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Immunomodulation by probiotics: a literature survey

 J Garssen, M Herreilers, H van Loveren, J Vos,
A Opperhuizen

Preface

A new trend in nutrition is the consumption of "functional food". Probiotics form an example of functional foods on which health claims based on experimental animal and clinical studies are being made. This literature survey shows that immunomodulation induced by probiotics could have beneficial as well as deleterious health effects. Unfortunately, only a few studies in the literature are focused on possible adverse effects of ingesting probiotics. In addition, a risk–benefit analysis of probiotics is hard to realise due to the diversity in probiotic products and their effects. Beneficial effects for different types of probiotics are reviewed here, and the need for risk–benefit analysis addressed. This emphasises the need for future research.

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Samenvatting

De belangrijkste functie van voeding is te voorzien in voldoende nutriënten en energie. Bij nutriënten moet gedacht worden aan zowel macronutriënten (eiwitten, vetten en koolhydraten) als micronutriënten zoals mineralen, vitaminen en spore-elementen. Een niet te onderschatten additionele functie is meer van sensorische aard zoals smaak en kleur van de voeding. Sinds enige jaren is er interesse in nog een derde functie ontstaan. Er komt steeds meer voeding op de markt waarvan geclaimd wordt dat ze fysiologische systemen zoals het immuunsysteem, endocrine systeem, hart- en vaatstelsel etc. kunnen verbeteren. Deze voedingsmiddelen worden functionele voedingsmiddelen genoemd. Dit rapport gaat met name over de immuunmodulerende werking van probiotische bacteriën. Het merendeel van de gepubliceerde literatuur is gericht op positive gezondheidseffecten. Over eventuele schadelijke gevolgen van probiotica gebruik is weinig bekend. Een eindconlcusie over probiotica als groep is zeker moeilijk te maken aangezien er grote verschillen bestaan tussen typen probiotica, met soms verschilllende en soms zelfs tegengestelde effecten. Daarom zijn meer gegevens nodig voordat een conclusie getrokken kan worden over de voors en tegens van het gebruik van een bepaald type probioticum.

Een adequate manier om to concluderen of blootstelling aan probiotica gezondheidsbevorderend of juist nadelig zullen zijn is het uitvoeren van experimenten gebruikmakend van ziektemodellen, zoals infectiemodellen, huid-, luchtweg-, of voedselallergiemodellen, en autoimmuunmodellen. Patronen in veranderingen in immuunparameters, geëvalueerd in de context van klinische effecten die worden waargenomen met dergelijke ziektemodellen, kunnen uiteindelijk indicatief zijn voor mogelijke gezondheidsbevorderende of juist nadelige effecten van probiotica, en dergelijke informatie kan de basis vormen voor een systeem van evaluatie van probiotica die op de markt worden gebracht.

Summary

The primary function of food in the human diet is to provide nutrients and energy. Nutrients include macronutrients (protein, fat and carbohydrates) and micronutrients (minerals, vitamins,and trace elements). Food also has the secondary function of giving sensory satisfaction by its flavour, taste and colour. Recently identified is a third function: the capacity of food to modulate physiological systems (immune, endocrine, nervous, circulatory and digestive) beyond the accepted nutritional effects. Food components having these functions are called "functional foods". This report reviews the immunomodulating properties of probiotics. Most literature on this topic is focused on beneficial health effects. In contrast, not much information is available on deleterious effects . Different probiotics may also have different effects, sometimes opposite, depending on the type. Therefore it is difficult to draw overall conclusions on the risks and benefits of probiotics. For this reason, new studies are required to investigate these risks and benefits.

The most appropriate way to conclude whether immune effects noted are beneficial or rather deleterious is to study effects of exposure in disease models. Such models include infection models, models of respiratory, skin, and food allergy and models of autoimmunity. Patterns of immune alteration due to the probiotics may be interpreted in the context of the clinical effects observed, so that eventually such patterns alone would be able to indicate possible beneficial or deleterious effects of probiotics. Information on patterns of effects indicative of positive or negative consequences of the use of probiotics will eventually form the basis of designing a protocol for the evaluation of probiotics to be put on the market.

1 Functional Foods

The primary function of food is to provide nutrients and energy. These nutrients can include macronutrients (protein, fat, and carbohydrates) and micronutrients (minerals, vitamins, and trace elements). Food also has a secondary function of giving sensory satisfaction in its flavour, taste, colour and texture. Recently, there has been renewed interest in a third function: the capacity of food to modulate physiological systems (immune, endocrine, nervous, circulatory, and digestive) beyond the accepted nutritional effects. Food components having these functions are called functional foods (NRLO report, 2000).

The interest in functional foods has increased strongly over the last few years.

Until the early 1980s, nutrition studies focused mainly on health risks. The most important issue before that time was that foods should not contain components that can be harmful for human health, and be pathogen-free.

In recent years more and more attention has been paid to health promoting effects of food. Probably the best studied example is that increased fruit and vegetable consumption is accompanied by a reduction in the risk of heart disease and cancer (Ohigashi et al, 1996 and Milner, 1999). This can be seen as the root for the widespread interest in functional (health promoting) foods. It was thought that there might be more food components, having a preventive aspect on disease, or being beneficial to human health. Examples of these components are 1) fat and sugar replacers to decrease fat and carbohydrate intake, thus reducing energy intake and reducing overweight, and diabetes, and 2) micronutrients with antioxidant activity. As a result, foods are now produced with increased amounts of these micronutrients.

There are many more reasons why public interest in functional foods is increasing. One of these reasons is the increase in health care costs. For the government it is important to reduce health care costs as much as possible. Additional reasons are the ageing of the population, which is accompanied by more disease. Recent legislation on functional foods further illustrates the interest (Hasler, 1996; Milner 2000).

Thus far, it has been unclear what criteria should be met before a food can be called 'functional'. A requirement is that the product is still a food, and not a drug. To make this clear the definitions for drugs and foods are given. A drug is any compound/agent that is intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease (Milner, 2000). Foods are compounds used primarily for nutrition but also for taste and/or

aroma (Hasler, 1996). Functional foods are foods that provide an additional physiological benefit that may help to prevent disease or promote health. Therefore, it can be said that functional foods can be categorised somewhere between foods and drugs. Several definitions have been published for functional foods:

- any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains (Institute of Medicine of the National Academy of Sciences (1994) (Milner, 1999)

- A food can be regarded as 'functional' if it has been demonstrated by sound scientific research to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease (ILSI (International Life Sciences Institute in Europe, 1999)

Functional foods must remain foods and they must demonstrate their effects in amounts that can normally be expected to be consumed in the diet: they are not pills or capsules, but part of a normal food pattern. A functional food can be a natural food, a food to which a component has been added, or a food from which a component has been removed by technological or biotechnological means. It can also be a food where the nature of one or more components has been modified, or a food in which the bioavailability of one or more components has been modified, or any combination of these possibilities. A functional food might be functional for all members of a population or for particular groups of the population, which might be defined, for example, by age or by genetic constitution.

From the definition given by ILSI it is important to note that functional foods are intended for a population generally in good health, and beneficial effects are only acceptable if they are obtained at a reasonable intake under normal consumption conditions. A reasonable intake must be effective and safe, and should not, result in an adverse effect on the indicators studied in any population group. Functional foods should be developed in the context of a total diet aimed at optimising health. Functional foods may contain either bioactive constituents in a higher quantity than present in corresponding conventional foods (such as for antioxidant vitamins) or may have reduced levels of undesirable components (such as saturated and trans-fatty acids) (NRLO report, 2000).

The ILSI definition claims that a functional food can be a natural food, or a food that has been modified. This modification can be achieved by using any of the 5 approaches as mentioned below (NRLO report, 2000):

- Eliminating a component known to cause or identified as causing a deleterious effect when consumed (e.g., an allergenic protein).

- Increasing the concentration of a component naturally present in food to a point at which it will induce predicted effects (e.g., fortification with a micronutrient to reach a daily intake higher than the recommended daily intake but compatible with the dietary guidelines for reducing risk of disease), or increasing the concentration of a non-nutritive component to a level known to produce a beneficial effect.

- Adding a component that is not normally present in most foods and is not necessarily a macronutrient or a micronutrient but for which beneficial effects have been shown (e.g., non-vitamin antioxidant or prebiotic fructans).

- Replacing a component, usually a macronutrient (e.g., fats), whose intake is usually excessive and thus a cause of deleterious effects, by a component for which beneficial effects have been shown.

- Increasing bioavailability or stability of a component known to produce a functional effect or to reduce the disease-risk potential of the food.

1.1 Possible effects of functional foods

Functional foods can effect different systems in the body. Thus far, the most important published effects include:

- gastrointestinal functions. These functions include those that are associated with a balanced colonic microflora, mediated by the endocrine activity of the gastrointestinal tract, dependent on the tract's immune activity, in control of nutrient (minerals in particular) bioavailability, in control of transit time and mucosal motility, and modulators of epithelial cell proliferation.

- redox and antioxidants systems. These systems require a balanced and satisfactory intake of antioxidant (pro-) vitamins as well as nonvitamin food components such as polyphenols and other natural antioxidants of plant origin. Redox activities and antioxidant protection are important for almost every cell and tissue, and their imbalance is associated with miscellaneous pathologies. Although well-founded hypotheses often exist regarding the mechanisms of action of dietary antioxidants, demonstration of their beneficial effects, except when they are consumed as components of fresh fruit and vegetables, remains problematic.

- metabolism of macronutrients. This target concerns metabolism of carbohydrates, amino acids, and fatty acids and, in particular, hormonal modulation of their metabolism via insulin and glucagon balance or the production of gastrointestinal peptides. The objective of this process is to reduce the risk of pathologic effects associated with insulin resistance and cardiovascular disease; doing so will require the study of interactions between nutrient intake and regulation of gene expression.

According to their effect, functional foods can be categorised in the following groups:

- Fat replacers: they may contribute to a reduction of fat intake. Replacing fat by the same amount of protein or carbohydrates already results in a reduction of energy intake. Fats can also be replaced by modified fats or synthesised fats. These are generally not digested and absorbed, which means a considerable reduction of the fat intake or fat content.

- Antioxidants: it is long known that free radicals can be a risk factor for the induction of degenerative diseases. There is a possibility in counteracting these radicals with antioxidants. Antioxidants are normally present in foods (e.g., certain vitamins). The question is now whether a food can be made functional by adding higher concentrations of antioxidants. Research has to be done to find optimal concentrations of antioxidants.

- Plant sterols and polyunsaturated fatty acids: it has been known for decades that a low level of serum cholesterol reduces the risk of coronary heart disease. To lower the level of cholesterol, the consumption of polyunsaturated fatty acids is recommended. Recently, the effects of plant sterols in reducing serum total and LDL cholesterol levels led to the introduction on the market of margarines containing specific plant sterols..

- Minerals and trace elements: the beneficial effects of minerals and trace elements have long been described. It is now thought that adding higher concentrations to foods can also make them more beneficial to human health.

- Sport foods and brain foods: this category includes foods that give an improvement on physical performance or of mental performance. Examples are creatinine, and caffeine respectively.

- Probiotics and prebiotics: these are dietary supplements that have a beneficial effect on the host by altering the intestinal microflora. The microflora can be altered either by adding certain microorganisms themselves to the intestine, or by adding certain

food ingredients that are capable of stimulating the growth or activity of a limited number of bacteria in the colon. The latter food ingredients are prebiotics, and the former probiotics. It is known that these probiotics, when administered to the intestine, can have many effects throughout the body (in other words systemic effects).

In the following sections of this report, probiotics and their effects will be discussed in detail.

2 Probiotics

In the beginning of the 20th century, Metchinkoff, was the first person who proposed that certain bacteria in yoghurt would have some beneficial effects on human health. He attributed the long life of Bulgarian peasants to their intake of yoghurt containing several Lactobacillus species (Sanders, 2000). In the 1930s, Minoru Shirota, a Japanese medical microbiologist, proposed that many diseases could be prevented if an optimal gut microflora was maintained (Alvarez-Olmos and Oberhelman, 2001). He selected strains of lactic acid bacteria from the human intestine that might be beneficial. He selected species that could survive passage through the digestive system. From his work the well known Yakult yoghurt product was developed.

This work by Metchinkoff and Shirota has been the start of the interest in microorganisms that might have a beneficial effect on human health. It was however not earlier than the 1980s, that the term "probiotics" has been introduced. Since the late 1990s a lot of research on this subject has been performed, and interest on the subject has increased even more since that time.

A probiotic is classically defined as a viable microbial dietary supplement that beneficially affects the host through its "local" effects in the intestinal tract. This definition was proposed by Fuller in 1989. It should be mentioned that it was initially intended for use in animal feed products. For human nutrition, the following definition has been proposed: "a live microbial food ingredient that is beneficial to health". Salminen gave this definition in 1998, and it is still used. The word "probiotic" is derived from the Greek, meaning "for life" (Schrezenmeir and de Vrese, 2001).

Because of the definition, there are several restrictions for a product to be called a probiotic. The first and major requirement is that the product contains living microorganisms. These microorganisms can be added to food components as freeze-dried cells or in fermented products like yoghurts and cheese. Today there is a debate as whether the microorganisms need to be alive, or that dead microorganisms can induce the same effect in the intestine. More research has to be performed on this subject, and for now it is felt that probiotics need to contain living organisms.

An important feature is that a probiotic can affect different mucosal surfaces: mouth, gastrointestinal tract, upper respiratory tract, and urogenital tract. Probiotics used in the respiratory tract can be applied as an aerosol, for the urogenital tract they can be applied

locally, which also applies for the mouth. Probiotics in foods are used to affect the intestinal microflora. These probiotics therefore have to survive passage through the stomach. Only very few bacteria, and some non-pathogenic, antibiotic-resistant yeasts qualify for this. For these bacteria and yeasts to be effective, they should have some basic characteristics. Preferably, the microorganisms are of human origin, and are innocuous. They should be able to withstand processing conditions. They should prevent diseases, e.g. by counteracting pathogens, yet should elicit no or minimal immune responses to themselves (Alvarez-Olmos and Oberhelman, 2001). Optimally they should be able to function with or without the use of antibiotics.

At present, the bacteria strains most commonly used as probiotics are lactobacilli, bifidobacteria, and to a lesser extent enterococcus and streptococcus. These bacteria are normal inhabitants of the gut. The representative species include *Lactobacillus acidophilus, Lactobacillus gasseri, Lactobacillus casei, Lactobacillus rhamnosus, Bifidobacterium longum*, and *Bifidobacterium bifidum*. Next to these strains of bacteria, the yeast *Saccharomyces boulardii* is also a potential probiotic agent (Rolfe, 2000). Microorganisms that are used as potential probiotic agents are listed in table 1 (reviewed by Alvarez-Olmos and Oberhelman, 2001).

Table 1: Microorganisms that are used as probiotic agents

Lactobacillus species	Bifidobacterium species	Others
L. acidophilus	*B. bifidum*	*Bacillus cereus*
L. casei	*B. longum*	*Eschericia Coli*
L. rhamnosus	*B. breve*	*Saccharomyces cerevisiae*
L. reuteri	*B. infantis*	*Saccharomyces boulardii*
L. bulgaricus	*B. lactis*	*Enterococcus faecalis*
L. plantarum	*B. adolescentis*	*Streptococcus thermophilus*
L. johnsonii		
L. lactis		
L. gasseri		

Lactobacilli are Gram-positive rod-shaped lactic acid bacteria. They are non-sporinating, catalase-negative organisms that are devoid of cytochromes and of non-aerobic habit but are aero-tolerant, acid-tolerant and fermentive (Holzapfel et al; 2001). Although there are some exceptions, most of the lactobacillus strains are not pathogenic and lactobacilli are major

constituents of the normal human and animal gut. Because of their harmless character they have the GRAS-status (generally regarded as safe), and are frequently used in bioprocessing and preservation of food.

The bacteria are almost exclusively consumed as fermented diary products such as enriched yoghurt, yoghurt-like products, milks or freeze-dried cultures. They are also marketed as capsules, and powders. In future, it is expected that they will also be found in fermented vegetables and meats.
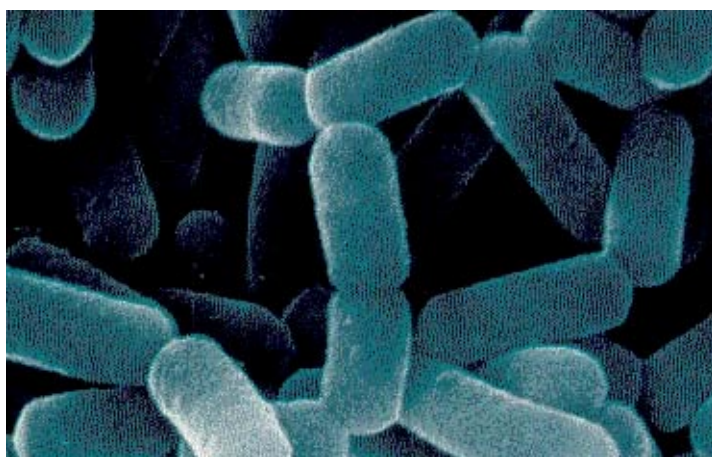
Figure 1: A scanning electron micrograph of Lactobacillus casei strain Shirota (Yakult Honsha Co., Ltd, 1999).

The health effects of probiotics reported include immune stimulation, cholesterol lowering, prevention of cancer recurrence, and relief in gastrointestinal disease. Some of these effects are associated with the immune system present in the gastro-intestinal tract. Therefore, first of all, the gastrointestinal immune system will be discussed, and after that the effects of probiotics.

3   The gastrointestinal tract and its immune system

The gastrointestinal tract is a lumen in the human body that is in continuum with the environment. The primary function of this tract is to digest and absorb nutrients to meet the metabolic requirements and demands for normal human growth and development. In addition, it also serves as a barrier against those bacteria, viruses and other pathogens that are harmful to the body. Factors that can protect against these harmful agents include saliva, gastric acid, peristalsis, mucus, and the intestinal flora (Isolauri et al., 2001).

Food is first taken into the mouth, where saliva is present to transport all components, including bacteria, to the stomach. The vast majority of all microorganisms are destroyed there by gastric acid. Species that can resist the acidity (e.g., lactobacilli, streptococci, and fungi) will be transported to the small bowel. Here they are exposed to further defence mechanisms. These mechanisms include intestinal motility, the inhibitory effect of bile salts, the hydrolytic activity of pancreatic enzymes, and the mucosal immune system such as high amounts of IgA. Finally the microorganisms reach the densely populated colon.

In general, when a pathogen has been able to enter the body, it will be cleared as soon as possible. The innate immune system is responsible for this first line of defense. It consists mainly of macrophages and neutrophils which have the ability to phagocytize and kill the pathogen. However, they cannot eliminate all infectious organisms and many pathogens are not even recognised. In this case the adaptive immune response is needed for clearance of the pathogen. This immune response is very specific and can be divided in a cellular and humoral immune response. The main cells of this response are T and B lymphocytes. T lymphocytes differentiate in the thymus, whereas B lymphocytes differentiate outside the thymus in different other tissues. B cells are associated with the humoral response, they are responsible for clearance of extracellular pathogens and do this via the production of specific antibodies. T cells are involved in both the cellular and humoral response. They serve as cells for antigen recognition and help to activate B cells. In the cellular response they serve as cytotoxic T cells. These cells recognise specific intracellular antigens and are able to phagocytise these infected cells. Several cytokines are produced to orchestra the different immune responses. These cytokines can trigger or skew immune responses to a more cellular response (IFNγ) or a humoral response (IL4). T cells that mediate a cellular immune response and T cells that helps a humoral immune response are defined as Th1 cells and Th2 cells respectively.

Certain pathogens have the capacity to translocate and cause systemic infection, and immune responses to these pathogens protect the host. As microbiological agents elicit immune responses, as do antigenic components of food, there is obviously a requirement of the intestinal mucosal immune system to discriminate between pathogens and the enormous diversity of dietary antigens to which it is exposed. This has resulted in the evolution of a unique and distinct intestinal immune system. Mucosal surfaces are specialised in two seemingly opposing functions: tolerance to environmental antigens (food antigens) and immunity to mucosal pathogenic microorganisms.

The gastrointestinal tract harbours it's own rich microflora of more than 500 different bacterial species. They include the so-called native inhabitants as well as transient organisms that are by change present in the gastro-intestinal tract. Native inhabitants are those organisms that colonise or fill specific niches in the body. The transient flora includes those microorganisms that colonise the body from the external environment and can persist if some niches are not filled with native flora (Alvarez-Olmos and Oberhelman, 2001). The precise function of microorganisms was long unknown.

In early stages it was thought that all bacteria were harmful, and everything was done to prevent bacteria from entering the body. Food was sterilised before use, and people were taking all kinds of antimicrobials. Today, it is known that a healthy individual has a multitude of microbial organisms associated to skin, upper respiratory tract, and gastro-intestinal surfaces. The majority is not pathogenic and essential to help maintain the gut luminal environment, to aid digestion, protect the host from invading harmful bacteria and viruses, and probably regulate the local immune system.

One of the most important determinants of virulence of a bacterial pathogen is the ability to adhere to the host surface (host intestinal epithelium). This is essential for the pathogen to resist the fluid flow of the luminal contents and the peristalsis of intestinal contraction (Lu and Walker, 2001). The first challenge to potential luminal pathogens is therefore to successfully attach to the intestinal surface. The indigenous intestinal microflora is known to interfere with this adherence and thus the colonisation of the intestine by pathogens. Competition for binding sites is likely to play an important role in the protective effects of non-pathogenic intestinal microorganisms.

The intestinal microflora also has an effect on the expression of glycoconjugates present on epithelial cells. The sugar moiety of these glycoconjugates can serve as receptors for several microorganisms, and can influence the binding possibilities of bacterial pathogens. The binding possibility can either be enhanced, increasing the susceptibility for disease (figure 2).

On the other hand the binding possibility can also be decreased, giving the pathogen no chance to effect the host.
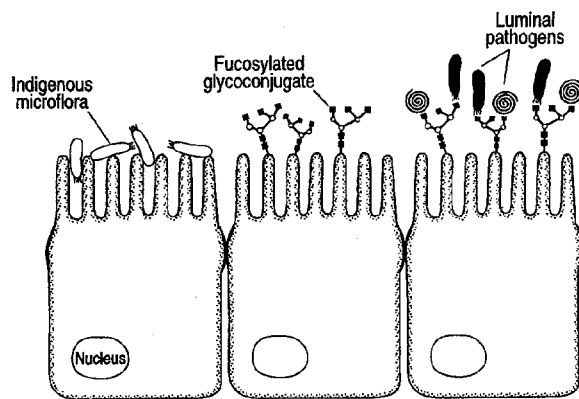

Figure 2: Crosstalk between intestinal bacteria and the host epithelium. (Colonisation by indigenous microflora induces the expression of fucosylated glycoconjugates on the host intestinal epithelium. The expression of the glycoconjugates provides lectin-like receptors for the attachment of luminal pathogens and eventually confers susceptibility to pathogen colonisation and disease. (Schauer, 1997))

Another important mechanism is the competition for nutrients between the intestinal microflora and the pathogenic microorganisms. A rich microflora will leave less nutrients for pathogens, preventing colonisation and possible disease (Alvarez-Olmos and Oberhelman, 2001). The mucosal flora can also produce toxins that can destroy certain pathogens. It can be concluded that different types of bacteria compete with one another and in doing so regulate the quantity and quality of microorganisms present. It is however evident that certain pathogens are not destroyed or inhibited by the intestinal flora.

The gut-associated lymphoid tissue (GALT) is part of the specialised mucosa-associated immune system, and represents the largest mass of lymphoid tissue in the entire human body. The immune response occurs in different physiologic compartments of this tissue: aggregated in follicles and Peyer's patches, and distributed within the mucosa, the intestinal epithelium, and secretory sites (Isolauri et al., 2001). After birth, T lymphocytes migrate from the thymus to the thymus-dependent areas of the Peyer's patches and to the epithelium. Exposure to microorganisms in the normal environment is necessary to develop the B cell population. As bacterial colonisation of the intestine starts immediately after birth this is rapidly established.

The intestinal epithelium overlying the Peyer's patches is specialised to allow the transport of antigens into the lymphoid tissue. Epithelial cells termed 'M' (microfold) cells, due to the numerous microfolds on their luminal surface, carry out this particular function. M cells are

able to absorb and transport antigens and, possibly, process and present them to subepithelial lymphoid cells (figure 3). The M cells overlay lymphoid follicles, and they lack mucus and glycocalyx thereby facilitating contact between molecules and particles and the M cells. Several microorganisms take advantage of the transcytotic function of M cells and use them to cross the otherwise impermeable epithelial lining of the gut. For example, this is true for several *Salmonella enteritidis* species.The interactions of pathogens with M cells may lead to changes in the host cell cytoskeleton which might result in bacterial entry into the cell. In addition, microbial entry into epithelial cells results in the production and release of proinflammatory cytokines. Therefore in response to a bacterial invasion an acute mucosal inflammatory response is initiated. This protects the host from these pathogens within a very short time period. Adherence to and uptake of microorganisms by M cells also results in secretion of protective polymeric IgA antibodies to prevent the uptake of antigens, thus limiting the intensity and duration of the disease. These mechanisms all help to keep infection with pathogens in the gut localized and as minimal as possible (Lu and Walker, 2001).
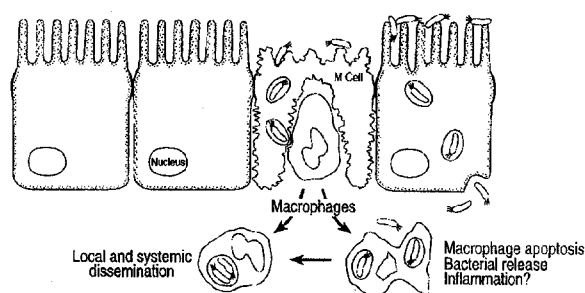

Figure 3: Pathogens crossing the epithelial barrier by entering via either M cells or enterocytes. (Subsequent events include M cell destruction and subepithelial invasion by pathogens of macrophages. Pathogens induce enhanced macrophage apoptosis and elicit a mucosal immune and inflammatory response (Sansonetti and Phalipon, 1999)).

Immunoglobulin A (IgA) is mainly secreted in the lamina propria. In contrast to IgA in serum, secretory IgA is present in a different form. This form is resistant to intraluminal proteolysis, and does not activate complement or inflammatory responses. This makes it ideal for protecting mucosal surfaces. There are differences between the upper and lower parts of the human GALT in the isotype distribution of immunoglobulin-producing cells. IgA-1 immunocytes predominate in the small intestine, whereas IgA-2 producing cells are most frequent in the colon, the latter being more resistant to bacterial proteases (Isolauri et al, 2001 and Doe, 1989). The secretory IgA antibodies in the gut are part of the common mucosal

immune system. That is the reason why these antibodies can also be effective at other mucosal surfaces. The specific antibody-secreting lymphocytes appear in peripheral blood 2-4 days after antigen exposure, reach a maximum concentration after 6-8 days, and persist in the blood for 2-3 weeks (Isolauri et al., 2001).

Regulatory pathways in the immune system include the dichotomy of Th1 and Th2 activity. T ceel provide help to immune responses, eg. The development of cellular responses. T cells that do this are characterized by interleukin production such as Interferon γ or Interleukin 2, and are designated T-helper 1 (Th1) cells. Other T helper cells, so called T helper 2 cells, are characterized rather by Interleukin 4 production, and provide help to the development of antibody production, notably IgE. These two T helper cell types regulate each others activity as a form of feed back, so that usually a proper balance exist between the two. Under cirtain circumstances, however, this balance may be disturbed, as is the case for instance in asthma. The intraepithelial T lymphocytes mainly exhibit a suppressor and cytotoxic phenotype, whereas the lamina propria cells exhibit a helper and inducer T cell phenotype. B cells are predominantly found in the lamina propria. T cells in the lamina propria have the ability of increased lymphokine and cytokine production. They even seem to have the potential to produce both T helper 1 as well as T helper 2 cell specific cytokines. If an intracellular infection (e.g. by Salmonella or Listeria bacteria) occurs, the cellular response can then be extended by T-helper-1 (Th1) cells. If only extracellular pathogens (e.g. Schistosoma or Trichinella infections) infiltrate the production of IL-4 and IL-10 will be enhanced, which are associated with a Th 2-response. However, it should be realised that during Th1 or Th2 dominated responses both Th subsets seem to be involved, at least partially or indirectly. Under physiological conditions, it is likely that cytokines only transiently shift the balance along the Th1-Th2 axis, without permanently fixing the Th phenotype. More extreme Th1 or Th2 phenotype is only seen in certain pathological conditions. For instance, in asthma predominantly Th2 type of immune responses are evident.

It is now clear that the gastrointestinal tract contains effective mechanisms to eliminate pathogens by a response to pathogenic antigens. Elimination of nutritive antigens is however not desired. This has led to the concept of "oral tolerance" by a mechanism that is not yet fully understood. (Isolauri et al., 2001; Doe, 1989). It is thought that anergy of antigen-specific T cells plays an important role. This anergy may be induced by class I restricted CD4+ T-cells and by the cytokines they produce. These cytokines may have an additional immunomodulatory role, in this case a suppressive effect.

Dose and frequency of antigen exposure may influence the tolerance and its mechanism. High doses of an antigen may result in clonal deletion or anergy, whereas low doses may result in active suppression subsequent to the induction of regulatory T cells, sometimes called Th3 cells, in Peyer's patches (figure 4). The regulatory T lymphocytes function through the production of suppressive cytokines, including IL-4, IL-10, and especially transforming growth factor β1. Clonal deletion or anergy is preceded by the local production of IL-12, IFN γ (with consequent suppression of IL-4 and transforming growth factor β generation), and involves the apoptosis of Th1 cells but also Th2 cells. It is therefore suggested that one of the major mechanisms by which the gut-associated lymphoid tissue maintains homeostasis is via local cytokine regulation, particularly transforming growth factor β-associated low-dose tolerance (Isolauri et al., 2001; Monteleone et al., 2001).
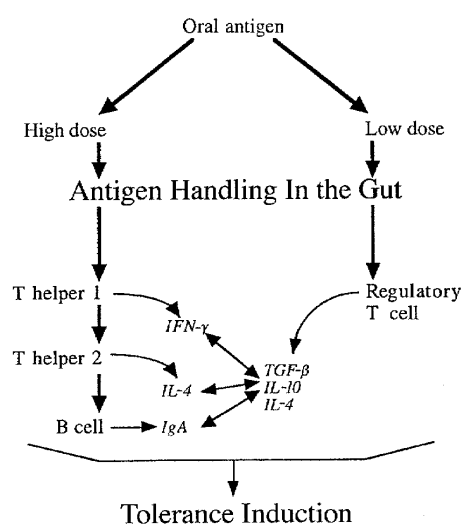

Figure 4: Mechanism of oral tolerance in relation to the dose of antigen given. (Isolauri et al., 2001).

4 Health effects of probiotics

In this section most reported effects of probiotics are listed. These include beneficial effects on lactose malabsorption, diarrhea, hypersensitivity reactions, candida-induced vaginitis, cancer, high blood cholesterol, hypertension and immunity. Some of the proposed mechanisms will be discussed below. Benefits of probiotic use have been reported especially for certain high-risk groups such as premature infants, travellers, and people receiving antibiotics.

4.1 Lactose intolerance

Lactobacillus species can have a positive effect in patients suffering from lactose malabsorption. Mammals are born with sufficient activity of lactase to metabolise lactose in the milk from their mothers and transfer it into it's metabolites: glucose and galactose (Saavedra, 2001). There is however a reduction in the activity of this enzyme in the intestinal brush border of mammals as they age. In some individuals, the ingestion of dairy products containing lactose leads to signs and symptoms of lactose intolerance, e.g., increased abnormal gas, bloating, flatus, abdominal pain, and diarrhea. In these patients, lactose behaves as an osmotic, nondigestible carbohydrate. When lactobacilli are taken as a supplement together with dairy products or as a part of cultured dairy foods, two mechanisms of action are proposed: 1) they can exert their β-galactosidase activity in vivo in the gut lumen, thus facilitating digestion and alleviating intolerance, and 2) they can increase the orocecal transit time, which gives more opportunity for digestion of the lactose (Sanders, 2000).

The effect of probiotics on lactose intolerance seems to be more cell-density dependent than strain specific, suggesting that, in general, most commercial strains of probiotic bacteria likely possess the physiologic and biochemical characteristics necessary for mediating the effect (Lin et al., 1991 and Martini et al., 1991).

4.2 Diarrhea

There are many different types of diarrhea, mostly due to different causes. The use of some types of probiotics may be beneficial, but this is not the case for all types. For this reason,

different types of diarrhea are discussed below. In figure 5, some modes of action of probiotics on intestinal function are schematically depicted.
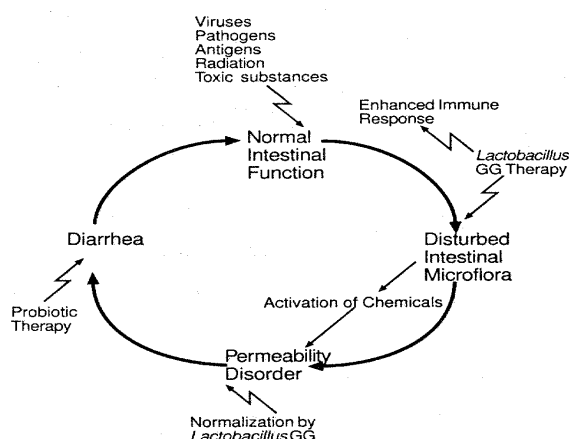

Figure 5: Modes of action of influences on intestinal function (Salminen, 1996).

4.2.1 Gastroenteritis

Gastroenteritis is the most frequent cause of acute diarrhea and normally heals within a few days. It can be caused by several viral or bacterial pathogens or parasites. The most common cause is infection with a rotavirus. This virus results in the hospitalization of approximately 55,000 children each year in the United States and the death of over 600,000 children annually worldwide. The disease is characterised by vomiting and watery diarrhea for 3-8 days; fever and abdominal pain occur frequently. The virus is stable in the environment and transmission can occur through ingestion of contaminated water or food and contact with contaminated surfaces. The highest rate of illness occurs among infants and young children. Adults can be infected also although the disease tends to be much milder and self limiting. There is no therapy for this disease, other than oral rehydration.

Several studies have therefore been performed that investigated the effects of probiotics on rotaviris associated diarrhea and on gastroenteritis, especially in infants (Marteau et al., 2001). Studies that showed a significant positive effect in randomnized controled trials are listed Table 2 . *Lactobacillus rhamnosus* GG is shown to be effective, especially in infants. This probiotic strain is thought to stimulate the rotavirus specific IgA response. The study also showed that viable *L. rhamnosus* GG is more effective in this stimulation than heat-inactivated *L. rhamnosus* GG. Other strains that have shown to be effective in reducing the duration of the diarrhea are *Enterococcus faecium SF68, Saccharomyces boulardii* and

Lactobacillus reuteri. Bifidobacterium bifidum and *Streptococcus thermophilus* have been shown to be able to prevent rotavirus associated diarrhea in infants also.

Table 2: Randomised controlled trials showing a significant therapeutic effect of probiotics on acute gastroenteritis.

	Probiotic	**Study population**	**Numbers**	**Reference**
Curative treatment				
Rotavirus-associated diarrhea	*Lactobacillus rhamnosus GG*	Infants	N = 71	Isolauri et al., 1991
	L. rhamnosus GG	Infants	N = 39	Kaila et al., 1992
	L. rhamnosus GG	Infants	N = 49	Majamaa et al; 1995
	L. rhamnosus GG	Infants	N = 42	Isolauri et al., 1994
	L. casei shirota	Infants	N = 32	Sugita, 1994
Gastroenteritis	*L. rhamnosus GG*	Infants	N = 32	Raza et al., 1996
	L. rhamnosus GG	Infants	N = 26	Pant et al., 1996
	L. rhamnosus GG	Infants	N = 100	Guarino et al., 1997
	L. rhamnosus GG	Infants	N = 123	Shornikova et al., 1997
	L. rhamnosus GG	Infants	N = 287	Guandalini et al., 2000
	Enterococcus faecium SF 68	Infants	N = 104	Bellomo et al., 1980
	E. faecium SF 68	Adults	N = 56	Camarri et al., 1981

	E. faecium SF 68	Adults	N = 78	Wunderlich et al., 1989
	E. faecium SF 68	Adults	N = 211	Buydens, 1996
	Yoghurt	Infants	N = 112	Unpublished
	Saccharomyces boulardii	Infants	N = 38	Chapoy, 1985
	Lactobacillus reuteri	Infants	N = 66	Shornikova et al., 1997
Prevention				
Acute diarrhea or rotavirus infection	*Bifidobacterium bifidum* and *Streptococcus thermophilus*	Infants	N = 55	Saavedra et al., 1994

The use of probiotics in diarrhea caused by other pathogens has also been studied. These include infections due to *Clostridium difficile* or *Helicobacter pylori. Clostridium difficile* is an intestinal pathogen, which can proliferate after breakdown of colonization resistance due to antibiotic administration or during episodes with a decreased immune response. This pathogen colonizes the intestine and releases two protein exotoxins, toxin A and toxin B, which lead to diarrhea, vomiting and colitis. After a first episode of *C. difficile* infection multiple relapses can occur and the relapses can be increasingly severe. The mechanism of relapse is unknown but is probably due to the survival of *C. difficile* as spores during the antibiotic treatment. When antibiotic treatment is discontinued, *C. difficile* may produce its toxins (Rolfe, 2000). A postulated treatment of this disease might be probiotics. Mc Farland et al., (1994) performed a placebo-controled study with *Saccharomyces boulardii*. They found that in patients with an initial episode of *C. difficile* there was no significant difference in the recurrence of the disease between the treated and the placebo group. However, in patients who had had recurrences, *S. boulardii* significantly inhibited further recurrences. The effects were shown in both adults and children.

Helicobacter pylori is a common infection of the stomach and intestine and might be treated with probiotics. Antagonistic actions of some *Lactobacillus* strains were reported in vitro, although in vivo these effects were not observed (Aiba et al., 1998; Kabir et al., 1997; Midolo et al., 1995).

4.2.2 Traveller's diarrhea

Traveller's diarrhea is defined as the passage of >3 unformed stools in a 24-h period in individuals liveing industrialized countries and who travel to tropical and semitropical areas. It affects 20-50% of travellers. The prevention of traveller's diarrhea by probiotics (a mixture of *Lactobacilli*, *bifidobacteria* and *streptococci* as well as *Saccharomyces boulardii* and *Lactobacillus rhamnosus GG)* could be a safe alternative to antibacterial drugs (De Roos and Katan, 2000). Based on these data, travellers may expect a 25-50% reduction in the risk of diarrheal illness. Other *lactobacilli* species have not shown any significant positive effects. The effects might not be uniform or consistent, and may depend on the geographic area or populations studied. The geographic dependency was studied by Oksanen et al., (1990), who investigated two groups of people travelling to different destinations in Turkey. In one group the use of probiotics showed a significant decrease in the occurrence of diarrhea, whereas in the other group no differences were found (Marteau et al., 2001). Studies that have evaluated the effect of probiotics on traveller's diarrhea are summarised in table 3.

Table 3: Effects of probiotics on traveller's diarrhea

Probiotic	Therapeutic effect: % diarrhea in probiotic treated volunteers compared with % in control volunteers	Numbers in study	Reference
Lactobacillus acidophilus + *Lactobacillus bulgaricus*	35% compared with 29% (NS)	N = 50	Pozo-Olano et al., 1978
Lactobacilli	55% compared with 51% (NS)	N = 212	Kollaritsch et al., 1983
Lactobacillus	23.8% compared	N = 282	Katelaris et al., 1995

fermentum strain KLD	with 23.8% (NS)		
L. acidophilus (unspecified strain*)*	25.7% compared with 23.8% (NS)	N = 282	Katelaris et al., 1995
Lactobacilli + bifidobacteria + streptococci	43% compared with 71% (P=0.02)	N = 81	Black et al., 1989
Saccharomyces boulardii	28.7% compared with 39.1% (P<0.05)	N = 1016	Kollaritsch et al., 1993
Lactobacillus rhamnosus GG	41.0% compared with 46.5% (P=0.065)	N = 756	Oksanen et al., 1990
L. rhamnosus GG	3.9%/ compared with 7.4%/ (P=0.05)	N = 245	Hilton et al.,1997

4.2.3 Antibiotic-associated diarrhea

Antibiotics can severely disrupt gut microbial ecology. Diarrhea occurs in up to 20% of patients who receive antibiotics. The endogenous flora decreases, which is usually responsible for colonisation resistance and fermentation capacity of the colon. With a decreased flora, there is less competition for binding sites and nutrients, giving a better chance for pathogens to survive. Ingestion of a probiotic with a prescribed antibiotic can reduce the effect of such microbial alteration and any resulting change in stool consistency and frequency. Strains that have been studied and have been reported to be effective are: *S. boulardii, Lactobacillus,* and *Bifidobacterium* (Marteau et al., 2001). These studies are summarised in table 4. *Streptococcus* has also been studied, but no positive effects on preventing antibiotic-associated diarrhea have been found.

Table 4: Effects of probiotics on antibiotic-associated diarrhea

Antibiotic	Probiotic	Blind study	Therapeutic effect[1]	Numbers in study	Reference
Ampicillin	*Lactobacillus acidophilus + Lactobacillus bulgaricus*	Yes	8.3% compared to 21%	N = 98	Gotz et al., 1979
Neomycin	*L. acidophilus + L. bulgaricus*	No	20% compared to 42%	N = 39	Clements et al., 1983
Amoxicillin-clavulanate	*L. acidophilus + L. bulgaricus*	No	Positive[2]	N = 27	Witsell et al.,1995
Antituberculous	*Enterococcus faecium SF 68*	No	5% compared to 18%	N = 200	Borgia et al., 1982
Miscellaneous	*E. faecium SF 68*	Yes	8.7% compared to 27.2%	N = 45	Wunderlich et al., 1989
Erythromycin	*Bifidobacterium longum*	Yes	Positive[2]	N = 10	Colombel et al., 1987
Erythromycin	*Lactobacillus rhamnosus GG*	No	Positive[2]	N = 16	Siitonen et al., 1990
Miscellaneous	*L. rhamnosus GG*	No	17% compared to 48%	N = 188	Young et al., 1997
Clindamycin	*B. longum + Lactobacillus*	Yes	Positive[2]	N = 10	Orrhage et al., 1994
β-lactamins or tetracyclins	*Saccharomyces boulardii*	Yes	4.5% compared to 17.5%	N = 388	Adam et al., 1977
Miscellaneous	*S. boulardii*	Yes	9.5% compared to 21.8%	N = 180	Surawicz et al; 1989
β-lactamins	*S. boulardii*	Yes	7.2% compared to 14.6%	N = 193	McFarland et al., 1995

[1]. Percentage of subjects with antibiotic-associated intestinal symptoms in the probiotic and control groups, respectively.

[2]. The authors reported a positive effect of the probiotic but did not provide the percentage of subjects with antibiotic-associated adverse effects in the 2 groups.

4.2.4 Treatment of diarrhea in developing countries

In developing countries, diarrhea is a common disease. It is responsible for many deaths annually because of dehydration. In the few published studies performed in these countries, the early administration of *Lactobacillus rhamnosus strain GG* in addition to oral rehydration therapy resulted in faster correction of acidosis and shorter duration of diarrhea, although not in persons with bloody diarrhea. This can be explained by the fact that these episodes of diarrhea may be caused by rotavirus which has been shown to be effected beneficially by the use of probiotics (Marteau et al., 2001).

4.2.5 Proposed mechanisms

One of the most important hypothesis regarding the effect of probiotoics on diarrhea might be the competition for binding sites on the intestinal epithelium. When *lactobacilli* are ingested, they will compete for binding sites, leaving less binding sites open for pathogens. Pathogens will pass through the gut and leave the body sooner if no binding site is available.

Another mechanism concerns competition for nutrients. This has been discussed shortly in the antibiotic-associated diarrhea (see above). When many harmless bacteria are present in the gut, they take many nutrients, leaving less nutrients for pathogenic bacteria, which may not be able to survive because of starvation.

In addition, entrance of probiotics in the gut may also stimulates the production of sIgA. The importance of the IgA production on the immune system has become clear from studies performed in mice who were kept germ-free after birth (Isolauri et al., 2001). In the absence of intestinal microflora the intestinal immune system is underdeveloped and intestinal morphology is disrupted. The mice have underdeveloped peyer's patches, decreased macrophage chemotaxis, and a lower capacity for intracellular killing of pathogens compared with macrophages from conventionalised animals. The number of lymphocytes in germ-free mice in the intestine is also greatly reduced. In most cases, they have a predominant production of IgM, little IgG, and no IgA at all. When these mice are given probiotics or are transferred to a conventional area, the immune system will develop into a normal regular immune system. They begin to produce a greater diversity of antibody isotypes, including antibodies specific for resident intestinal bacteria.

A specific effect of probiotics on rotavirus is decrease of fecal urease. Urease is a proinflammatory mediator that predisposes gut mucosa to ammonia-induced destruction and thus to the overgrowth of urease-producing bacteria, and is stimulated by rotavirus. Oral

probiotics appeared to normalise fecal urease concentration, thereby stabilising the gut microbial environment (Isolauri et al., 2000).

4.3 Hypersensitivity reactions

At the beginning of the third millennium, allergic diseases, atopic eczema, allergic rhinitis, and asthma, together with chronic inflammatory bowel disease, Crohn disease, ulcerative colitis, diabetes, and arthritis, represent chronic diseases of rising importance in industrialised countries worldwide. The causes of these inflammatory conditions remain unknown. One of the explanations is the "hygiene hypothesis": because of the increased hygiene children have a reduced exposure to microorganisms and an underdeveloped immune system. Another hypothesis is impairment of the gut-barrier function. It is suggested that the host defence mechanisms in the gut, primed to assimilate potentially harmful challenges, have decreased in Western societies during the past decades (Isolauri, 2001).

Gastrointestinal microflora promote potentially antiallergenic processes in the following ways: 1) it activates and or skews towards T-helper-1-type immunity, 2) it activates the generation of transforming growth factor β (probably produced by Th 3 cells), which has an essential role in suppression of T-helper-2 induced allergic inflammation and induction of oral tolerance. T-helper-3 cells might be a separate group of T helper cells responsible for inhibiton of certain types of immune responses and the induction of oral tolerance; and 3) it increases IgA production, an essential component of mucosal immune defence. Therefore, the microflora might be able to alter the immune reaction, and consequently reduce hypersensitivity reactions, including food allergy.

In a double-blind, randomised placebo-controlled trial *Lactobacillus GG* was given prenatally to mothers who had at least one first-degree relative (or partner) with atopic eczema, allergic rhinitis, or asthma, and postnatally for 6 months to their infants. Chronic recurring atopic eczema, which is the main sign of atopic disease in the first years of life, was the primary endpoint. Atopic eczema was diagnosed in 46 of 132 (35%) children aged 2 years. Asthma was diagnosed in 6 of these children and allergic rhinitis in one. The frequency of atopic eczema in the probiotic group was half that of the placebo group (15/64 (23%) vs. 31/68 (46%)). The conclusion was therefore that *Lactobacillus GG* was effective in prevention of early atopic disease in children at high risk. (Kalliomaki, 2001). It should be realized that in this study the asthma prevalence in the non-treated group was exceptional high.

The immunomodulatory effect of probiotics in allergic inflammation was characterised in a study whereby the effect on the cytokine production was determined. This study showed that unhydrolyzed casein increased the production of IL4, whereas *L. rhamnosus strain GG*-hydrolyzed casein reduced it. IL4 is one of the most important cytokines involved in Th2 responses, whereas IFNγ is the crucial cytokine for a Th1 response. By reducing IL4, a possible role of probiotics might be the shift of a Th2 mediated response towards a Th1 mediated immune response (Majamaa and Isolauri, 1997).

Further, it is important to note that an increased sIgA production due to the use of probiotics also effects the Th1/Th2 balance. IgA is a postulated feature of a Th3-regulated immune response in mucosal tissues. It is associated with an increase in certain cytokines, like IFNγ, IL12 and IL18. These cytokines downregulate the Th2-response. The Th2-response, known to induce the production of IgE-antibodies, is evident during food allergy. Certain probiotics can induce a shift to the Th1-response, for example *Lactobacillus casei* Shirota strain (De Waard et al., 2001, 2002) which may eventually alleviating allergic reactions.

4.4 Candida-induced vaginitis

Candida species are the most common cause of vaginal infection, with frequent recurrence and chronic infection. Because of the characteristics of this mucosal infection it was suggested that probiotics may be useful for treatment.

L. acidophilus has been reported to have an effect on the duration of vaginal infection caused by candida. This bacterial strain was able to reduce the duration. Other strains did not show this effect, and more studies are needed to confirm the findings (Sanders, 2000).

Studies have shown that the presence of H_2O_2-producing *lactobacilli* is important in the prevention of vaginal disease. These *lactobacilli* can be isolated from the vaginal mucosa of most healthy women but are present in <23% of women with bacterial vaginosis. H_2O_2-producing *lactobacilli* are toxic to *Gardenella vaginalis* at high concentrations, but their absence may allow an overgrowth of catalase-negative organisms present in women with bacterial vaginosis. Because bacterial vaginosis is a risk factor for sexually transmitted diseases, probiotic agents that contain *Lactobacillus* species may have a potential protective role against these diseases (after local treatments). Also, H_2O_2-producing lactobacilli may inhibit growth of *Neisseria gonorrhoea* by several mechanisms, including acidification of the environment, H_2O_2 secretion, and production of protein inhibitors (Alvarez-Olmos and Oberhelman, 2001).

4.5 Cancer

Probiotics are claimed to have the ability to reduce the risk of several forms of cancer. The best studied form is colon cancer which will be discussed separately in this report. First the other forms of cancer will be discussed.

The normal intestinal flora can influence carcinogenesis by producing enzymes that transform precarcinogens into active carcinogens. Microbial metabolites may also possess genotoxic, mutagenic or carcinogenic activity and contribute subsequently to the development of cancer over a period of long-term exposure. It is thought that some microorganisms (probiotics) may protect the host from this carcinogenic activity. There are 3 proposed mechanisms: (1) the probiotic may inhibit the bacteria that are responsible for converting precarcinogens into carcinogens; (2) probiotics might inhibit tumor cell formation directly; (3) some bacteria can bind and/or inactivate carcinogens (Orrhage et al, 1994 and Rowland and Grasso, 1975). Also immunomodulatory mechanisms are frequently suggested as possible mechanisms.

Studies have been performed to investigate the effect of probiotics on carcinogenesis. Three studies have confirmed that volunteers receiving either *L. acidophilus* or *L. casei* have reduced levels of enzymes that convert precarcinogens to carcinogens, which relates to the first mechanism (Hayatsu 1993; Lee and Salminen 1995; Lidbeck et al., 1992). Whether this also results in a reduced incidence of cancer is unknown.

A study performed by Tagaki et al., (2001), showed that tumor onset is inhibited in part by NK cytotoxicity of the host. The study demonstrated that NK cytotoxicity is enhanced by *Lactobacilli strain Shirota* before tumor onset. Moreover, the proportion of splenic NK cells was increased but functional enhancement of splenic NK cells was not detected at a cellular level. Therefore, the enhanced NK cytotoxicity in spleen cells may simply reflect a higher number of cells in the NK subset able to express lytic activity. It is speculated that the enhancement of NK cell functions by *Lactobacillus strain Shirota* is involved in detection and removal of transformed cells at the site of carcinogenesis, and these processes would result in retardation of tumor onset. It is, however, still unclear how the administered *lactobacilli* increase the number of NK cells.

A few case-controlled studies have been conducted to evaluate the effects of yoghurt or fermented milks on some cancer rates. Monique et al., (1986) found an inverse relationship between frequency of yoghurt consumption and risk of breast cancer in France.

Peters et al., (1992) found yoghurt to be a protective factor in a case-controlled study of colon cancer incidence in Los Angeles County. A case-controlled study of breast cancer in the Netherlands (van't Veer et al 1989) also suggested that fermented dairy products could be protective, although Kampman et al. (1994) did not find a similar relationship between fermented dairy products and colorectal cancer.

Two human studies have shown an effect of the consumption of probiotics on tumor growth. Daily intake of a viable *L. casei* strain postponed recurrence of bladder tumors in a randomized, controlled, multicenter study in 48 Japanese patients. Patients were enrolled within 2 weeks after removal of one or more bladder tumors. *L. casei* was taken for 1 year or until tumor recurrence. After 1 year, tumors recurred in 19 of 23 (83%) patients in the control group compared with 12 of 21 (57%) patients in the *L. casei* group. 4 patients were lost to follow up. In a multivariate analysis including age and tumor characteristics, treatment with *L. casei* significantly postponed tumor recurrence (Aso et al., 1992).

4.5.1 Colon cancer

The development of chemical-induced colon cancer starts with an initiating step, in which a carcinogen produces an alteration in the DNA. It is believed that several mutations must occur for a tumor to develop. The post-initiation steps are much less clear, but usually involve changes in signal transduction pathways. The next step is the development of aberrant crypts. A certain small but unknown fraction of these aberrant crypts will progress to polyps and eventually to tumors (Brady et al., 2000). Because colon tumors can be induced in rats with administration of dimethylhydrazine (DMH) or azoxymethane (AOM), these are considered to be a good animal model to represent human colon carcinogenesis. Tumors produced by this method closely resemble human disease in histologic type, distribution within the large bowel, metastasis, and cell turnover (Brady et al., 2000). A number of animal studies using probiotics in animal models of colon cancer have been performed, these are summarised in table 5.

The conclusion from these studies was that probiotics, with or without prebiotics, have an inhibitory effect on the development of aberrant crypts and tumors in animal models after induction with DMH or AOM. The effect is not always reproducible consistent and is small in some studies, but this perhaps represents a dose effect.

The proposed mechanisms by which probiotic bacteria may influence the incidence of colon cancer are the following: (Sanders, 2000):

1. Enhancement of the immune response

2. Suppression of growth and activities of intestinal microbes that produce carcinogens and promoters by competitive colonization or production of inhibitors (short-chain fatty acids or bacteriocins)

3. Binding and removal of carcinogens

4. Production of antimutagenic compounds

5. Production of butyrate to stimulate programmed cell death of abnormal cells

6. Inhibition of the conversion of bile salts to secondary bile salts

Table 5: Effects of probiotics on colon cancer:

Probiotics	Assessment	Effect	Study
Lactobacilli	Tumor formation in rats	Colon tumorigenesis induced by 1,2 dimethylhydrazine (DMH) was reduced	Takano et al., 1985
Streptococcus thermophilus and *Lactobacillus bulgaricus*	Survival rate and induction of colon tumors in rats	Survival rate was greater in the treated group. Numbers of tumors were not different between the groups.	Shackleford et al., 1983
Bifidobacterium	Incidence of aberrant crypts in rats	The incidence of aberrant crypts was reduced by 50%	Abdelali et al., 1995
Bifidobacterium longum and lactoferrin	Incidence of aberrant crypts	Both lactoferrin and B. longum reduced aberrant crypt foci	Tsuda et al., 1998
Bifidobacterium pseudolongum and 5% neosugar (FOS)	Aberrant crypts in mice given DMH	50% reduction in the occurrence of aberrant crypts compared to the control group.	Koo and Rao; 1991
Bifidobacteria	Incidence of colonic aberrant crypts after treatment with azoxymethane (AOM)	Consumption of bifidobacteria inhibited the development of the crypts by 50%	Kulkarni and Reddy, 1994
10% oligofructose* or 10% inulin*	Incidence of colonic aberrant crypts after treatment with AOM in rats	Total numbers of crypts were significantly less compared to the control group (120 ± 28 for control; 92 ± 28 for oligofructose; 78 ± 37 for inulin	Reddy et al., 1997

Bifidobacterium longum and lactulose*	AOM-induced aberrant crypts in rats	Both B. longum and lactulose singly and together reduced the formation of crypts	Challa et al., 1997
Bifidobacteria or inulin*	AOM-induced aberrant crypts in rats	Both bifidobacteria and inulin singly and together reduced the formation of crypts	Rowland et al., 1998
Bifidobacteria, *Lactobacillus acidophilus, perfringens*	Effect of numbers of intestinal bacteria on *C.* AOM-induced aberrant crypts	Bifidobacteria had no effect, whereas both L. acidophilus and C, perfringens decreased crypt formation significantly. Culture supernatants were found to mediate the effect, suggesting a metabolite product	Arimochi et al., 1997

* prebiotics

4.6 Cholesterol

The first indication that probiotics might influence cholesterol levels came from the observation that conventional animals excrete higher levels of cholesterol in faeces than germ-free animals. This suggests that colonizing microbes may influence serum cholesterol levels. Since 1974, 13 studies have been published evaluating blood lipids in human subjects consuming fermented milk products, with a total of 465 subjects (302 of those subjects were in 3 studies). Statistically significant lowering of total cholesterol as well as LDL cholesterol was found. These early data, however, have been challenged by the results of more recent studies, almost all of which did not report any significant effect (Sanders, 2000). The conclusions from these earlier studies have been revised because of some major limitations. These limitations include the fact that excessive volumes (0.5-8.4 L) of yoghurt were consumed daily in most of the positive studies. There was no possibility to control for the background diet and exercise patterns of the subjects studied. They failed to randomise groups for confounding factors. No basement measurements were performed, and volunteers were not adapted to the diet. Finally, experimental evidence does not support a significant hypocholesterolemic effect for probiotics when consumed in easily achievable quantities.

The mechanism by which effects can be achieved are unknown although there are certain hypotheses. One is that some *Lactobacillus* strains assimilate the cholesterol molecule, but this has not been confirmed (Gilliland et al., 1985). Another proposed mechanism is based on

the ability of certain probiotic *lactobacilli* and *bifidobacteria* to deconjugate bile acids enzymatically, increasing their rates of excretion (De Smet et al.,1994). Because cholesterol is a precursor of bile acids, this could lead to reduction in serum cholesterol because cholesterol molecules are converted to bile acids to replace those lost through excretion.

4.7 Hypertension

One line of research has suggested that bioactive peptides resulting from the proteolytic action of probiotic bacteria on casein (milk protein) during milk fermentation may suppress the blood pressure of hypertensive individuals. Preliminary studies with spontaneously hypertensive rats (Nakamura et al., 1995 and 1996) and one human clinical study (Hata et al., 1996) provide evidence. Two tripeptides, valine-proline-proline and isoleucine-proline-proline, isolated from a dairy-based fermentation of milk by *Saccharomyces cerevisea* and *Lactobacillus helveticus* have been identified as the active components. These tripeptides function as angiotensin I converting enzyme inhibitors and reduce blood pressure (Sanders, 2000). It is important to note that unlike other probiotic-induced effects, the effect is mediated by a fermentation end product and not the viable probiotic cells themselves. Powdered cell extracts from *Lactobacillus casei* Shirota strain (Sawada et al., 1990) also showed a decrease in blood pressure in a placebo-controlled trial with 28 human hypertensive subjects.

4.8 Effects on immunity

It is clear that probiotics might have immunomodulatory effects, but it is still unknown how these effects are achieved. There have been several reports recently describing the effects of probiotics on sIgA in both rodents and humans. Generally an enhanced sIgA production was observed during probiotic treatment. *Lactobacillus casei* and *Lactobacillus acidophilus* enhanced the number of IgA-producing plasma cells in a dose-dependent manner. In general IgA production was enhanced whereas IgE production was decreased.

Effects on cytokines: Several studies have shown that cytokine production by cells of the immune system can be altered by probiotic use. This accounts for cells cultured in vitro, where the production of IL6 and to a lesser extent TNFα was increased after the cells were stimulated with a mitogen.

Murine studies have been performed to determine the effect of *Lactobacillus casei strain Shirota* on resistance and immune parameters. This has been studied in host resistance models to the *Trichinella spiralis* parasite in different mouse strains. These studies showed no effect on severity of the infection when the lactobacilli were orally administered in different mouse strains, but another study showed that there was an effect when the administration was performed parenterally (Erickson and Hubbard, 2000). The studies did show an effect on several immune parameters. The Th1/Th2 balance was shifted more towards the Th1-side. This same was found in a study using rats, performed by de Waard et al.,2001). In this study the effects of orally administered viable *Lactobacillus casei Shirota* on the immune system in two rat strains was studied. The T. spiralis specific DTH (delayed type hypersensitivity) response was enhanced in both strains compared to the control groups. In both rat strains fed *L. casei*, serum T. spiralis specific IgG2b concentrations were also significantly increased. Other serum specific antibodies were not altered. Orally administered *Bifidobacterium breve* or *Bifidobacterium bifidum* had no effects on the immune system in both rat strains. This clerly demonstrates differences in mechanisms, and hence in outcome of various probiotics. Since the rat DTH response is considered to be a manifestation of Th1 cell-mediated immunity and the IgG2b isotype has been associated with Th1 activity, it was concluded that Th1 cells could play an active role in the immunomodulatory effects of orally administered
 L. casei (De Waard et al., 2001, 2002, 2003).

Many more studies (reviewed by Erickson and Hubbard, 2000) have been performed and they are summarised in table 6 to 10. From these results can be concluded that probiotics can increase the production of sIgA and IFNγ and enhance phagocytosis.

Table 6: Probiotic modulation of humoral immunity:

Probiotics	Species	Assessment	Effect
Lactobacillus casei Shirota, oral (heat killed)	Rodent	Systemic antibody response to ovalbumin	Inhibited splenocyte IgE in vitro and serum IgE
L. casei, oral (live)	Rodent	Infection and antibody production in malnourished animals	Increased sIgA and reduced enteric infection
L. acidophilus + *Peptostreptococcus*, oral (live)	Rodent	Translocation of *E. Coli* and serum total anti-*E. Coli* IgG, IgE and IgM	Decreased translocation and increased IgM and IgE
Bifidobacterium bifidus, oral (live)	Human	Total IgA and response to polio virus	Increased sIgA

Table 7: Probiotic effects on cytokine production:

Probiotics	Species	Assessment	Effect
Lactobacillus casei, oral (dry)	Human	Serum IFNγ	Increased
Lactobacillus GG, oral (live)	Human	TNFα in patients with food allergy	Decreased fecal TNFα
Lactobacillus, Bifidobacterium, and streptococcus (several strains), oral (live)	Rodent	Mitogen-induced IL6, IL12, IFNγ, and TNFα production by intestinal lymphoid cells	Enhanced IL6 and IL12 (*L. casei* and *acidophilus*) Enhanced IFNγ and NO (*L. acidophilus*)

Table 8: Effects of probiotics on nonspecific immunity:

Probiotics	Species	Assessment	Effect
Lactobacillus casei Shirota, intravenous	Rodent	Peritoneal macrophages	Increased phagocytosis
L. acidophilus or Bifidobacterium bifidum, oral (live)	Rodent	Peritoneal or peripheral blood macrophages	Enhanced phagocytosis
L. acidophilus or casei, oral (live)	Rodent	Resident peritoneal macrophages	Enhanced phagocytosis
L. casei Shirota, oral (live)	Human	Peripheral blood	No effect on natural killer cell cytolysis in vitro

Table 9: Probiotic effects in rodent models of human disease:

Disease model	Probiotic	Assessment	Effect
Insulin-dependent diabetes mellitus	*Lactobacillus casei*, oral (live)	T-cell markers, splenic cytokines	Decreased CD4+ cells, IFNγ and IL2
Insulin-dependent diabetes mellitus	*L. casei*, oral (heat-killed)	Splenic B and T cell number and production of IFNγ, IL2, IL4, and IL5. IL6, IL10	Decreased incidence of diabetes, increased CD45+ B-cells, decreased CD8+ T-cells. Decreased IFNγ and increased IL2.
Collagen-induced arthritis (CIA)	*L. casei Shirota*, oral (live)	Joint swelling, DTH, collagen stimulated IL4 and IFNγ production by spleen cells	Decreased CIA, anticollagen antibodies
Influenza immunization	*Bifidobacterium bifidus*, oral	Respiratory tract infection and anti-influenza virus IgG	Protection against lower resp. tract infections. Higher serum IgG levels

Table 10: Effect of probiotics on select human disease:

Disease	Probiotic	Assessment	Effect
Asthma	*Lactobacillus acidophilus*, oral (live)	Serum IgE and IL4 lymphocyte proliferation	No alterations
Rotavirus infection	L. GG, oral (live)	Diarrhea Serum total IgA and IgM and anti-rotavirus IgM and IgG	Duration of diarrhea decreased; Increased anti-rotavirus IgA
Crohn's disease and juvenile arthritis	*L. casei*, oral (live)	Anti-β-lactoglobulin IgA-secreting cells	Number increased

In sum, probiotics are generally thought to affect the gastrointestinal tract and the associated (local) immune system. Several studies have been performed to investigate the effects of different probiotic bacteria. From these studies it has become clear that different strains of lactobacilli induce very different effects. In addition, effects seen in a certain human population with one strain of bacteria can often not be reproduced. This makes a final overall conclusion very difficult. The mechanisms underlying the observed effects are often not understood which also demands for further investigations.

5 Safety of probiotics: are probiotics (immunologically) safe?

In the previous section several beneficial effects of probiotics and their proposed mechanisms have been discussed. By the same token that probiotics may enhace health due to immunologic mechanisms, influences on the immune system may also have deleterious effects on health. Unfortunately, little information is available on deleterious effects of probiotic use. Only a few studies have dealth with pathological features of probiotics (Bayer et al., 1978; Sharpe et al., 1973; Harty et al., 1994). In these papers infections, endocartitis, dental caries, rheumatic vascular disease, septicaemia were described due to the ingestion of lactobacilli. Thus, one issue that clerly needs more attention is safety of probiotics. This pertains to the entire population as well as subgroups, such as children, the elderly, allergy prone individuals, immunocompromized individuals, especially as there is a tendency to promote the use of probiotics for specific groups (for instance in baby formula with the hope to decrease the chance of developing asthma later on in life).

Alterations in specific immune parameters cannot be interpreted unidirectionally as beneficial or deleterious. For instance: an increased Th1 response may enhance resistance to an infection, yet also increase the expression of autoimmunity. Increased natural killer activity may enhance the resistance to viral infections, yet through enhanced interferon production also enhance the severity of inflammatory responses to bacterial infections. Hence, immune alterations may be indicative of biological effects of exposure to probiotics, they are not necessarily indicative of the direction of the health effect.

Further research to characterize effects of exposure to probiotics, including the unravelling of mechanistic pathways, the assessment of dose-response relationships, and the comparison of effects of different types of probiotics will provide a first step in understanding the balance between positive and negative effects. The most appropriate way to conclude whether immune effects noted are beneficial or rather deleterious is to study effects of exposure in disease models. Such models include infection models, models of respiratory, skin, and food allergy and models of autoimmunity. Patterns of immune alteration due to the probiotics may interpreted in the context of the clinical effects observed, so that eventually such patterns alone would be able to indicate possible beneficial or deleterious effects of probiotics.

Even if there is an array of immune parameters available that can be measured, it is doubtful whether these alone will suffice to indicate patterns of beneficial or disadvantageous effects. Especially as many different cell types, interleukins, cell receptors, transcription factors, etc.

may be involved, that can never all be evaluated simultaneously in one or a limited number of experiments, a natural next step to analyze effects of exposure of probiotics is to investigate effects on gene expression. Such parameters too should be validated against clinical effects found in disease models.

Information on patterns of effects indicative of positive or negative consequences of the use of probiotics will eventually form the basis of designing a protocol for the evaluation of probiotics to be put on the market.

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