, Enterococcus, Peptostreptococcus, Bifidobacterium, Clostridium* and *Bacteroides* spp. (Bateup *et al.,* 1995; Grill *et al.,* 1995). The BSH hypothesis has not definitely been proved. Recent observations indicate that free bile acids are less effectively absorbed by the active transport system in the ileum, but are more effectively reabsorbed in the intestine and colon by passive diffusion (Marteau *et al.,* 1990).

De Smet *et al.* (1998) have shown that feeding pigs with cells of *Lactobacillus reuteri* containing active BSH resulted in significant lowering of serum total and LDL-cholesterol concentrations, accompanied by a gradual increase in *Lactobacillus* cell numbers. No change in HDL cholesterol concentration was observed. The authors have also shown that during the final three weeks of changing from a high fat diet to a regular diet, the cholesterol levels significantly decreased and the differences in total and LDL-cholesterol concentrations between the treated and untreated animals largely disappeared. *L. reuteri* cells were gradually washed out and they did not succeed in permanently colonising the intestinal tract.

Taranto *et al.* (2000) have shown that administration of *L. reuteri* CRL 1098 (10^4 cells/day) to mice for 7 days effectively prevented hypercholesterolemia. A 17% increase in the ratio of high-density lipoprotein to low-density lipoprotein was observed. The total serum cholesterol and triglyceride levels decreased by 22 and 33%, respectively, in mice that received *L. reuteri*.

Alleviation of lactose intolerance

Many individuals, especially of Asian and African descent, lack the intestinal mucosal enzyme β-galactosidase (lactase) or suffer from a reduction in lactase activity caused by intestinal infection (e.g. rotavirus gastroenteritis). *Streptococcus salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus* produce high levels of β-galactosidase. Both species are sensitive to bile salts, leading to the release of high levels of β-galactosidase in the GIT. *L. acidophilus* is bile resistant and has lower levels of β-galactosidase compared to *S. salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, but grows in the GIT and may produce β-galactosidase over a longer period (Driessen and De Boer, 1989). Lactose from yoghurt and milk containing the probiotic *L. acidophilus* were better absorbed by subjects with low β-galactosidase activity (Sanders, 1993). There were fewer symptoms of lactose intolerance and bacterial fermentation of undigested lactose was also evident in breath hydrogen concentrations.

Normalisation of stool transit

Lactobacillus GG accelerated the recovery of acute watery diarrhoea in young children (Colombel *et al.,* 1987). In another study (Siitonen *et al.,* 1990) volunteers with diarrhoea and on erythromycin treatment reacted positively when they received *Lactobacillus* GG. Symptoms of diarrhoea, stomach pain, abdominal pain and nausea were less frequent and recovery much quicker. Similar findings have been reported by Isolauri *et al.* (1991) and Majamaa and Isolauri (1997). *E. faecium* SF68 proved as effective in children with paediatric diarrhoea (Bellomo *et al.,* 1980). In other studies *E. faecium* SF68 also reduced the duration of diarrhoea in adults (Buydens and Debeuckelaere, 1996).

Another form of diarrhoea more difficult to treat is that caused by *Clostridium difficile*. Symptoms usually occur after antibiotic treatment, which makes treatment of this disorder with antibiotics less optimal. Treatment with *L. rhamnosus* GG improved symptoms of intestinal disorders (Bennett *et al.,* 1996).

Salminen *et al.* (1988) studied the effect of *L. acidophilus* NCFB 1748 on 21 female cancer patients that received pelvic radiotherapy. Patients who consumed milk fermented by the strain experienced less diarrhoea. The effect of different LAB on different types of diarrhoea has been reviewed by Gorbach (2002). Further research is needed to determine which mechanisms LAB use to relieve diarrhoea. *L. acidophilus* and *Bifidobacterium* spp. have been shown to relieve constipation, but more conclusive data are needed (De Vrese and Marteau, 2007; Fuller,

1989). A study conducted by Koebnick *et al.* (2003) has shown that *L. casei* strain Shirota (LcS) of Yakult resulted in a significant improvement in self-reported severity of constipation and stool consistency, from the second week of treatment onwards. Eighty-nine percent of the patients that received LcS versus 56% of the placebo group reacted positively to the treatment. Marteau *et al.* (2002) have shown that a fermented milk product containing *Bifidobacterium animalis* strain DN-173 010 of Danone shortened the colonic transit time in healthy women.

Hepatic encephalopathy

Patients with liver failure have higher levels of ammonia, leading to encephalopathy. Ingestion of *L. acidophilus* lowered levels of faecal urease and blood ammonia (Salminen *et al.,* 1993). *E. faecium* SF68 also proved effective in lowering blood ammonia levels (Loguercio *et al.,* 1987). Probiotic preparations containing *E. faecium* SF68 proved to be as effective as lactulose in lowering blood ammonia and in improving mental state and psychometric performance (Loguercio *et al.,* 1987). The effects of strain SF68, contrary to that of lactulose, persisted longer after treatment withdrawal.

Treatment of peptic ulcers

Lactic acid produced by *L. acidophilus* inhibited the growth of *Helicobacter pylori* in *in vitro* tests (Bhatia *et al.,* 1989). Only strains of *L. acidophilus* and *L. rhamnosus,* obtained from the Commonwealth Scientific and Industrial Research Organisation (CSIRO) starter culture collection, Australia, inhibited the growth of *H. pylori* (Midolo *et al.,* 1995). Metabolic products other than lactic acid may also play a role. *Lactobacillus salivarius* inhibited the attachment of *H. pylori* and interleukin-8 release *in vitro* (Kabir *et al.,* 1997). Probiotic lactobacilli are acid tolerant and may survive conditions in the stomach. They may thus be good candidates for treatment of peptic ulcers.

3. Characteristics of probiotic LAB and mode of action

Acid tolerance

Lactic and acetic acids are the main metabolic end products produced by LAB. Both acids are more effective in their undissociated form as they penetrate the microbial cell and interfere with essential cell functions and reduce intracellular pH (Holzapfel *et al.,* 1995). Acetic acid is more effective against Gram-negative bacteria, moulds and yeasts (Gilliland, 1989). Bifidobacteria produce acetic and lactic acids at a ratio of 3:2 and may be more effective in the control of Gram-negative pathogens and yeasts in the GIT compared to *Lactobacillus* spp.

Resistance of LAB to acid is strain dependent and is influenced by pH and exposure time. Strains with good colonising properties were less resistant to low pH in *in vitro* studies (Mishra and Prasad, 2005). Strains of *Lactobacillus paracasei* showed low resistance to gastric acid *in vitro*, although survival in a simulation of the gastro-intestinal tract was excellent (from a starting dose of approximately 10^8 cfu/ml to 10^7 cfu/ml even after 3 hours of incubation at pH 3; Mishra and Prasad, 2005). Although *in vitro* assays may provide information on acid tolerance, the method should be refined to correlate with *in vivo* tests, or validated by human clinical trials. The survival of *B. bifidum*, *L. acidophilus*, *L. bulgaricus* and *S. thermophilus* was measured under physiological conditions (e.g. peristalsis, changes in pH, and changes in concentrations of enzymes and bile) in a stomach and small intestine model (Marteau *et al.*, 1997). In these studies, normal (physiological) levels of bile and low bile secretion were simulated. The bile exerted a strong influence on the survival of the bacteria, with survival varying within a small range of bile concentrations. Apart from initial pH stress, bile concentrations and residence times vary in each compartment of the GIT. Bacteria already sublethally injured by high acid concentrations often behave in different and unpredictable ways to new stress factors. The dynamic model of Marteau *et al.* (1997) proved more valuable in predicting the *in vivo* effects of bile on microorganisms than a static model with a constant concentration of bile.

Adhesion to mucus and epithelial cells

Adhesion to epithelial cells and mucus is also considered important. However, adhesion may be reduced by exposure to low pH, suggesting that adherence may be reduced after passage through the stomach (Ouwehand *et al.*, 2001). Over the past 25 years the Caco-2 cell lines were used extensively in experiments to determine adhesion properties of probiotic bacteria (Sambuy *et al.*, 2005). An alternative *in vitro* adhesion model is the colonic cell line HT-29 which also shows typical characteristics of enterocytic differentiation (Gopal *et al.*, 2001). Other cell lines include fetal I-407 and the more recently developed IPEC-J2 isolated from the jejunum of piglets (Schierack *et al.*, 2005). The HT29-MTX cell line from the small intestine of humans was developed to simulate a mucus-secreting environment (Lesuffleur *et al.,* 1990). Adhesion of LAB to human intestinal tissue and mucus is strain dependent (Kirjavainen *et al.*, 1998) and depends on the number of adhesion sites on the target cell (Tuomola *et al.,* 1999). Moreover, adhesion *in vivo* is bound to be influenced by changes in cell-surface properties caused by, for instance, proteases from pancreatic juice and thus *in vitro* experiments with cells grown in laboratory media may not be predictive at all.

Several *in vivo* studies with mice and pigs were conducted to study the adhesion of probiotic strains. Gnotobiotic mice

were orally fed with *L. rhamnosus* GG and *L. johnsonii* La1 and the bacteria were established in all segments of the gut (Bernet-Camard *et al.,* 1997). *L. salivarius* and *L. plantarum* strain 299v colonised the gut of gnotobiotic mice (Kabir *et al.,* 1997; Matsumoto *et al.*, 2001). *Lactobacillus* spp. orally administrated to gnotobiotic pigs colonised the jejunum and ileum (Bomba *et al.,* 1996). In a few well-designed human trials, colonization of *L. rhamnosus* GG in the intestinal tract was proved by colonic biopsies from patients who consumed a whey drink fermented with the strain (Alander *et al.,* 1997; Isolauri *et al.,* 1994). *L. johnsonii* La1 and *L. casei* Shirota survived intestinal transit in adults (Donnet-Hughes *et al.,* 1999). Under normal circumstances, probiotic bacteria are in competition with other intestinal microbiota and probiotics have to be taken on a daily basis to provide a continuous exogenous probiotic effect (Bezkorovainy, 2001).

L. rhamnosus GG adheres to epithelial cells via hydrophobic interaction and inhibits pathogens from attaching to the enterocytic receptor (Lee *et al.*, 2003). A pathogen may also be displaced by steric hindrance and eventually detach from the enterocytic receptor. Another example of steric hindrance is the adhesion of heat-killed cells of *L. acidophilus* LB to Caco-2 cells. This inhibited the adhesion of diarrheogenic *Escherichia coli* (Chauviére *et al.,* 1992). Multiple surface adhesins were found on *L. casei* Shirota. Four of these adhesins bound to the mucosal surface (Lee and Puong, 2002) and affected competition and exclusion interactions with pathogens. One cell of *L. casei* subsp. *rhamnosus* could out-compete as many as four pathogen cells (Lee *et al.*, 2003). In another study by Forestier *et al.* (2001), the presence of *L. rhamnosus* (Lcr35) decreased adhesion of three pathogens, enteropathogenic and enterotoxigenic *E. coli* and *Klebsiella pneumoniae*. The access of pathogens to receptor sites was possibly impeded by the addition of Lcr35. A second explanation is the interaction of Lcr35 with the level of mucins produced by Caco-2 cells. Although Caco-2 cells express significant levels of mucins, expression is further elicited in the presence of a probiotic (Mack *et al.*, 1999). Bacteriocins produced by lactobacilli may also play a role in the competitive exclusion of pathogens. *L. johnsonii* La1 inhibited the growth of *Giardia intestinalis* and its attachment to Caco-2 cells. The factors involved were heat-labile peptides of low molecular mass (Perez *et al.,* 2001). Spent culture supernatant of LB blocked the intracellular life cycle of *Salmonella enterica* serovar Typhimurium SL1344 and inhibited cell damage induced by *Salmonella* and *E. coli* (Coconnier *et al.*, 2000).

Many bacteria have a crystalline layer on their cell surface (S-layer), which changes with the environment (Boot *et al.,* 1996). This layer consists of single protein or glycoprotein species with relative molecular weights of 40,000 to 200,000 Dalton, representing 10-15% of the total protein of the bacterial cell (Sára and Sleytr, 2000). Bacteria have efficient expression of genes, synthesis and secretion of S-layer proteins (Boot and Pouwels, 1996). The S-layer structure contains pores of identical size and morphology that comprise up to 70% of the lattice surface area (Sleytr and Beveridge, 1999). S-layer proteins are hypothesised to be involved in cell protection, adhesion, trapping molecules and ions, and virulence (Åvall-Jääskeläinen and Palva, 2005). S-layer proteins isolated from the *Lactobacillus* species *acidophilus, amylovorus, brevis, buchneri, casei, crispatus, fermentum, gallinarum, helveticus, kefir* and *parakefir* vary from 25 to 71 kDa in size (Åvall-Jääskeläinen *et al.*, 2002; Jakava-Viljanen *et al.*, 2002; Kos *et al.*, 2003). Many of the genes encoding these proteins have been sequenced. S-layer proteins in lactobacilli have been shown to function in adhesion to epithelial cells (Frece *et al.*, 2005) and mammalian extracellular matrix (Hynonen *et al.*, 2002; Toba *et al.,* 1995). However, removal of the S-layer proteins by treatment with LiCl revealed that they are not involved in the adhesion of lactobacilli to Caco-2 cells (Greene and Klaenhammer, 1994). S-layer proteins of *L. crispatus* ZJ001 are involved in adhesion to epithelial cells and competitive exclusion of pathogens such as *E. coli* O157:H7 and *Salmonella typhimurium* (Chen *et al.*, 2007).

Little research has been done on the adhesion of enterococci to mucus and their competitive exclusion of pathogens (Franz *et al.,* 1999). The adhesion of *E. faecium* M74 and *E. faecium* SF68 to mucus was 3% and 18%, respectively (Pultz *et al.*, 2006). The adhesion is normal when compared to 9.2% adhesion recorded for strain LGG. Approximately 9% of *E. faecium* 18C23 cells adhered to small intestine mucus of piglets and effectively inhibited the adhesion of *E. coli* K88ac and K88MB to the mucus. Adhesion of more than 90% of *E. coli* K88 was inhibited by the addition of 10^9 cfu/ml or higher cell numbers of *E. faecium* 18C23 or pH-neutralised supernatant to mucus. Treatment of mucus with pronase and proteinase reduced the adhesion of *E. coli* K88ac and increased the adhesion of *E. faecium* 18C23. The mucus receptors of the two strains may be different and inhibition of *E. coli* K88ac is possibly through steric hindrance (Jin *et al.*, 2000a,b).

Production of antimicrobial compounds

Apart from competition for binding sites, production of hydrogen peroxide and bacteriocins (antimicrobial peptides) play a key role in competitive exclusion and probiotic properties (Boris and Barbes, 2000; Lepargneur and Rousseau, 2002; Reid and Burton, 2002; Velraeds *et al.,* 1998). Hydrogen peroxide, produced by some strains of LAB, effectively inhibits *Staphylococcus aureus* and *Pseudomonas* spp. (Holzapfel *et al.,* 1995). Hydrogen peroxide is only active in the upper part of the GIT and mouth where oxygen is available. It has a strong oxidising effect on the bacterial cell, sulfhydryl groups of cell proteins and membrane lipids. Hydrogen peroxide-producing lactobacilli, which colonise the urogenital tract, decrease

the acquisition of human immune deficiency virus (HIV) infection, gonorrhoea and urinary tract infections (Vallor *et al.*, 2001). The main antimicrobial effect is the blocking of glycolysis. Glucose transport, hexokinase activity, and glyceraldehyde-3-phosphate dehydrogenase activity are inhibited due to the oxidation of sulfhydryl groups in metabolic enzymes (Carlsson *et al.,* 1983).

Lactobacillus GG, isolated from humans, is incorporated in fermented milk products and is used as a probiotic agent to prevent different types of diarrhoea (Lee and Salminen, 1995). An unidentified antimicrobial substance (<1000 Da in size and soluble in acetone-water) produced by strain GG inhibited the growth of Gram-positive anaerobic bacteria (*Clostridium, Bifidobacterium*), *Staphylococcus, Streptococcus* and Gram-negative bacteria (Enterobacteriaceae, *Pseudomonas in vitro*) (Silva *et al.*, 1987). Bifidobacteria that produce broad-spectrum antimicrobial substances active *in vitro* against *Salmonella, Listeria, Campylobacter* and *Shigella* spp. have also been reported (Gibson and Wang, 1994). *L. acidophilus* LA1, also isolated from humans, produces a non-bacteriocin antimicrobial substance active against Gram-positive and Gram-negative pathogens, both *in vitro* and *in vivo* (Bernet-Camard *et al.,* 1997).

L. reuteri produces a low molecular-weight antimicrobial substance, reuterin, when grown anaerobically in the presence of glucose and glycerol or glyceraldehydes (Axelsson *et al.,* 1989). When in contact with target cells, *L. reuteri* is stimulated to produce reuterin which is active against bacteria, fungi, protozoa and viruses (Axelsson *et al.,* 1989; Dobrogosz *et al.,* 1989). Reuterin acts against sulfhydryl enzymes and interferes with DNA-synthesis by inhibiting the binding of substrates to the subunit of ribonucleotide reductase (Dobrogosz *et al.,* 1989). Reutericyclin, also produced by *L. reuteri*, has a low molecular weight (349 Da), a negative charge and high hydrophobicity (Höltzel *et al.*, 2000). Only Gram-positive bacteria are sensitive to reutericyclin (Gänzle *et al.*, 2000). It works as a proton ionophore, forming pores in the membranes of target cells. Due to its hydrophobicity, it intercalates into the cytoplasmic membrane and selectively dissipates the transmembrane ΔpH (Gänzle and Vogel, 2003).

Pyroglutamic acid (PCA), present in fruits, vegetables and grasses, is also produced by *L. casei* subsp. *casei*, *L. casei* subsp. *pseudoplantarum* and *Streptococcus bovis* (Huttunen *et al.,* 1995). Pyroglutamic acid has a stronger antimicrobial activity than lactic acid and its mechanism of action is similar to that of organic acids and inhibits *Bacillus subtilis, Enterobacter cloacae, Pseudomonas putida* and *Pseudomonas fluorescens* (Yang *et al.,* 1997).

Some bacteria produce antimicrobial peptides (bacteriocins) that inhibit strains closely related to the producer strain. Since the discovery of bacteriocins the interest in such compounds as possible preservative agents for food, and potential supplements or as replacements for therapeutic antibiotics has increased (Ouwehand and Vesterlund, 2004). These small (2-6 kDa) peptides are ribosomally produced as a defence mechanism against other organisms (Hansen *et al.,* 1989). Gram-positive bacteria produce bacteriocins in competitive environments, resulting in peptides bearing unique structural features and varied modes of action. Recently, Cotter *et al.* (2005) reclassified bacteriocins into two groups, i.e. Class I, lantibiotics and Class II, non lantibiotics (Table 1).

Although the role of bacteriocins and their significance in controlling the proliferation of pathogenic bacteria in the intestinal tract is questionable (Brink *et al.*, 2006), recent reports on bacteriocins active against Gram-negative bacteria (Caridi, 2002; Ivanova *et al.*, 1998; Messi *et al.*, 2001; Todorov and Dicks, 2005a,b) placed a renewed interest in these peptides and their interaction with intestinal pathogens. However, activity against Gram-negative bacteria has been recorded *in vitro* and does not mean the same results will be obtained in the gastro-intestinal tract.

Table 1. Classification of bacteriocins (Cotter *et al*., 2005).

Class	Characteristics	Example	Reference
Class I	Small (<5 kDa), heat stable post-translationally modified peptides containing lanthionine and methyllanthionine amino acids		Chatterjee *et al.* (2005); Jack and Sahl (1995)
Class Ia	Elongated, flexible positively charged peptides. Forms pores in cytoplasmic membranes of sensitive cells	nisin	Breukink and De Kruijff (1999)
Class Ib	Globular, rigid peptides with a negative or no net charge. Interfere with essential enzymatic reactions of sensitive cells	mersacidin	Bierbaum *et al.* (1995)
Class II	Small (<10 kDa) heat stable nonlanthionine containing peptides		
Class IIa	Pediocin-like peptides with conserved YGNGVXCXXXXXCXV region in N-terminal domain	pediocin AcH/PA-1	Eijsink *et al.* (1998)
Class IIb	Two-component bacteriocins requiring two peptides for activity	lactococcin G	Nissen-Meyer *et al.* (1992)

Bacteriocins are produced by many LAB, including species normally found in the GIT, *viz.* the *L. acidophilus*-group (*L. acidophilus*, *Lactobacillus amylovorus*, *L.crispatus*, *Lactobacillus gallinarum*, *Lactobacillus gasseri* and *L. johnsonii*), *L. reuteri, L. casei, L. fermentum* and *L. plantarum* (De Vuyst *et al.,* 1996; Tahara and Kanatani, 1997). Most bacteriocins produced by these species are active against food-borne pathogens such as *Listeria, Clostridium, Bacillus* and *Staphylococcus* spp. (Schillinger *et al.,* 1996).

Bacteriocin production by enterococci isolated from healthy humans or animals may be an important additional feature of future probiotic products which may be applied to exclude enteric and food-borne pathogens. There are several excellent reviews on bacteriocin production by LAB (Cleveland *et al.*, 2001; Drider *et al.*, 2006; Ennahar *et al.*, 2000; Riley and Wertz, 2002).

Immune stimulation

Different pathways have been identified by which probiotics modulate immune cells (Figure 1). Probiotics are directly taken up through transcytosis by microfold epithelial cells and are engulfed by macrophages or dendritic cells, which eventually triggers an immune response. Cytokines modulates the immune functions of dendritics, T and B cells (Shida and Nanno, 2008). In the case of *L. casei* Shirota and *L. rhamnosus* Lr23, TLR2-mediated signalling stimulates macrophages to secrete TNF-α or promote development of regulatory dendritic cells (Matsuguchi *et al.*, 2003). Several *in vitro* studies with Caco-2 cells and mice have shown that probiotic strains such as *L. casei* DN-114 001, LGG and VSL#3 act directly with intestinal epithelial cells and maintain the integrity of the epithelial barrier. Mechanisms identified include the suppression of NF-κ B activation and activation of the antiapoptotic protein Akt to prevent apoptosis and enhance mucin secretion by epithelial cells (Yan and Polk, 2002).

The main cytokines produced by macrophages and dendritic cells are IL-12 and IL-10. *Lactobacillus* strains such as *L. casei* Shirota, *L. rhamnosus* GG and *L. paracasei* KW3110 activate macrophages to produce more IL-12 in murine models (Ichikawa *et al.*, 2007). The MyD88-dependent receptor might recognise these probiotic strains to trigger IL-12 production (Gratz *et al.*, 2008). Stimulation of IL-12 production may also promote differentiation of naïve $CD4^+$ T cells (nThs) into Th1 cells, leading to natural killer (NK) cell activity. Dietary intake of *L. rhamnosus*

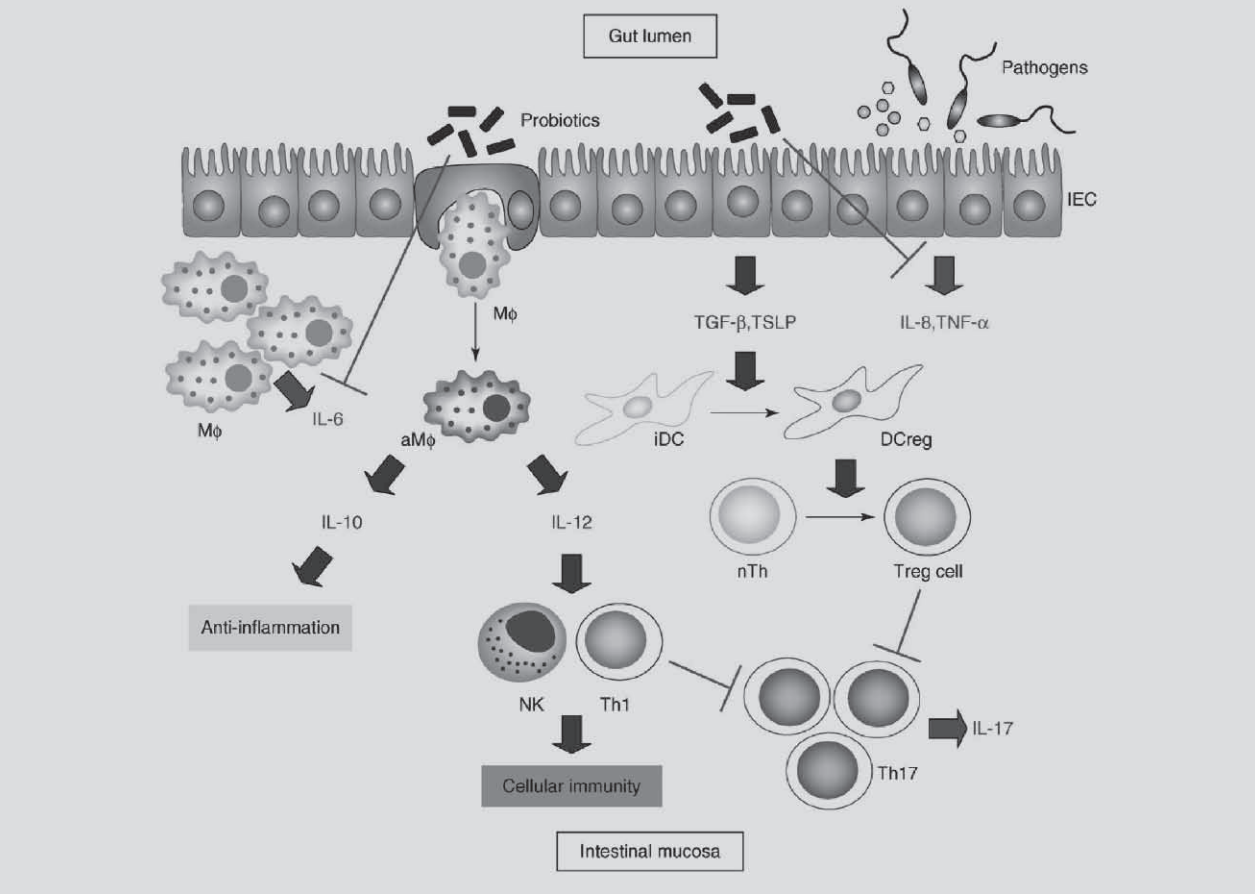

Figure 1. Proposed immunomodulation pathways by probiotics (Shida and Nanno, 2008).

HN001 and *L. casei* Shirota-fermented milk increased NK cell numbers and enhanced cytotoxic activity targeting K562 cells in humans (Takeda and Okumura, 2007). Future research is needed to identify host receptors and ligands of probiotics involved in the stimulation of IL-10 production. Administration of high doses *Lactobacillus* strains in mice provoked IL-10 production and decreased IL-12 production (Shida and Nanno, 2008).

Pro-inflammatory cytokines play a pivotal, yet paradoxical role, in inflammation. Experiments in cytokine transgenic knockout mice show that a harmless immune response to commensal gut microbiota becomes a harmful inflammatory state in the absence of IL-10, TGF-β and IL-2 and can lead to chronic gastro-intestinal tract inflammatory diseases (Dugas *et al.,* 1999). This indicates that an unbalanced local or systemic cytokine environment induces inflammation. Ingestion of probiotic bacteria can potentially stabilise the immunological barrier in the gut mucosa by reducing the generation of local pro-inflammatory cytokines. The regulatory role of specific strains of the gut microbiota has been proved by a suppressive effect of immune responses to dietary antigens in allergic individuals. This is partly attributable to enhanced production of anti-inflammatory cytokines IL-10 and TGF-β, whereas the capacity to stimulate non-specific immune responses was retained. It has recently been shown that probiotics modulated the host's immune responses to foreign antigens with a potential to dampen hypersensitivity reactions. Unheated and heat-treated homogenates were prepared from probiotic strains, including *L. rhamnosus* GG, *B. lactis, L. acidophilus, L. delbrueckii subsp. bulgaricus* and *S. thermophilus*. The phytohemagglutinin-induced proliferation of mononuclear cells was suppressed by these homogenates compared with controls with no homogenates, indicating that probiotic bacteria possess heat-stable, antiproliferative components, which could be therapeutically exploited in inflammatory conditions. Probiotics play a role in down-regulating inflammation associated with hypersensitivity reactions in patients with atopic eczema and food allergies (Kaur *et al.*, 2002; Parvez *et al.*, 2006; Young and Huffman, 2003). Probiotics also showed up-regulation of anti-inflammatory cytokines, such as IL-10, in atopic children (Young and Huffman, 2003). In this way, probiotics play a role both in immunostimulation in healthy individuals and down-regulation of immunoinflammatory responses in hypersensitive individuals.

L. bulgaricus 100158 and *S. thermophilus* 001158 fed to rats increased lymphocyte proliferation in the spleen, peripheral blood and Peyer's patches and elevated IFN-γ production in the Peyer's patches and spleen (Aatourri *et al.*, 2002). Splenocytes cultured *in vitro* displayed an increase in the inflammatory immune response associated with IL-12 when given an oral application of *L. casei* strain Shirota (Tagaki *et al.*, 2001). Macrophage cell lines and murine cultures composed of peritoneal, spleen and Peyer's patch cells were used to examine the effect of heat-killed cells, cell walls and cytoplasmic extracts of *Bifidobacterium, L. acidophilus, L. bulgaricus, L. casei, L. gasseri, L. helveticus, L. reuteri* and *S. thermophilus* effects on cell proliferation and cytokine and nitric oxide (NO) production (Kaur *et al.*, 2002). Both the cell wall and cytoplasmic fractions were able to stimulate cloned macrophages to produce significant amounts of TNF-α, IL-6 and NO. Increased IFN-α activity was observed in virally-challenged peripheral blood samples from humans that consumed *L. brevis* subsp. *coagulans* (Kishi *et al.,* 1996). A decrease in TNF-α production was observed in human ileal specimens from Crohn's disease patients treated *ex vivo* with *L. casei* DN114001 and *L. bulgaricus* LB10. TNF-α changes were not observed in non-inflamed mucosa indicating a down-regulation of Th1-like cytokines associated inflammation by probiotics. The high secretion of TNF- α in patients with Crohn's disease was also down-regulated by *L. casei* DN-114 (Borruel *et al.*, 2002). Paradoxically, ingestion of lactobacilli in fermented milk products or as live attenuated bacteria was shown to increase IFN-γ production by peripheral blood mononuclear cells. IFN-γ can promote the uptake of antigens in Peyer's patches where specific IgA-committed cells are generated. An increase in systemic and mucosal IgA response to dietary antigens was shown after oral administration of lactobacilli. Similarly, feeding an extensively hydrolysed whey formula supplemented with *L. rhamnosus* GG improved the clinical score of atopic dermatitis and decreased the intestinal excretion of α1-antitrypsin and TNF-α compared with children fed the extensively hydrolysed formula alone. A mixture of bovine caseins, hydrolyzed with *L. rhamnosus* GG, yield enzymes that may suppress lymphocyte proliferation and down-regulate IL-4 production *in vitro* (Dugas *et al.,* 1999). Bacterial VSL#3 has shown to down-regulate proinflammatory cytokine secretion by attenuation of the nuclear factor-κB pathway in intestinal an epithelial cell chemically induced colitis model in mice (Marteau and Shanahan, 2003). The polysaccharide-peptidoglycan complex of *L. casei* Shirota (Matsumoto *et al.*, 2005) inhibited IL-6 production by lipopolysaccharide-stimulated peripheral blood mononuclear cells in ulcerative colitis patients. *In vitro* studies showed that probiotic bacteria play a role in the up-regulation of Th1 cytokines (IL-12, IL-18 and IFN-γ) in the Th1:Th2 balance (Ezendam and Van Loveren, 2006). *L. casei* Shirota promoted the development of Th1 cells in mice and *L. rhamnosus* GG inhibited Th2 cytokine production in allergic patients (Pochard *et al.*, 2002). Treg cells play a role in immunopathology in many inflammatory diseases and the anti-inflammatory cytokine transforming growth factor (TGF) β is important for the development and function (Shevach, 2006). *Lactobacillus* strains may stimulate the production of TGF-β and thymic stromal lymphoprotein (TSLP) which promote the differentiation of immature dendritic cells to regulatory

dendritic cells and subsequently induce TGF-β-secreting Treg cells (Zeuthen *et al.*, 2008). This reaction may play a very important role in controlling inflammatory diseases in the gut. Treg cells and Th1 cells may also inhibit the activity of Th17 cells which are involved in autoimmune diseases (Shida and Nanno, 2008).

Several *in vivo* studies and clinical trials were performed to determine a possible increase in the frequency of circulating immunoglobulin-secreting cells of the IgG, IgM and IgA classes after probiotic intake. *Saccharomyces boulardii* increase secretory IgA levels in the gastro-intestinal tract (Parvez *et al.*, 2006). *L. rhamnosus* GG modulates intestinal immunity by increasing the levels of IgA and other immunoglobulin secreting cells in the intestinal mucosa (Kaur *et al.*, 2002). This strain increased the rotavirus-specific IgA response in children with rotavirus diarrhoea (Isolauri *et al.*, 2001). The increase in rotavirus-specific IgA was significantly higher with strain GG compared to a combination of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. Consumption of *L. rhamnosus* GG shortened the phase of diarrhoea from an average of 3.5 days to 2.5 days in children being treated for rotavirus infection (Guarino *et al.*, 1997). Both active and non-active preparations of strain GG reduced the duration of rotavirus diarrhoea, however only active forms increase the level of specific-IgA secreting cells to rotavirus. Infants that received *L. rhamnosus* GG showed temporary increases in IgG, IgA and IgM levels and cells secreting specific-IgA were present at higher numbers compared to controls, thereby providing protection against re-infection (De Roos and Katan, 2000).

Volunteers that ingested *L. johnsonii* La1 and milk fermented with bifidobacteria showed a 4-fold increase in specific IgA after ingesting an attenuated strain of *Salmonella typhi,* compared to a control group (Link-Amster *et al.*, 1994). When pre-feeding of probiotics was initiated 21 days before vaccination with the attenuated Ty21A strain of *S. typhi,* there was a significant increase in the pathogen-specific IgA response, while 7 days pre-feeding induced a non-significant trend toward an increase in the same parameter (Fang *et al.*, 2000). The specific IgA titre to *S. typhi* Ty21a in human volunteers was increased by fermented milk containing *L. johnsonii* La1 and bifidobacteria. *L. rhamnosus* GG stimulated IgA antigen secreting cell responses against *S. typhi* Ty21a in greater numbers compared to *Lactobacillus lactis* and a placebo control. *L. rhamnosus* GG increases antigen transfer across Peyer's patches to underlying lymphoid cells. This may explain the different responses observed (Kaur *et al.*, 2002). In two groups of adult volunteers that received typhoid vaccine, the antibody titres were significantly higher in the group that received *L. rhamnosus* GG (Young and Huffman, 2003). Strain GG also increased rotavirus-specific IgM secreting cells in infants that received an oral rotavirus vaccine (Isolauri *et al.,* 1995). Yoghurt supplemented with *L. acidophilus, B. bifidum* and *B. infantis* enhanced mucosal and systemic IgA responses to cholera toxin immunogen in mice (Kaur *et al.*, 2002). Feeding mice with *L. casei* Shirota prior to influenza virus challenge also significantly increased protection of the upper respiratory tract (Cross, 2002). *L. casei* Shirota and *E. coli* O157:H7 fed to infant rabbits exhibited a lower incidence of severe diarrhoea and lower levels of Shiga toxins 1 and 2 were present in the gastro-intestinal tract compared to infant rabbits fed only with *E. coli* O157:H7. Anti-*E. coli* and anti-toxin IgA levels were higher in the gastro-intestinal tract tissue of the animals that were fed probiotics compared to control animals. When mice were fed a combination of *L. acidophilus/L. casei* an increase in survival against pathogens was observed and both serum and gastro-intestinal tract mucosal anti-*Salmonella* antibody titres were elevated (Perdigon *et al.,* 1990). These results were also observed for anti-Shigella antibodies when mice were challenged with *Shigella sonnei*. These results indicate that some LAB have the ability to persist in the intestinal tract and act as adjuvants to the humoral immune response (Cross, 2002).

Oral introduction of *L. casei* and *L. bulgaricus* activates the production of macrophages and administration of *L. casei* and *L. acidophilus* activates phagocytosis in mice (Isolauri *et al.*, 2001). Increases in polymorphonucleas and/or macrophage phagocytic activity were reported after supplementation with *L. casei* DN114001, *L. rhamnosus* HN001 and a combination of *B. animalis* ssp. *lactis* 420 and *L. acidophilus* 74-2 or *L. paracasei* Lpc-37. Probiotic bacteria modulate phagocytosis differently in healthy and allergic subjects. An immunostimulatory effect was observed in healthy persons, whereas in allergic persons, down-regulation of the inflammatory response was detected. In a study of the immunomodulation following the consumption of milks fermented with *B. bifidum* or *L. johnsonii* LA1, human blood samples showed an increased phagocytosis of *E. coli in vitro*. When *L. rhamnosus* GG was given to volunteers the number of white blood cells with phagocytic activity doubled (De Roos and Katan, 2000). *L. acidophilus* and *B. bifidum* had little effect on immunity in elderly volunteers, only B lymphocytes increased significantly. This suggests that host characteristics also contribute to the different effects exerted by probiotics. In a study to determine the effect of consuming different doses and different strains of LAB on immune indices, one group of volunteers consumed 10^{10} cfu *B. bifidum* Bb12 and a second group received 7×10^{10} cfu *L. johnsonii* La1 daily for 3 weeks (Pfeifer and Rosat, 1999). Two other groups each consumed either 10^9 cfu or 10^8 cfu of strain La1 daily. The increase in leukocyte phagocytic activity was significant in both groups after the ingestion of LAB, but was more evident in the group ingesting bifidobacterium. Overall, phagocytic activity decreased 6 weeks after probiotic ingestion was stopped, but the group that consumed La1 retained the highest activity. The respiratory burst and phagocytic

activity were significantly enhanced in volunteers who consumed 10^9 cfu La1, but not in those who consumed a lower dose of 10^8 cfu. In animals, LAB also exhibit immune stimulating capacity. Different strains of *Lactobacillus* and *S. thermophilus* were capable of stimulating non-specific (macrophages) and specific (lymphocytes B and T) immunity in mice (Heyman, 2000). *L. acidophilus* UFV-H2b20 stimulates a non-specific immune response in germ-free Swiss mice as indicated by stimulation of the host mononuclear phagocytic activity (Kaur *et al.*, 2002). There was a two-fold increase in the number of Kupffer cells, responsible for the clearance of circulating bacteria. Enhanced phagocytosis was substantiated in humans by *L. johnsonii* La1 and *L. rhamnosus* GG (Isolauri *et al.*, 2001). In addition to enhanced pathogen-specific antibody production, strains of *Lactobacillus* and *Bifidobacterium* spp. have also resulted in an increase in non-specific *in vivo* phagocytic activity of peritoneal macrophages and blood-borne neutrophils following pathogenic challenge (Cross, 2002). This suggests that enhanced cell mediated immunity may also contribute to increased protection. Rats fed with *L. casei* Shirota prior to oral challenge with *L. monocytogenes* showed reduced pathogen burdens in several excised GI tract tissues and lower pathogen translocation to the spleen and liver (De Waard *et al.*, 2002). The probiotic-fed rats showed an increased level of *in vivo* lymphocyte sensitisation to microbial antigens. Intestinal microorganisms could down-regulate the allergic inflammation by counter-balancing T-helper cell Type-2 responses and by enhancing antigen exclusion through induction of an IgA response.

A few probiotic strains show potential in influencing both intestinal epithelial cells and immune cells of the gut. These responses can be identified to select a specific probiotic to exhibit a health-promoting activity or relieve symptoms of a disease triggered by a disordered immune response.

4. Interactions of probiotic cells with the GIT

Survival of probiotic bacteria in the intestinal tract remains the most important characteristic of a probiotic. Survival is determined by a number of factors, including stress response and transcriptional regulation, cell wall maintenance, metabolism, amino acid transport (which plays a putative role in pH homogenesis), and fatty acid or isoprenoid biosynthesis, which is important in bile-rich environments (Breton *et al.*, 2002). Cholic acid and deoxycholic acid (DCA) have a stronger inhibitory effect on intestinal aerobic and anaerobic bacteria compared to conjugated bile acids (Floch *et al.*, 1972). The first evidence of DCA toxicity in LAB was reported by Taranto *et al.* (2006). Electron microscopy conducted on *L. reuteri* CRL 1098 showed severe distortion of the cell envelope, complete permeabilisation of the cells and prevention of glucose uptake. Significant changes in lipid composition of the membrane, including the ratio of phospholipids to glycolipids, were observed. Changes in the integrity of the cell wall was shown by sugar transport and permeability assays.

The reaction of bacteria to intestinal conditions is not fully understood (De Vriese *et al.*, 2006). Genome sequencing could be a new resolution and therefore the complete genome sequences of probiotic bacteria such as *L. plantarum* WCFS1, *L. acidophilus* NCFM and *L. johnsonii* NCC 533 were determined (Alterman *et al.*, 2005; Kleerebezem *et al.*, 2003; Pridmore *et al.*, 2004). Genomic analysis together with studies done on their behaviour in the gastro-intestinal tract could give more insight into the mechanisms behind probiotic functions (De Vos *et al.*, 2004).

Mechanisms used by Gram-positive bacteria in resistance to acid include proton pumps, amino acid decarboxylation, electrogenic transport systems, chaperones involved in repair/degradation of damaged proteins, incremental expression of regulators promoting local or global responses and changes in the structure of the cell envelope (Cotter and Hill, 2003). The F_1F_0-system encoded by the *atp* operon and ornithine decarboxylase (La996) was studied for *L. acidophilus* NCFM (Azcarate-Peril *et al.*, 2004). A thioredoxin system and genes encoding glutathione reductase, NADH-oxidase and NADH-peroxidase were also identified (Altermann *et al.*, 2005). *L. johnsonii* NCC 533 has three genes encoding BSH, one less than *L. plantarum* WCFS1 (Pridmore *et al.*, 2004). The large number of BSH-encoding genes emphasises the importance of this characteristic to the survival of strains in the gastro-intestinal tract (Pridmore *et al.*, 2004).

Genes that are switched on under conditions simulating the gastro-intestinal tract were detected in several *in vitro* studies. Fragments of the *L. plantarum* WCFS1 genome were cloned upstream of a promoterless alanine racemace (*alr*) gene of *Lactococcus lactis* in a low copy number plasmid. The plasmid library, which covered 98% of the genome, was introduced into a *alr* deletion mutant (*L. plantarum Δalr*) (Bron *et al.*, 2004c). Clones that could complement the D-alanine auxotroph phenotype in the presence of 0.8 M NaCl were screened. Significantly higher production of *alr* in eight clones was detected that contained *L. plantarum* promoters preceding genes coding for different functions. These functions included an integral membrane protein, glycerate kinase, permease, short chain dehydrogenase and different hypothetical proteins. Four promoters with the same conserved motive, present on the plasmid (Bron *et al.*, 2004c), indicated a specific regulation of genes. Thirty-one genes, including 11 membrane- and cell wall-associated functions, five functions involved in redox reactions and five regulatory factors were induced in the presence of 0.1% porcine bile (Bron *et al.*, 2006). Growth

of *L. plantarum* WCF1 on MRS agar, with or without 0.1% porcine bile, was compared by using DNA micro-arrays. Stress proteins, cell-envelope located proteins and proteins involved in redox reactions were up-regulated (Bron *et al.*, 2004b, 2006). These studies showed alterations in the cell wall that could protect the cell from harsh conditions (Bron *et al.*, 2004b). Genes involved in redox reactions which are up-regulated might be explained by different metabolic reactions under intestinal conditions.

A resolvase-based *in vivo* expression technology (R-IVET) was used to screen for genes of *L. plantarum* WCFS1 switched on in the intestinal tract of mice (Bron *et al.*, 2004a). The genes coded for sugar-related functions, acquisition and synthesis of amino acids, nucleotides, cofactors, vitamins and stress-related functions. Deletion of these genes resulted in reduced survival of *L. plantarum* WCFS1 in the gastro-intestinal tract. This indicated that *L. plantarum* WCFS1 adapts to different environmental conditions and that the series of functions are concentrated in a defined genomic region (Bron *et al.*, 2004a).

DNA micro-arrays were used to determine gene expression of *L. plantarum* 299v in surgically removed intestinal segments of potential colon cancer patients (De Vriese *et al.*, 2006). The patients were fed a fermented oatmeal drink with *L. plantarum* 299v. Genes encoding sugar uptake and metabolism, amino acid biosynthesis, cell division and stress were up-regulated. This indicated survival, metabolic activity and growth of *L. plantarum* attached to the human intestine (De Vriese *et al.*, 2006). DNA micro-arrays combined with clinical studies may provide insight into and new perspectives on *in vivo* host-microbe interactions (De Vriese *et al.*, 2006).

5. Conclusions and perspectives

The probiotic concept, developed in recent years, involves the selection of LAB for probiotic supplements and functional foods. Regulatory guidelines were implemented in 2002 (FAO/WHO) to ensure safe and reliable products. A probiotic should be resistant to gastric and bile acid, adhere to mucus and/or human epithelial cells and inhibit the growth and colonisation of pathogens.

Probiotic bacteria may lead to maturation of the humoral immune mechanisms, particularly circulation of the IgA and IgM-secreting cells. LAB may also induce the production of proinflammatory cytokines, tumor necrosis factor alpha and interleukin-6, thereby stimulating non-specific immunity. Many more in-depth studies are needed to understand the stimulation of the host's immune system.

Adhesion and colonisation of probiotic bacteria in the gastro-intestinal tract of the host is believed to be one of the essential features required for delivering health benefits. Most of these tests are performed *in vitro*. Studies with human cell lines such as Caco-2, HT-29 and HT29-MTX provide an opportunity to study adhesion to mucus, glycoproteins and epithelial cells. Adhesion to epithelial cells differs with the use of different cell lines, e.g. some bacterial strains have a higher affinity to HT29-MTX cells than Caco-2 cells, and *vice versa*. Other factors of importance are cell-surface proteins, carbohydrates, haemaglutins, S-layer proteins and lipoteichoic acids. Studies with cell lines will continue to be important in the evaluation of probiotics.

In vitro studies on cell lines are important in that they bridge the gap between 'test-tube' research and animal or human studies. *In vitro* models, simulating the gastrointestinal tract in humans, have been developed to represent *in vivo* conditions, some with predictive power (Marteau *et al.*, 1997). This provides some insight into the metabolism, adherence and antimicrobial activity of probiotic cells. However, *in vivo* trials have to be performed in final evaluations.

Lactobacilli, pediococci and *Leuconostoc* spp. have a high natural resistance to vancomycin, which is different for other Gram-positive bacteria. Some lactobacilli have a high natural resistance to bacitracin, cefoxitin, ciprofloxacin, fusidic acid, kanamycin, gentamicin, metronidazole, nitrofurantoin, norfloxacin, streptomycin, sulphadiazine, teicoplanin, trimethoprim/sulphamethoxazole, and vancomycin (Danielsen and Wind, 2003). In the case of *Lactobacillus* spp., susceptibility to antimicrobial agents is species-dependent (Danielsen and Wind, 2003).

Reports on virulence and the possible exchange of genes encoding antibiotic resistance have emphasised the importance of having the correct safety precautions in place when selecting a probiotic. Probiotic properties are strain specific and these strains do not meet all the probiotic criteria.

Genomic analyses have proved valuable in probiotic studies. Examples are the F1F0-ATPase system, encoded by the *atp* operon, ornithine decarboxylase (La996) and the role it plays in acid tolerance of *L. acidophilus* NCFM (Alterman *et al.*, 2005), bile salt hydrolase (BSH) and bile transport by *L. johnsonii* NCC 533 (Pridmore *et al.*, 2004). Bron and co-workers (2006) described fourteen genes and gene clusters encoding cytoplasmic membrane and cell-wall-associated functions involved in bile tolerance of *L. plantarum* WCFS1.

In summary, *in vitro* assays can give an indication of probiotic properties and can be the first part of the selection process. This process might be refined in future by using genomic analysis. However, clinical trials are the most reliable method of ensuring probiotic validity. Regulations proposed by the FAO/WHO should be strictly followed

to improve the image of probiotics as safe and reliable in promoting human health.

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