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Developing and approving health claims for functional dairy products

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12.1 Introduction

There is an increasing commercial interest in *Lactobacillus* cultures that aim to improve human health. These cultures and their products are generally called probiotics, although the exact definition of probiotics has changed over the course of time. Initially, Fuller (1989) stated that probiotics are live microbial feed supplements that beneficially affect the host animal by improving its intestinal microbial balance. Later, Guarner and Schaafsma (1998) defined probiotics as living microorganisms which upon ingestion exert health effects beyond basic nutrition (in humans and animals). It is increasingly recognised that inactivated probiotic microorganisms may also have beneficial effects on human health. Therefore, Salminen and co-workers (1999) proposed that probiotics are microbial cell preparations, or components of microbial cells, that have a beneficial effect on health and well-being of the host. This variety in definitions is mainly due to the discussions about applications other than in food or feed, the necessity to use living bacteria, and whether the health-promoting mechanism is related to the functionality of the microflora as a total.

The majority of the current probiotics belong to the genera *Lactobacillus* and *Bifidobacterium*. The health benefits of these strains have been investigated using *in vitro* approaches, as well as animal and human studies. On the basis of human studies, it is demonstrated that fermented products containing these probiotic strains can benefit human health in many ways. This could be shortening the duration of rotavirus-induced diarrhoea (Shornikova *et al.*, 1997; Guandalini *et al.*, 2000), improving control of atopic eczema (Isolauri *et al.*, 2000), reduction of colonisation by *Helicobacter pylori* (Felley *et al.*, 2001),

relieving the symptoms of irritable bowel syndrome (Niedzielin *et al.*, 2001), and delaying the recurrence of superficial bladder cancer (Aso *et al.*, 1995). For consumer products, the most prevalent claim deals with increasing the natural resistance of the body.

There is a general agreement that health claims on probiotics for the human market should be validated in human studies. Since the health benefits are strain specific, results obtained with one strain cannot directly be extrapolated to other strains. A good probiotic strain should also possess technologically interesting properties, such as the possibility to cultivate the organism on an industrial scale. Strains should be able to grow on a fermentable substrate (e.g. milk) and the final product should have an attractive colour, taste, aroma and texture. Furthermore, a sufficient number of probiotic bacteria should be present in the final product to induce the health benefit.

This chapter will focus on how lactic acid bacteria, either dead or alive, can contribute to health by increasing resistance towards pathogenic microorganisms. Since infection experiments cannot easily be performed in humans, other ways to substantiate health claims are necessary. *In vitro* and animal studies can provide the mechanism(s) by which a probiotic strain is active. Human volunteer studies and clinical trials should provide evidence that the same mechanisms play a role in humans too. Here, we will describe how these studies can be translated into health claims.

12.2 The body's defence mechanisms

In order to enable efficient absorption of nutrients from the food flow, the intestinal mucosal surface is greatly increased through the formation of villi and microvilli. However, the enormous surface area gets easily colonised by a variety of microbes, either commensal or pathogenic. This makes the gut a major site of entrance for several bacterial pathogens such as *Escherichia coli*, *Salmonella*, or *Campylobacter* species (Wells *et al.*, 1988). Fortunately, commensal bacteria usually outnumber pathogens and form a stable ecosystem that hampers colonisation by pathogens.

Pathogenic microorganisms are excluded from the body by various barriers. After ingestion, the microorganisms are exposed to digestive juices in the stomach and small intestine that form a first line of defence that interferes with survival of the pathogen. The low pH and the presence of pepsin in the stomach, and the bile salts and proteolytic enzymes of the small intestine, efficiently kill many newly ingested bacteria.

Also the microflora contribute to a large extent to the host defence system by preventing colonisation of pathogens. Commensal bacteria can compete for nutrients that are necessary for growth of the pathogens. Furthermore, pathogens depend on adhesion sites on the mucosa to maintain themselves in the intestinal tract. Commensal bacteria can prevent adhesion of pathogens by specific blocking of the receptor or by steric hindrance. Next to that, the microflora

contain bacteria that produce antimicrobial substances such as bacteriocins or volatile fatty acids that can kill other bacteria or reduce their growth.

Evidence for the role of the microflora in resistance to infections is obtained from comparison of germ-free (lacking any microbes) and conventional animals (Freter and Abrams, 1972; Berg and Savage, 1975). These studies have raised the belief that the addition of selected probiotic strains may change the composition of the microflora from potentially harmful to beneficial for the host. Probiotic bacteria that survive gastrointestinal transit and are active in the gut may compete for nutrients and adhesion sites, and produce antimicrobial factors. Ideally, this leads to a reduction in potentially pathogenic bacteria. A more thorough understanding of probiotic mechanisms has revealed that a change in microflora composition by itself is not a guarantee of better resistance against pathogens. For immunomodulating properties of probiotic strains, the effects are probably caused by direct interactions between probiotic bacteria and the host rather than by a change in microflora composition.

The intestinal epithelium forms a second line of defence, aiming to prevent pathogens from translocation through the gut wall to peripheral organs such as the spleen and liver. This single-cell layer contains enterocytes, and goblet cells producing mucins that cover the surface of the epithelium. The stability of this mucus layer is improved by trefoil peptides. Tight junctions form strong bonds between epithelial cells and prevent migration of bacteria through the paracellular pathway. At the bottom of the small intestinal crypts, Paneth cells are found that produce antimicrobial peptides called defensins (Selsted *et al.*, 1992).

Invasive pathogens that enter the body are recognised by the mucosal immune system that serves as a third line of defence. Initially, translocating pathogens evoke an innate immune reaction by macrophages and neutrophilic granulocytes that destroy pathogens after phagocytosis, and natural killer cells that destroy infected host cells. Although these are fast actions that can kill pathogens within minutes, these immune cells produce reactive molecules such as NO and oxygen radicals that are harmful to the host as well. Therefore, an adaptive immune response is triggered if the pathogens escape the innate immune response or if they infect the host for a second time. This adaptive immune response takes several days to build up, and is based on the specific recognition of the pathogen by antibody proteins and T-lymphocytes (Kuby, 1997).

The mucosal immune system needs to discriminate between nutrients, non-pathogenic commensal bacteria and potentially harmful pathogens. Although the microflora stimulates the alertness of the mucosal immune system (Berg and Savage, 1975), it usually does not induce immune responses against commensal bacteria or nutrients. These components induce so-called immunological tolerance. The first step in this process is continuous sampling of bacteria and macromolecules from the intestinal contents by dendritic cells that are just below the epithelial barrier, and by M-cells. M-cells form a specialised type of epithelial cells that covers the lymphoid follicles in the small intestine called

Peyer's patches. How the balance between tolerance against nutrients and commensal bacteria and immune reactivity against pathogens is maintained is still unclear, especially since pathogenic and commensal microorganisms share multiple antigens (MacDonald, 1995).

There are numerous mechanisms by which probiotics potentially can stimulate host resistance against pathogens in humans. Basically, all three barriers mentioned above could be improved, since probiotics are active in the intestinal contents and have direct interactions with the epithelium and mucosal immune system. However, hard evidence is limited, obviously due to a lack of controlled infection models in humans. Mechanistic studies using animals or *in vitro* models have demonstrated the potential of probiotics to improve host defence.

12.3 *In vitro* studies

In vitro studies are very suitable to select for new candidate strains that can be evaluated in further animal or human trials. Using human or animal faeces as a source of lactic acid bacteria, several thousand strains have been isolated and screened for *in vitro* characteristics (Dunne *et al.*, 2001). Advanced *in vitro* models have been described that are provided with a complete microflora (Alander *et al.*, 1999), or contain dynamic devices to mimic gastrointestinal conditions as closely as possible (Marteau *et al.*, 1997). However, it is important to note that results from such studies never reflect the *in vivo* situation. For example, many *in vitro* systems lack the absorption of probiotic metabolites. Also the development of an immune response cannot be mimicked *in vitro*, and interactions with mucin-producing goblet cells and other epithelial cells are usually studied in cancer cell lines with culture medium rather than digestive juices. Nevertheless, *in vitro* screening is the only solution to select probiotic candidates for further *in vivo* evaluation on a large scale.

Many screening strategies primarily focus on intestinal survival and temporary colonisation, since it is strongly believed that probiotics should survive gastrointestinal conditions in order to be active in the gut. Important factors are the ability to deal with the low pH of the stomach, the presence of bile salts and adherence to epithelial surfaces or mucus. These prior conditions, however, do not predict a health benefit of a candidate probiotic and should be extended by investigation of the health promoting activity *in vivo*.

Screening of probiotics for adhesion to epithelial cell lines such as Caco-2 or HT29 cells has become an important selection criterion, although a good correlation between *in vitro* adherence characteristics and *in vivo* colonisation has not been made. A comparison of 12 probiotic strains revealed that there is a considerable difference between strains that are currently on the market (Tuomola and Salminen, 1998). Since the mucus layer forms the first contact of probiotic bacteria with the intestinal mucosa, the adhesion to mucus has also gained interest (Kirjavainen *et al.*, 1998).

The basic hypothesis has been for a long time that a good interaction between epithelial cells and probiotic bacteria is important for a functional health effect. Although that might be true, the cell line studies show no saturation in the adhesion: adding more bacteria to the cell culture does not change the fraction of bacteria that adhere (Tuomola and Salminen, 1998). This suggests that the adhesion is rather non-specific without any receptor–ligand interactions. Therefore, strong but non-specific adhesion to cell lines is unlikely to predict any health benefit.

Epithelial cells in culture can be used to demonstrate an effect of probiotic bacteria on the adhesion of pathogens. It has been shown that probiotics and enteropathogens share binding sites for adhesion to epithelium (Neeser *et al.*, 2000). In a similar concept, it was shown that probiotics can also interfere with the adhesion of probiotics to mucus (Tuomola *et al.*, 1999). A mathematical approach to analyse the interaction between probiotics and pathogen for adhesion to cells and mucus has been described (Lee *et al.*, 2000). The inhibition of adhesion might also be related to production of antimicrobial substances by probiotics. It has been shown that lactobacilli can produce antimicrobial factors with an inhibiting activity against Gram-positve as well as Gram-negative bacteria (Talarico and Dobrogosz, 1989).

Several studies have investigated the interactions between probiotics and cells of the immune system to explore an immunomodulating effect. These could be either transformed macrophage or T-cell lines (Marin *et al.*, 1997), or cells obtained from blood or lymphoid organs (Kitazawa *et al.*, 1994). From these studies, it becomes clear that strains of lactic acid bacteria have a differential effect on cytokine production by immune cells. However, since probiotics are generally not invasive, it seems unlikely that immune cells from blood or spleen get in direct contact with probiotic cells. This makes the direct extrapolation of the results to humans questionable. To circumvent this problem, immunological studies with lymphoid cells from murine Peyer's patches have been described (Yasui and Ohwaki, 1991). Since the relative amounts of different cell types in Peyer's patches is highly variable, it might be difficult to reproduce the outcome of experiments. Therefore, this method has not gained much attention yet.

Two recent developments have created breakthroughs for *in vitro* screening on immunomodulating properties. A first important discovery is that co-cultivation of human Caco-2 cells and mouse lymphocytes results in the formation of an M-cell-like cell type (Kerneis *et al.*, 1997). M-cells are part of Peyer's patches, and are important for antigen sampling. It is thought that these cells can bring probiotics in contact with the immune system. At this time, the first probiotic studies using this system need to be published. A second promising approach is the use of dendritic cells, since these cells are also used for antigen sampling from the gut (Rescigno *et al.*, 2001). Intestinal dendritic cells cannot be used for *in vitro* stimulation with probiotics since they have already been stimulated by various antigens from the microflora. Using naive bone marrow-derived dendritic cells that were stimulated with various probiotic

lactobacilli, Christensen *et al*. (2002) showed a differential cytokine expression favouring either immunological tolerance, or cellular or antibody immune responses depending on the strain of *Lactobacillus*.

12.4 Animal studies

Animal studies in which enteric pathogens are orally administered offer realistic controlled models for food-borne infections. They have the advantage that the infection process can be followed in time with a focus on the mechanism behind the health benefit. The intact intestinal physiology and presence of a microflora make extrapolation to humans much better than the *in vitro* studies.

Probiotics can in principle contribute to three different stages of the *in vivo* host defence. They can improve colonisation resistance, strengthen the mucosal barrier or improve the response of the body by stimulating the immune system. We have developed a *Salmonella enteritidis* infection model that allows a simultaneous study of all three barriers of host defence (Bovee-Oudenhoven *et al.*, 1999). Colonisation resistance is measured by monitoring faecal excretion of *Salmonella* in time. Nitric oxide-derived nitrite and nitrate in urine serves as a marker for translocation. Furthermore, antibody titres provide information on the humoral immune response. The first probiotics that have been studied with this model demonstrate the ability of some strains to improve colonisation resistance (unpublished data).

In the absence of an endogenous microflora, lactic acid bacteria can grow out to high colonisation levels and efficiently compete with enteric pathogenic bacteria. For example, colonisation of germ-free animals with a *Lactobacillus casei* strain delayed mortality and reduced colonisation of pathogens in mice infected with a lethal *Salmonella* dose (Hudault *et al.*, 1997). Similar effects were shown for *L. salivarius* and resistance to *Helicobacter pylori* in mice (Kabir *et al.*, 1997), and for *L. plantarum* and resistance against *E. coli* infection in rats (Herias *et al.*, 1999). Although such studies are very useful for elucidating mechanisms by which probiotics may act under gastrointestinal conditions, the use of germ-free animals is rather artificial since the functionality of probiotics is always complementary to the normal microflora.

Several studies have provided evidence that lactic acid bacteria can suppress colonisation of pathogens in animal feeding trials. A study in which yoghurt containing *L. bulgaricus* and *Streptococcus thermophilus* was fed to mice infected with *Salmonella typhimurium* demonstrated a reduction of the pathogen (De Simone *et al.*, 1988). Since both strains of lactic acid bacteria cannot survive gastrointestinal conditions, the effect is probably explained by the presence of large amounts of lactate in the yoghurt. Lactate that is produced by all lactic acid bacteria has a strong antimicrobial activity against Gram-negative bacteria such as *Salmonella*. Less clear is another study that used a *L. acidophilus* strain (Coconnier *et al.*, 1997). Spent supernatant of this strain showed antimicrobial

activity against a broad range of Gram-positive and Gram-negative bacteria. A *Salmonella typhimurium* infection experiment in mice demonstrated that feeding probiotic bacteria together with the spent culture supernatant inhibited colonisation of the pathogen. It is uncertain, however, whether lactate might have played a role in this study too, which demonstrates the necessity of choosing proper controls.

Studies with a specific focus on intestinal permeability are rare. It has been demonstrated that *L. casei* can decrease intestinal permeability in suckling rats (Isolauri *et al.*, 1993). Nevertheless, this study intended to unravel the mechanism by which this strain can contribute to prevent antigenic uptake and the subsequent development of cow's milk allergy. The relevance for infections still needs to be demonstrated.

In order to study an immunomodulatory effect of probiotics, several models have been described in which animals are infected by oral or parenteral routes with a broad variety of pathogens. We have previously chosen to use an oral infection model with the helminth *Trichinella spiralis*. Encapsulated muscle larvae of this parasite get released in the stomach after consumption of contaminated meat, pass towards the jejunum, and mature within 3–4 days. Viviparous females penetrate the small intestinal epithelium and produce larvae. New-born larvae migrate through the intestinal mucosa via lymphatic and blood vessels towards host striated muscle tissue, where they are encapsulated within a host-derived structure. A rat model making use of this parasite has the advantage that the immunity to *T. spiralis* depends on multiple cell types at the mucosal and systemic, specific and non-specific, and humoral and cellular levels. A *Lactobacillus casei* strain, but not two *Bifidobacterium* strains, was shown to enhance cellular immunity, although no difference in helminth load was observed (De Waard *et al.*, 2001). Since an infection with *Listeria monocytogenes* is highly dependent on cellular immunity, a follow-up study was performed with this pathogen. This revealed that the probiotic *Lactobacillus casei* strain improved cellular immunity and reduced the numbers of *Listeria* in the intestinal tract as well as in liver and spleen (De Waard *et al.*, 2002).

12.5 Human studies

Since most probiotics are marketed for healthy consumers, it is important to demonstrate the benefits in healthy subjects. On the other hand, as long as probiotics are marketed as food products, it is not recommended that probiotics largely change the normal physiology of the body since this might turn probiotics into pharmaceutical preparations. One of the first human studies aiming to show an effect in healthy adult volunteers demonstrated that a *Lactobacillus casei* strain was able to modulate the composition and metabolic activity of the microflora (Spanhaak *et al.*, 1998). Demonstration of the presence of live probiotic bacteria in the microflora might demonstrate that a probiotic bacterium is active, but it cannot be considered as a functional health effect. This

L. casei strain did not alter any of the immune parameters that were measured, although the same strain has shown a strong immunomodulating activity in rat experiments (De Waard *et al.*, 2002).

Some probiotics have an immunomodulating effect that can be detected in healthy volunteers. As an example, it has been reported that elderly volunteers consuming *L. rhamnosus* had a stimulation of natural killer cell activity as determined *ex vivo* (Gill *et al.*, 2001). Whether this can be translated into a functional health effect requires further study. Although it is possible to determine a direct immunomodulation in healthy subjects by monitoring immune parameters such as natural antibodies or leukocyte differentiation, several volunteer studies focus on the body's reaction against some kind of challenge, e.g. vaccination, that induce a change in normal immune parameters.

A vaccination approach was used to demonstrate the effect of a probiotic product containing *L. acidophilus* and bifidobacteria (Link-Amster *et al.*, 1994). Volunteers consumed this product for three weeks after which an attenuated *Salmonella typhi* was administered for oral vaccination. An increased count of faecal *Lactobacillus* and *Bifidobacterium* was found during the fermented milk intake. Furthermore, a four-fold increase of specific serum-IgA antibodies was observed. Extrapolation of these results predicts a better protection after infection with a virulent *Salmonella* strain for persons who have consumed the probiotic product.

A realistic approach focusing on a direct health effect on pathogenic microorganisms is to demonstrate benefits in patients recovering from spontaneous infections. The first evidence for health benefits from probiotics during infection comes from a Finnish study on recovery of young children from acute diarrhoea caused by rotavirus (Isolauri *et al.*, 1991). These authors showed that a probiotic strain is effective in shortening the hospitalisation time from 2.4 to 1.4 days. Subsequently, a multicentre trial confirmed a shorter duration of diarrhoea, less chance of a protracted course, and earlier discharge from the hospital (Guandalini *et al.*, 2000).

Another target group that might be useful for probiotic health studies are people at risk, such as persons getting antibiotic treatment or patients suffering from inflammatory bowel diseases. A meta-analysis showed that certain probiotic lactobacilli can be used for prevention or treatment of antibiotic-associated diarrhoea (D'Souza *et al.*, 2002). A first pilot study gives indications for an effect of *L. rhamnosus* in prevention of Crohn's disease, although a permanent clinical trial should give final evidence (Gupta *et al.*, 2000). A study using patients suffering from irritable bowel syndrome reported an improvement of the symptoms in 95% of the patients treated with *L. plantarum* versus 15% in the placebo-treated group (Niedzielin *et al.*, 2001).

A drawback of many human studies is their focus on a therapeutic effect whereas the main benefit for the consumer is a preventive effect. Although controlled human infection studies with pathogenic microorganisms would be the gold standard to show whether probiotic strains are indeed beneficial, the possibilities of doing this are for obvious ethical reasons highly limited, if not

impossible. Recently, we have overcome this problem by developing a new model in which healthy human volunteers were infected with a modified *E. coli* derived from a strain causing traveller's diarrhoea. This mutant strain is non-invasive and lacks the ability to produce toxins, but survives intestinal conditions and causes mild, short-lived symptoms. Using this approach, we have found that subjects consuming calcium-rich dairy products developed significantly less diarrhoea (Bovee-Oudenhoven *et al.*, 2002). Probiotic studies have not been performed with this model yet.

12.6 Making health claims

There is no doubt that health claims on probiotics should be supported by scientific evidence. The studies described in the previous sections of this chapter, however, illustrate that there is presently a wide variety of approaches. The only way to keep the confidence of consumers in probiotic products is for the scientific community to accept health claims. A thorough understanding of the working mechanism facilitates the acceptance, but at least two independent positive human studies seem to be required. The difficulty of demonstrating health effects in healthy humans as described earlier is a major handicap for general acceptance. Several countries including The Netherlands, Belgium, Sweden and the United Kingdom have introduced organisations that should judge the scientific information on functional foods (Feord, 2002). Leading scientists participate in committees that evaluate the evidence on probiotic strains in relation to the claims that are made.

Probiotic health claims have long been hampered by the notion that the activity of lactic acid bacteria is strain-dependent. This means that health benefits obtained with one strain cannot be directly extrapolated to another strain. As a consequence, all strains should have their own dossier to support health claims, although the strength of the scientific support varies from strain to strain.

At the moment, strains are mostly promoted for a general benefit to digestive health. In the perception of the consumer, this is a generic characteristic for all probiotic products, not for specific strains. As there are many strains of probiotics available, data need to be generated on the specific health benefits related to a specific strain, or final product. It will become increasingly important to associate a specific strain with a specific claim, and possibly with a specific target group of consumers. Only in this way can probiotic strains be marketed for their unique health benefits.

The legislative relation between claims and health benefits is far from clear (for a review, see Feord, 2002). An important problem is that medicinal legislation worldwide prohibits associating medicinal claims with food products. In several countries even health claims are prohibited. The basic rule is that food laws dictate that food labelling must not mislead the consumer. It is, however, difficult to objectively judge misleading of the consumer in the field of

probiotics and improved resistance since it is impossible to perform controlled infection experiments in humans.

12.7 Future trends

Currently, the search for new probiotic strains is handicapped by our limited knowledge of the mechanism behind the health benefit. Studying health benefits for probiotics on a trial and error basis for each single strain remains a costly operation that can only be funded by large companies and governments. Our increasing knowledge of probiotic mechanisms and the rapid progress in genomic techniques enables the identification of biochemical structures or bacterial genes that are essential for health benefit. This allows rational selection and subsequent validation of promising new strains.

An example of a potential target for *in vitro* selection of probiotics is the ability to adhere to carbohydrate binding sites at the epithelium that are also used for adhesion by enteropathogenic bacteria (Neeser *et al.*, 2000). These probiotics can reduce effects of food-borne infections. A more thorough understanding of bacterial cell characteristics could result in strain selection for immunomodulatory purposes (Christensen *et al.*, 2002). This might be useful for the development of probiotics preventing relapses of inflammatory bowel diseases, or for reduction of allergy symptoms. The major advantage of a first focus on a functional activity is that the potential as well as the limitations of the health benefits are known. The second focus should therefore be the optimisation of the health benefit of a strain. The addition of prebiotics to a probiotic product might give the strain a selective advantage in the gut, and encapsulation could help to pass the stomach.

A new promising area of research on probiotics is the field of genomics. The availability of complete genome sequences of several lactic acid bacteria (Klaenhammer *et al.*, 2002) will facilitate the identification of bacterial genes that are responsible for the probiotic effect. DNA microarray analysis and real-time PCR enable the study of genes at the transcription level under controlled conditions. Molecular techniques are available to study gene expression of bacteria under *in vivo* circumstances such as in the gastrointestinal tract (Slauch and Camilli, 2000). We strongly believe that in-depth studies in this direction will improve our understanding of probiotic effects and generate new leads for the identification and selection of probiotic strains. For those reasons, our research efforts are increasingly focused on genomic approaches.

The wide variety of mechanisms by which probiotics can potentially improve health make it likely that some strains can be used for certain applications, whereas other strains have different health benefits. This has raised the belief that a differentiation of strains for specific purposes will develop. As an example, *Lactobacillus* GG has been demonstrated to be active against rotavirus diarrhoea in young children (Isolauri *et al.*, 1991; Guandalini *et al.*, 2000). Nevertheless, this same strain failed to reduce the incidence of urinary tract

infections, bacterial sepsis and necrotising enterocolitis in preterm infants (Dani *et al.*, 2002). Apparently, the mechanism by which this strain contributes to resistance against rotavirus cannot be generalised to other infections. Therefore, we expect that in future strains are marketed specifically for allergy reduction, food-poisoning, prevention of traveller's diarrhoea, resistance to flu and common colds, and so on. Furthermore, there will be a focus on specific consumer groups, such as patients suffering from inflammatory bowel diseases, infants and the elderly.

Although most probiotics are used for intestinal applications, there is an increasing interest in alternative applications such as the treatment or prevention of urinary tract infections (Reid and Bruce, 2001). For this application, the ability to produce hydrogen peroxide is considered to be an important feature for a probiotic strain (Ocana *et al.*, 1999). Another application for probiotics might be the reduction of *Streptococcus mutans* in the oral cavity, which is responsible for dental caries, or the improvement of skin health. These applications have received little attention until now (Ouwehand *et al.*, 2002).

An unexplored new field for probiotics is the use of recombinant lactic acid bacteria as vehicles for the delivery of active molecules. It has been shown that immunisation of mice with recombinant *Lactococcus lactis* expressing tetanus toxin fragment C elicits a protective immune response against a challenge with the complete toxin (Robinson *et al.*, 1997). Using a similar approach, others showed that the delivery of an interleukin-10 producing *L. lactis* to mice could reduce the symptoms of TNBS-induced colitis and spontaneous colitis in IL-10-/- mice (Steidler *et al.*, 2000). Despite the straightforward approach and promising results, it is questionable to what extent the use of recombinant microorganisms is accepted by the consumer.

The scientific community, as well as consumers, increasingly accepts the health benefits of selected probiotic strains. The use of lactic acid bacteria to enhance the resistance to infections has become a major application of probiotics. Several double-blind placebo-controlled studies have been performed to demonstrate the effects in humans. In particular, shortening of rotavirus-induced diarrhoea is well established. Nevertheless, although many mechanisms behind the health effects have been proposed, the selection of new strains takes place mainly on a trial and error basis.

12.8 Sources of further information and advice

A recent overview of probiotic strains that are currently on the market and their documented health benefits in human clinical trials is given by Ouwehand and colleagues (Ouwehand *et al.*, 2002). Further information with respect to legal affairs, describing differences between the European, American and Japanese market, is given in a recent overview by Feord (2002).

12.9 References

ALANDER, M., DE SMET, I., NOLLET, L., VERSTRAETE, W., VON WRIGHT, A. and MATTILA-SANDHOLM, T. (1999) The effect of probiotic strains on the microbiota of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). *Int J Food Microbiol* 46: 71–79.

ASO, Y., AKAZA, H., KOTAKE, T., TSUKAMOTO, T., IMAI, K. and NAITO, S. (1995) Preventive effect of a *Lactobacillus casei* preparation on the recurrence of superficial bladder cancer in a double-blind trial. The BLP Study Group. *Eur Urol* 27: 104–109.

BERG, R.D. and SAVAGE, D.C. (1975) Immune responses of specific pathogen-free and gnotobiotic mice to antigens of indigenous and nonindigenous microorganisms. *Infect Immun* 11: 320–329.

BOVEE-OUDENHOVEN, I.M., WISSINK, M.L., WOUTERS, J.T. and VAN DER MEER, R. (1999) Dietary calcium phosphate stimulates intestinal lactobacilli and decreases the severity of a salmonella infection in rats. *J Nutr* 129: 607–612.

BOVEE-OUDENHOVEN, I., LETTINK-WISSINK, M., VAN DOESBURG, W. and VANDER MEER, R. (2002) Abstract 347: Dietary calcium inhibits traveler's diarrhea in humans.

CHRISTENSEN, H.R., FROKIAER, H. and PESTKA, J.J. (2002) Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. *J Immunol* 168: 171–178.

COCONNIER, M.H., LIEVIN, V., BERNET-CAMARD, M.F., HUDAULT, S. and SERVIN, A.L. (1997) Antibacterial effect of the adhering human *Lactobacillus acidophilus* strain LB. *Antimicrob Agents Chemother* 41: 1046–1052.

DANI, C., BIADAIOLI, R., BERTINI, G., MARTELLI, E. and RUBALTELLI, F.F. (2002) Probiotics feeding in prevention of urinary tract infection, bacterial sepsis and necrotizing enterocolitis in preterm infants. A prospective double-blind study. *Biol Neonate* 82: 103–108.

DE SIMONE, C., TZANTZOGLOU, S., BALDINELLI, L., DI FABIO, S., BIANCHI-SALVADORI, B., JIRILLO, E. and VESELY, R. (1988) Enhancement of host resistance against *Salmonella typhimurium* infection by a diet supplemented with yogurt. *Immunopharmacol Immunotoxicol* 10: 399–415.

DE WAARD, R., GARSSEN, J., SNEL, J., BOKKEN, G.C., SAKO, T., VELD, J.H. and VOS, J.G. (2001) Enhanced antigen-specific delayed-type hypersensitivity and immunoglobulin G2b responses after oral administration of viable *Lactobacillus casei* YIT9029 in Wistar and Brown Norway rats. *Clin Diagn Lab Immunol* 8: 762–767.

DE WAARD, R., GARSSEN, J., BOKKEN, G.C. and VOS, J.G. (2002) Antagonistic activity of *Lactobacillus casei* strain *shirota* against gastrointestinal *Listeria monocytogenes* infection in rats. *Int J Food Microbiol* 73: 93–100.

D'SOUZA, A.L., RAJKUMAR, C., COOKE, J. and BULPITT, C.J. (2002) Probiotics in prevention of antibiotic associated diarrhoea: meta-analysis. *BMJ* 324: 1361.

DUNNE, C., O'MAHONY, L., MURPHY, L., THORNTON, G., MORRISSEY, D., O'HALLORAN, S., FEENEY, M., FLYNN, S., FITZGERALD, G., DALY, C., KIELY, B., O'SULLIVAN, G.C., SHANAHAN, F. and COLLINS, J.K. (2001) *In vitro* selection criteria for probiotic bacteria of human origin: correlation with *in vivo* findings. *Am J Clin Nutr* 73: 386S–392S.

FELLEY, C.P., CORTHESY-THEULAZ, I., RIVERO, J.L., SIPPONEN, P., KAUFMANN, M., BAUERFEIND, P., WIESEL, P.H., BRASSART, D., PFEIFER, A., BLUM, A.L. and MICHETTI, P. (2001) Favourable effect of an acidified milk (LC-1) on *Helicobacter pylori* gastritis in man. *Eur J Gastroenterol Hepatol* 13: 25–29.

FEORD, J. (2002) Lactic acid bacteria in a changing legislative environment. *Antonie van Leeuwenhoek* 82: 353–360.

FRETER, R. and ABRAMS, G.D. (1972) Function of various intestinal bacteria in converting germfree mice to the normal state. *Infect Immun* 6: 119–126.

FULLER, R. (1989) Probiotics in man and animals. *J Appl Bacteriol* 66: 365–378.

GILL, H.S., RUTHERFURD, K.J. and CROSS, M.L. (2001) Dietary probiotic supplementation enhances natural killer cell activity in the elderly: an investigation of age-related immunological changes. *J Clin Immunol* 21: 264–271.

GUANDALINI, S., PENSABENE, L., ZIKRI, M.A., DIAS, J.A., CASALI, L.G., HOEKSTRA, H., KOLACEK, S., MASSAR, K., MICETIC-TURK, D., PAPADOPOULOU, A., DE SOUSA, J.S., SANDHU, B., SZAJEWSKA, H. and WEIZMAN, Z. (2000) Lactobacillus GG administered in oral rehydration solution to children with acute diarrhea: a multicenter European trial. *J Pediatr Gastroenterol Nutr* 30: 54–60.

GUARNER, F. and SCHAAFSMA, G.J. (1998) Probiotics. *Int J Food Microbiol* 39: 237–238.

GUPTA, P., ANDREW, H., KIRSCHNER, B.S. and GUANDALINI, S. (2000) Is lactobacillus GG helpful in children with Crohn's disease? Results of a preliminary, open-label study. *J Pediatr Gastroenterol Nutr* 31: 453–457.

HERIAS, M.V., HESSLE, C., TELEMO, E., MIDTVEDT, T., HANSON, L.A. and WOLD, A.E. (1999) Immunomodulatory effects of *Lactobacillus plantarum* colonizing the intestine of gnotobiotic rats. *Clin Exp Immunol* 116: 283–290.

HUDAULT, S., LIEVIN, V., BERNET-CAMARD, M.F. and SERVIN, A.L. (1997) Antagonistic activity exerted *in vitro* and *in vivo* by *Lactobacillus casei* (strain GG) against *Salmonella typhimurium* C5 infection. *Appl Environ Microbiol* 63: 513–518.

ISOLAURI, E., JUNTUNEN, M., RAUTANEN, T., SILLANAUKEE, P. and KOIVULA, T. (1991) A human *Lactobacillus* strain (*Lactobacillus casei* sp strain GG) promotes recovery from acute diarrhea in children. *Pediatrics* 88: 90–97.

ISOLAURI, E., MAJAMAA, H., ARVOLA, T., RANTALA, I., VIRTANEN, E. and ARVILOMMI, H. (1993) *Lactobacillus casei* strain GG reverses increased intestinal permeability induced by cow milk in suckling rats. *Gastroenterology* 105: 1643–1650.

ISOLAURI, E., ARVOLA, T., SUTAS, Y., MOILANEN, E. and SALMINEN, S. (2000) Probiotics in the management of atopic eczema. *Clin Exp Allergy* 30: 1604–1610.

KABIR, A.M., AIBA, Y., TAKAGI, A., KAMIYA, S., MIWA, T. and KOGA, Y. (1997) Prevention of *Helicobacter pylori* infection by lactobacilli in a gnotobiotic murine model. *Gut* 41: 49–55.

KERNEIS, S., BOGDANOVA, A., KRAEHENBUHL, J.P. and PRINGAULT, E. (1997) Conversion by Peyer's patch lymphocytes of human enterocytes into M cells that transport bacteria. *Science* 277: 949–952.

KIRJAVAINEN, P.V., OUWEHAND, A.C., ISOLAURI, E. and SALMINEN, S.J. (1998) The ability of probiotic bacteria to bind to human intestinal mucus. *FEMS Microbiol Lett* 167: 185–189.

KITAZAWA, H., TOMIOKA, Y., MATSUMURA, K., ASO, H., MIZUGAKI, M., ITOH, T. and YAMAGUCHI, T. (1994) Expression of mRNA encoding IFN alpha in macrophages stimulated with *Lactobacillus gasseri. FEMS Microbiol Lett* 120: 315–321.

KLAENHAMMER, T., ALTERMANN, E., ARIGONI, F., BOLOTIN, A., BREIDT, F., BROADBENT, J., CANO, R., CHAILLOU, S., DEUTSCHER, J., GASSON, M., VAN DEN GUCHTE, M., GUZZO, J., HARTKE, A., HAWKINS, T., HOLS, P., HUTKINS, R., KLEEREBEZEM, M., KOK, J., KUIPERS, O., LUBBERS, M., MAGUIN, E., MCKAY, L., MILLS, D., NAUTA, A., OVERBEEK, R., PEL, H., PRIDMORE, D., SAIER, M., VAN SINDERSEN, D., SOROKIN, A., STEELE, J., O'SULLIVAN, D., DE VOS, W., WEIMER, B., ZAGOREC, M. and SIEZEN, R. (2002) Discovering lactic acid

bacteria by genomics. *Antonie van Leeuwenhoek* 82(1–4): 29–58.

KUBY, J. (1997) *Immunology*, Third edition. W.H. Freeman, New York.

LEE, Y.K., LIM, C.Y., TENG, W.L., OUWEHAND, A.C., TUOMOLA, E.M. and SALMINEN, S. (2000) Quantitative approach in the study of adhesion of lactic acid bacteria to intestinal cells and their competition with enterobacteria. *Appl Environ Microbiol* 66: 3692–3697.

LINK-AMSTER, H., ROCHAT, F., SAUDAN, K.Y., MIGNOT, O. and AESCHLIMANN, J.M. (1994) Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intake. *FEMS Immunol Med Microbiol* 10: 55–63.

MACDONALD, T.T. (1995) Breakdown of tolerance to the intestinal bacterial flora in inflammatory bowel disease (IBD) [editorial; comment]. *Clin Exp Immunol* 102: 445–447.

MARIN, M.L., LEE, J.H., MURTHA, J., USTUNOL, Z. and PESTKA, J.J. (1997) Differential cytokine production in clonal macrophage and T-cell lines cultured with bifidobacteria. *J Dairy Sci* 80: 2713–2720.

MARTEAU, P., MINEKUS, M., HAVENAAR, R. and HUIS IN'T VELD, J.H. (1997) Survival of lactic acid bacteria in a dynamic model of the stomach and small intestine: validation and the effects of bile. *J Dairy Sci* 80: 1031–1037.

NEESER, J.R., GRANATO, D., ROUVET, M., SERVIN, A., TENEBERG, S. and KARLSSON, K.A. (2000) *Lactobacillus johnsonii* La1 shares carbohydrate-binding specificities with several enteropathogenic bacteria. *Glycobiology* 10: 1193–1199.

NIEDZIELIN, K., KORDECKI, H. and BIRKENFELD, B. (2001) A controlled, double-blind, randomized study on the efficacy of *Lactobacillus plantarum* 299V in patients with irritable bowel syndrome. *Eur J Gastroenterol Hepatol* 13: 1143–1147.

OCANA, V.S., PESCE DE RUIZ HOLGADO, A.A. and NADER-MACIAS, M.E. (1999) Selection of vaginal H_2O_2-generating *Lactobacillus* species for probiotic use. *Curr Microbiol* 38: 279–284.

OUWEHAND, A.C., SALMINEN, S. and ISOLAURI, E. (2002) Probiotics: an overview of beneficial effects. *Antonie van Leeuwenhoek* 82: 279–289.

REID, G. and BRUCE, A.W. (2001) Selection of lactobacillus strains for urogenital probiotic applications. *J Infect Dis* 183 Suppl 1: S77–S80.

RESCIGNO, M., URBANO, M., VALZASINA, B., FRANCOLINI, M., ROTTA, G., BONASIO, R., GRANUCCI, F., KRAEHENBUHL, J.P. and RICCIARDI-CASTAGNOLI, P. (2001) Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat Immunol* 2: 361–367.

ROBINSON, K., CHAMBERLAIN, L.M., SCHOFIELD, K.M., WELLS, J.M. and LE PAGE, R.W. (1997) Oral vaccination of mice against tetanus with recombinant *Lactococcus lactis. Nat Biotechnol* 15: 653–657.

SALMINEN, S., OUWEHAND, A., BENNO, Y. and LEE, K.Y. (1999) Probiotics: how should they be defined? *Trends in Food Science and Technology* 10: 107–110.

SELSTED, M.E., MILLER, S.I., HENSCHEN, A.H. and OUELLETTE, A.J. (1992) Enteric defensins: antibiotic peptide components of intestinal host defense. *J Cell Biol* 118: 929–936.

SHORNIKOVA, A.V., CASAS, I.A., MYKKANEN, H., SALO, E. and VESIKARI, T. (1997) Bacteriotherapy with *Lactobacillus reuteri* in rotavirus gastroenteritis. *Pediatr Infect Dis J* 16: 1103–1107.

SLAUCH, J.M. and CAMILLI, A. (2000) IVET and RIVET: use of gene fusions to identify bacterial virulence factors specifically induced in host tissues. *Methods Enzymol* 326: 73–96.

SPANHAAK, S., HAVENAAR, R. and SCHAAFSMA, G. (1998) The effect of consumption of milk fermented by *Lactobacillus casei* strain *Shirota* on the intestinal microflora and immune parameters in humans. *Eur J Clin Nutr* 52: 899–907.

STEIDLER, L., HANS, W., SCHOTTE, L., NEIRYNCK, S., OBERMEIER, F., FALK, W., FIERS, W. and REMAUT, E. (2000) Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. *Science* 289: 1352–1355.

TALARICO, T.L. and DOBROGOSZ, W.J. (1989) Chemical characterization of an antimicrobial substance produced by *Lactobacillus reuteri*. *Antimicrob Agents Chemother* 33: 674–679.

TUOMOLA, E.M. and SALMINEN, S.J. (1998) Adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures. *Int J Food Microbiol* 41: 45–51.

TUOMOLA, E.M., OUWEHAND, A.C. and SALMINEN, S.J. (1999) The effect of probiotic bacteria on the adhesion of pathogens to human intestinal mucus. *FEMS Immunol Med Microbiol* 26: 137–142.

WELLS, C.L., MADDAUS, M.A. and SIMMONS, R.L. (1988) Proposed mechanisms for the translocation of intestinal bacteria. *Rev Infect Dis* 10: 958–979.

YASUI, H. and OHWAKI, M. (1991) Enhancement of immune response in Peyer's patch cells cultured with *Bifidobacterium breve*. *J Dairy Sci* 74: 1187–1195.