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Immunomodulation by probiotics: efficacy and safety evaluation

J. Ezendam, A. Opperhuizen, H. van Loveren

Contact: J. Ezendam

Laboratory for Toxicology, Pathology and Genetics (TOX)

E-mail: Janine.Ezendam@rivm.nl

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RIVM, P.O. Box 1, 3720 BA Bilthoven, telephone: 31 - 30 - 274 91 11; telefax: 31 - 30 -274 29 71

Abstract

Immunomodulation by probiotics: efficacy and safety evaluation

Probiotics are non-pathogenic bacteria that are used as functional food components with claimed health-promoting effects. In the European Union probiotics are regulated via the Novel Foods Regulation (258/97/EC). This regulation is only applied to strains that were not used before 1997 and concerns novel foods or food ingredients. However, probiotic strains used before 1997 may have health effects that so far are not being regulated.

These beneficial effects on health are based on experimental animal studies and clinical trials. As yet there is no unequivocal conclusion as to whether probiotics are generally capable of health promotion, especially since evidence from clinical trials is scarce. In addition, most of the studies focus on beneficial effects, whereas there is a lack of experimental data on possible adverse effects, which makes a benefit-risk assessment of these products very difficult.

Beneficial effects of probiotic intake include stimulation of resistance and suppression of allergies, but the down side of such effects may be stimulation of autoimmune diseases or contact hypersensitivity.

In this report, a scheme for the evaluation of probiotics is proposed. This scheme may form the basis to evaluate efficacy and safety of newly marketed probiotic strains or products.

Keywords: probiotics, immunomodulation, efficacy, safety

Rapport in het kort

Immunomodulation by probiotics : efficacy and safety evaluation

In dit rapport wordt een voorstel gedaan voor een schema om veiligheid en werkzaamheid van probiotica te beoordelen. Probiotica zijn niet-pathogene bacteriën die onder andere worden toegevoegd aan zuivelproducten. De verwachting wordt daarbij gewekt dat consumptie van deze producten gezondheidsbevorderend is bijvoorbeeld door beïnvloeding van het immuunsysteem. Informatie over werkzaamheid is voornamelijk verkregen door middel van dierexperimenteel onderzoek, terwijl informatie verkregen uit klinisch onderzoek nog beperkt is. Verder is er nog weinig bekend over de eventuele schadelijke gevolgen van probiotica, hoewel toch zeker aanleiding bestaat tot zorg hierover. Stimulatie van immuunresponsen lijkt voordelig in termen van weerstand, maar de keerzijde is wellicht inductie van autoimmuniteit. Tegenwoordig is babyvoeding verkrijgbaar waaraan probiotica zijn toegevoegd. Fabrikanten claimen een positief effect op darmflora en weerstand en mogelijk preventie van allergieën. Echter, ook voor babyvoeding geldt dat werkzaamheid en veiligheid van dit soort producten wetenschappelijk niet onderbouwd is. Aangezien baby's gevoeliger zijn voor immuunmodulatie, kan consumptie van probiotica wellicht schadelijke (lange-termijn) effecten veroorzaken. In de Europese Unie worden probiotica gereguleerd door de 'Novel Foods Regulation' (258/97/EC). Deze regelgeving is alleen van toepassing op bacteriestammen die voor 1997 niet werden gebruikt in de voeding. Hiervoor werden al probiotische stammen toegepast in de voeding, waarvoor geen regelgeving bestaat, maar die wel gezondheidseffecten kunnen hebben. Het in dit rapport voorgestelde schema om probiotica te beoordelen op zowel veiligheid als werkzaamheid, kan wellicht bijdragen aan het formuleren van regelgeving ten aanzien van bestaande en nieuw op de markt te brengen probiotica.

Trefwoorden: probiotica, immuunmodulatie, werkzaamheid, veiligheid

Contents

Summary

Probiotics are defined as “living micro-organisms, which upon ingestion of sufficient numbers exert beneficial health effects”. Partly, such beneficial effects may be mediated by immunomodulation. Probiotic products are considered to be safe and have the GRAS (Generally Regarded As Safe) status. Many probiotics have this status because they are commensal non-pathogenic bacteria of human origin. Probiotics may have beneficial consequences for human health, in part because of effects on the immune system. However, effects on the immune system may under certain circumstances also be detrimental for health.

This report focuses on the efficacy and safety evaluation of immunomodulation by probiotics. Immunomodulatory effects of certain probiotic strains have been confirmed in animal models for allergy, autoimmunity and host resistance. In humans, information from clinical trials is scarce, although several papers show that probiotics can improve atopic eczema. It is important to note that information available on this topic might be biased, because research is predominantly performed by the food industry that produces probiotics. The focus of these studies is on the beneficial effects and experimental data on possible adverse effects are lacking. Furthermore, baby formulas supplemented with probiotics are currently marketed, although there is hardly any scientific information available on possible adverse effects. The immune system of infants, that has not fully developed, is especially susceptible to influences of immunomodulatory agents. As such, infants may form a group at risk of adverse effects due to immunomodulation.

In the European Union probiotics are regulated via the Novel Foods Regulation (258/97/EC). This regulation is only applied to strains that were not used before 1997 and concerns novel foods or food ingredients. However, probiotic strains used before 1997 may have health effects that so far are not being regulated. In view of the potential adverse effects of probiotics, approaches for the evaluation of safety need to be considered.

In this report, a scheme for the evaluation of efficacy and safety is proposed. This scheme provides guidelines that can be used to evaluate existing and newly marketed probiotics.

Samenvatting

Probiotica worden gedefinieerd als “levende micro-organismen die bij voldoende inname gezondheidsbevorderende effecten hebben”. Deze gezondheidsbevorderende effecten worden deels tot stand gebracht door immuunmodulatie. Probiotische producten worden beschouwd als veilig en hebben de zogenoemde GRAS status (Generally Regarded As Safe; algemeen beschouwd als veilig). Veel probiotica hebben deze status omdat ze commensale non-pathogene bacteriën van humane afkomst zijn. Probiotica kunnen gezondheidsbevorderende effecten hebben door middel van beïnvloeding van het immuunsysteem. Echter, effecten op het immuunsysteem kunnen onder bepaalde omstandigheden ook ongewenste effecten teweeg brengen.

Dit rapport richt zich op evaluatie van werkzaamheid en veiligheid van immuunmodulatie door probiotica. De effecten van probiotica op het immuunsysteem zijn aangetoond in verschillende diermodellen voor allergie, autoimmuniteit en infecties. Informatie over de effecten in mensen is nog beperkt, hoewel in enkele clinical trials is aangetoond dat inname van probiotica de symptomen van atopisch eczeem kunnen verbeteren. Echter, de informatie die aanwezig is over dit onderwerp kan minder objectief zijn omdat het onderzoek voor een belangrijk deel wordt uitgevoerd door de voedingsindustrie die probiotica produceert. Deze studies zijn gericht op het vinden van gezondheidsbevorderende effecten en experimentele data over mogelijke nadelige effecten zijn niet voorhanden of worden niet gepubliceerd. Tegenwoordig worden probiotica ook toegevoegd aan flesvoeding voor baby’s, terwijl wetenschappelijke informatie over de mogelijke nadelige effecten ontbreekt. Aangezien baby’s een nog niet volledig ontwikkeld immuunsysteem hebben en gevoeliger zijn voor immuunmodulatie kan consumptie van probiotica wellicht schadelijke (lange-termijn) effecten veroorzaken. Baby’s kunnen als zodanig een risicogroep vormen voor ongewenste effecten veroorzaakt door immuunmodulatie.

In de Europese Unie worden probiotica gereguleerd door de ‘Novel Foods Regulation’ (258/97/EC). Deze regelgeving is alleen van toepassing op bacteriestammen die voor 1997 niet werden gebruikt in de voeding, en betreft ‘novel foods’ of ‘novel food’ ingrediënten. Echter, probiotische stammen die voor 1997 toegepast werden kunnen ook gezondheidseffecten hebben, waarvoor geen regelgeving bestaat. Met het oog op mogelijke ongewenste effecten van probiotica, dient een aanpak voor evaluatie van veiligheid overwogen te worden. In dit rapport wordt een schema voor evaluatie van werkzaamheid en veiligheid voorgesteld. Dit schema levert richtlijnen die kunnen worden gebruikt voor de evaluatie van bestaande en nieuwe probiotica.

1. INTRODUCTION

Probiotics are dietary supplements that are by definition "living microorganisms, which, upon ingestion in sufficient numbers, exert health benefits' (1). The most commonly used probiotics are lactic acid bacteria, mainly *Lactobacillus* and *Bifidobacterium* strains (some examples are given in Table 1).

Table 1: Overview of microorganisms applied in probiotic products

Lactobacillus species	Bifidobacterium species
L. acidophilus	*B. bifidum*
L. casei	*B. longum*
L. rhamnosus	*B. breve*
L. gasseri	*B. infanti*
L. reuteri	*B. lactis*
L. bulgaricus	*B. adolescentis*
L. plantarum	
L. johnsonii	
L. salivarius	
L. lactis	

Abbreviations: *L.: Lactobacillus, B.: Bifidobacterium*

Some general microbiological criteria apply to probiotics and concern issues on safety and survival. Probiotics need to be non-pathogenic, preferably of human origin and genetically stable. Due to these characteristics these products have the GRAS-status (generally regarded as save) (2). It is important to note that GRAS status pertains only to their specified use, i.e. the microbes themselves are not considered GRAS, but their traditional use in dairy foods is. In addition, probiotics must be resistant to a low pH, enzymatic degradation and bile to survive passage to the gastrointestinal tract and to reach the colon alive. Several products containing probiotics are commercially available e.g. fermented foods such as yoghurt and cheese. In addition, probiotics can be added to food components as freeze-dried cells or can be consumed as dietary supplements, for instance as capsules or powders. Some companies, such as Nestlé and MeadJohnson, also sell baby formulas that contain probiotics. Well-known products that are available for consumers are summarized in Table 2.

Table 2: Summary of some probiotic products available in the Netherlands

Product	Producer	Probiotic strain
Yakult®	Yakult	*L. casei* Shirota
Activia	Danone	Bifidus Essensis® (*B*. sp. DN 173 010)
Actimel	Danone	*L. casei* Defensis
Vitamel	Campina Melkunie	*L. casei* Rhamnosus
NAN hypoallergeen 2*	Nestlé	*B. lactis* Bb12+ *Streptococcus thermophilus*
Nutragimen 2*	MeadJohnson	*L. rhamnosus* GG

* Baby formulas. Abbreviations: *L.: Lactobacillus, B.: Bifidobacterium*

Partly, beneficial effects of probiotics may be mediated by immunomodulation. The focus of these studies has been on the beneficial effects and experimental data on possible adverse effects are lacking. It is important to note that information available on this topic might be biased, because research is predominantly performed by the food industry that produces probiotics. Currently, baby formulas supplemented with probiotics are being marketed, although there is hardly any scientific information available on possible adverse effects. The immune system of infants, that has not fully developed, is especially susceptible to influences of immunomodulatory agents. As such, infants may form a group at risk of adverse effects due to immunomodulation.

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In addition, appendices provide additional information on the mucosal immune system (Appendix 1), health effects of probiotics (Appendix 2), Toll-like receptors (Appendix 3), Toll-like receptor signaling pathways (Appendix 4) and probiotics and Toll-like receptors (Appendix 5).

2. ASSESSMENT OF EFFICACY AND SAFETY

An overview of the beneficial effects of probiotics on gastrointestinal and immune-mediated diseases can be found in Appendix 2. Exact mechanisms are not completely clear but from these studies it can be concluded that probiotic strains can affect the immune system under certain circumstances. However, stimulation of the immune system can also have harmful effects, but until now this issue received little attention. Regarding safety issues, the literature only focuses on the pathological features of probiotics (2-6). In these papers infections, endocarditis, dental caries, rheumatic vascular disease and sepsis were described due to ingestion of lactobacilli. However, these effects are only observed in isolated cases and affect mostly immunocompromised individuals.

The current regulation of probiotics (Novel Foods Regulation (258/97/EC)) applies only to strains that were not used before 1997 and concerns novel foods or food ingredients. The definition of novel foods is “foods and foods ingredients which have not hitherto been used for human consumption to a significant degree within the community”. To date, only in Denmark the relevant authority should be notified by the manufacturer prior to the use of new probiotic strains. In France, a premarket approval system for novel strain is being considered and proposed recommendations are published by Agence Francaise de Securite Sanitaire des Aliments (AFFSA). In the US, a probiotic used in food could be classified either as an additive, in which it has to be approved by the Food and Drug Administration (FDA) on basis of safety and efficacy data, or it can generally be recognized as safe (GRAS notification). The GRAS status is given to a probiotic when it has a history of safe use in food dating before 1958 or it has been identified as safe by expert judgment under the condition of intended use (7, 8).

To evaluate newly discovered probiotics the approach proposed by the Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) might be useful (9). According to this scheme (Figure 1) phenotype and genotype of probiotic strains should first be established. Thereafter, assessment of safety and efficacy and functional characterization of probiotics should be performed with *in vitro* assays and animal studies. *In vitro* assays can be used to gain knowledge of probiotic strains and mechanisms of probiotic effect, e.g. adherence to epithelial cell lines or ability to reduce pathogen adhesion to surfaces. If possible, *in vitro* effects should be confirmed in animal models. Then, probiotics have to be tested in standard methods for clinical evaluation studies: Phase 1 (safety assessment) and Phase 2 (efficacy assessment) studies. If these clinical studies confirm efficacy and safety of a probiotic strain, this strain can be marketed as a probiotic food. When a claim is made that a probiotic can alter disease state a Phase 3 study must be performed. This claim can only be

made when it is based on sound scientific evidence. Finally, the scheme provides recommendations for labeling of probiotic food (9).

A similar approach has been proposed by Salminen *et al*. (2) (Figure 2) and according to this approach safety of probiotics should be assessed by combining three approaches. In the first approach the intrinsic properties of the strain should be studied, for instance enzymatic properties of a strain. In the second approach safety and stability should be evaluated.

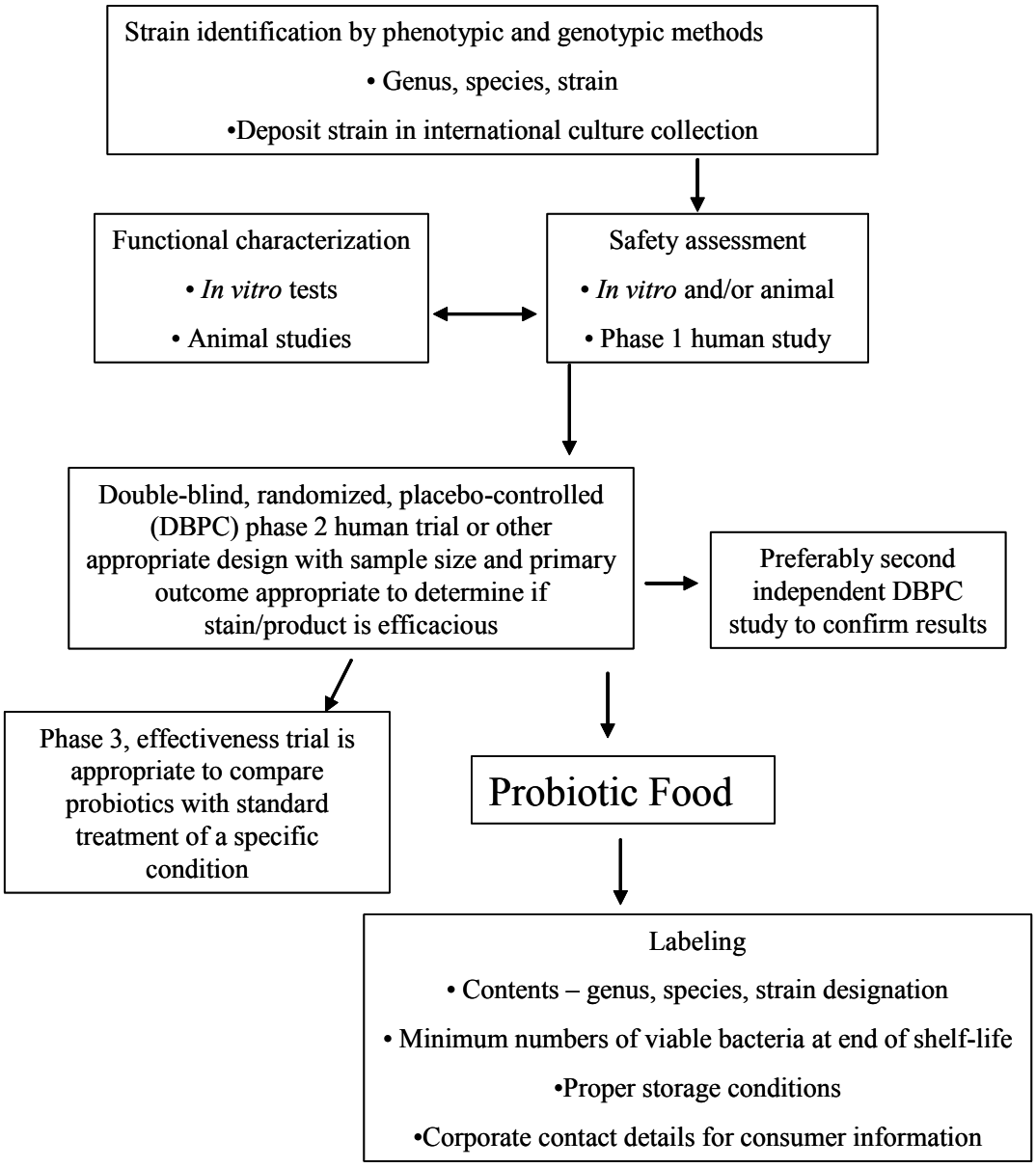

Figure 1: FAO/WHO guidelines for the evaluation of probiotics in food

For example, survival in the gastrointestinal tract is a prerequisite for a probiotic strain, but strains that can translocate and invade the host might cause unwanted side effects. Finally, in the third approach interactions between strain and host are studied. In this approach several

functional and physiological aspects of probiotic strains should be studied, either with *in vitro* assays and/or animal models. Adherence to intestinal epithelium, for instance, can be studied with epithelial cell lines and translocation can be studied in animal models. Ultimately, clinical side effects should be studied in healthy volunteers and patients. In this scheme stimulation and suppression of immune responses is mentioned. Clearly, immunomodulation should be studied, because several studies have shown the immunomodulatory effects of

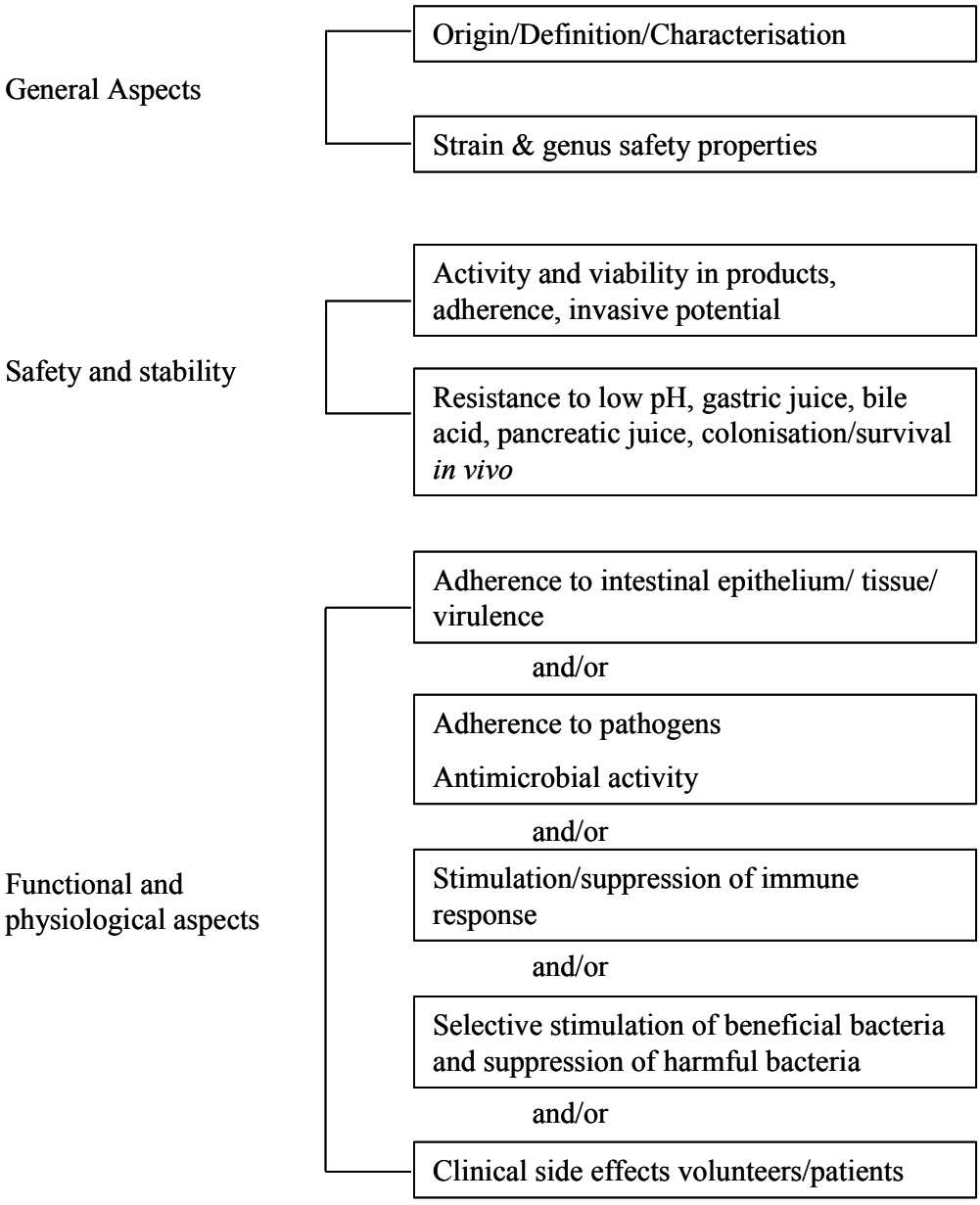

Figure 2: Approach to assess safety of a probiotic strain as proposed by Salminen et al. (ref. 2).

probiotics. Immunomodulation might induce unwanted effects on the host. Accordingly, in the efficacy and safety assessment of probiotics, evaluation of the immunomodulatory

properties of a probiotic strain should be included. Immunomodulatory effects of probiotics vary with different types of bacteria and in different experimental models. Hence, immunomodulation cannot be assessed with one single assay, but requires a panel of assays.
Therefore, we propose a decision scheme based on the scheme presented by Salminen *et al*. (Figure 3). To assess immunomodulation, effects of probiotics should be studied with *in vitro* assays, experimental animal models and clinical trials. Initially, *in vitro* assays should be used to determine cytokine profiles. Effects of probiotics should be studied in monocytes, macrophages or PBMCs, because previously it has been shown that probiotics can induce production of pro-inflammatory cytokines in macrophages (10) and Th1 cytokines in peripheral blood monocytes (11). In addition, effects on DC maturation and activation should be included, because probiotics can differentially affect DC maturation (12, 13) and this can influence the type of immune response generated (14). Together, cytokine profiles may be predictive for the outcome of immunomodulatory effects of a probiotic strain.
Additional information should be obtained from experimental animal studies. Effects of probiotics should be tested in several experimental disease models in order to establish efficacy and possible unwanted effects. Preferably, probiotics should be tested in host resistance models (cellular immunity), allergy models (Th2-mediated immune responses), autoimmunity models and contact hypersensitivity models (both Th1-mediated immune responses). Ultimately, the probiotic strain should be tested in clinical trials with the approach that was proposed by the FAO/WHO (Figure 1), using standard Phase 1 and 2 studies, and if necessary Phase 3 studies (9).
Data from human studies is important in the evaluation of probiotic strains or products, because data from experimental animals is not sufficient. Extrapolation is difficult because of species and microflora differences. Finally, all data available on a probiotic strain or probiotic product should be evaluated by expert judgment. Important issues at this point are the plausibility of the health claim and the possibility of adverse effects. Furthermore, intended use should be taken into account. This approach has similarities with the GRAS notification used in the US that is usually restricted to a specific application and not to a general use of a probiotic strain or product. For example, the FDA has accepted the use of *B. lactis* Bb12 and *S. thermophilus* strain Th3 as ingredients for Nestlé's infant formula, under the condition that it is intended for consumption by infants of four months and older that are not immunocompromised (15).
Thus, acceptance of probiotic products is approved under certain restrictions, e.g. age and immune status. In the European Union there is no special regulation for supplementation of infant formulas with probiotics. The Scientific Committee on Food of the European Commission has recommended that infant formulas supplemented with probiotics should only be marketed if their benefit and safety have been evaluated according to principles outlined by

the same Committee (16). In addition, the ESPGHAN (European Society for Paediatric Gastroenterology, Hepatology and Nutrition) Committee on Nutrition concluded that further evaluation of safety and efficacy of probiotic supplementation of dietetic products for infants is necessary. Concerns are raised that available scientific data are not sufficient to support safety of probiotics in healthy newborn and very young infants with immature defense systems (17). Finally, according to the scheme presented in Figure 3, surveillance of probiotic products on the market could provide more insight in both efficacy and in side effects after long-term consumption.

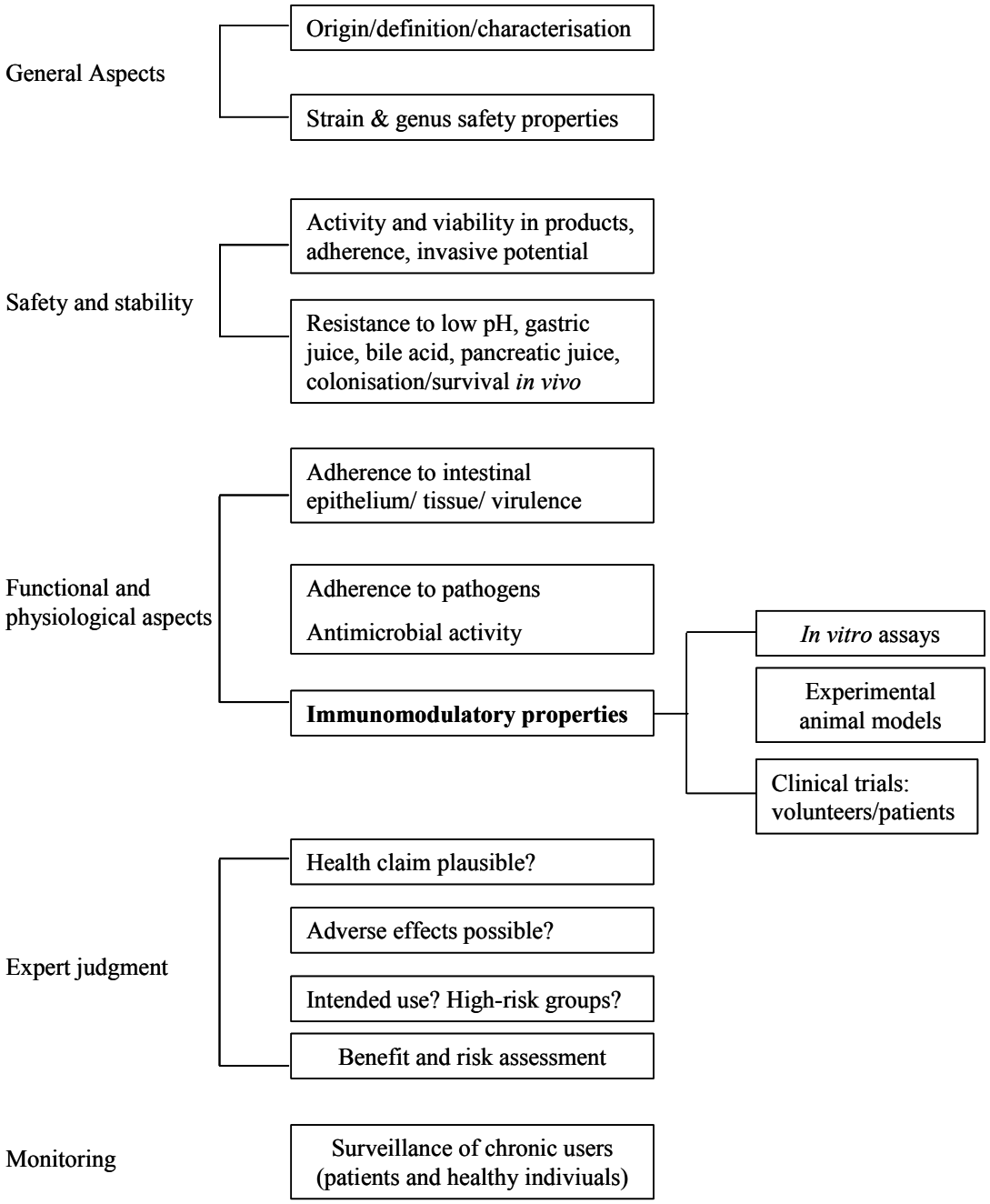

Figure 3: Scheme for efficacy and safety evaluation of probiotics (adapted from Salminen et al., 1998)

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APPENDIX 1: Mucosal immune sytem

A balanced intestinal microflora is important in order to maintain good health. The gastrointestinal tract protects the host against ingested harmful compounds, e.g. pathogens. The intestinal microflora, the mucosal barrier and the mucosal immune system (the so-called gut-associated lymphoid tissue (GALT)) are all involved in this protection. The mucosal microflora protects the host against colonization of ingested bacteria by a phenomenon called colonization resistance. Several mechanisms are involved, including competition for substrates and adhesion sites (19). In addition, the mucosal barrier prevents passage of most antigens. Antigens that are able to penetrate the mucosa are removed by lysosomal degradation, resulting in immune elimination. Some antigens are taken up by M cells of Peyer's patches and in this way processed by the mucosal immune system. Mucosal immunity is of importance in discriminating between harmless antigens from food and dangerous antigens form exogenous pathogens. Several mechanisms are involved in maintaining a balance between immune responses against pathogens and systemic unresponsiveness, called oral tolerance, against food antigens (Figure 4). After mucosal exposure to a dietary antigen a local IgA antibody response can be generated, almost always inducing systemic immunologic hyporesponsiveness to this antigen. Furthermore, it is thought that anergy of antigen-specific cells is important in oral tolerance. The dose of antigen exposure influences the mechanism underlying unresponsiveness. High dose exposure may result in clonal deletion and anergy of T cells, whereas low dose exposure may result in active suppression regulated by regulatory T cells. These regulatory T cells produce suppressive cytokines, including IL-4, IL-10 and transforming growth factor (TGF)-β. Thus, homeostasis in the gut is maintained via local immune regulation (20, 21).

Microbial colonization starts at birth and plays an important role in the development of both intestinal microflora, gut barrier and the GALT. Colonization depends on several external factors. Breast-feeding encourages the growth of bifidobacteria, whereas formula-fed infants have a more complex microflora with less bifidobacteria. After weaning, the composition of microflora resembles the adult flora (22). Intestinal colonization is also involved in the maturation of the GALT. For example, germ-free mice display numerous defects in the generation of an appropriate immune response (23). Also, germfree mice are more prone to develop Th2-type immune sensitization to oral administered proteins, due to a lack of oral tolerance. The tolerance can be fully restored after reconstitution of the gut with probiotic (*Bifidobacteria*) strains (24). Thus, the intestinal bacterial flora plays a crucial role in the generation of an appropriate functioning immune system. Therefore, health effects of probiotics are often attributed to beneficial effects on the intestinal microflora and mucosal

and systemic immune system. Probiotics normally do not colonize the gut permanently, due to colonization resistance. However, some probiotics, for instance *L. rhamnosus* GG (LGG) (25) and *L. casei* shirota (LcS) (26) can colonize the gut temporary. This feature is dependent on the ability of microorganisms to adhere to mucosal cells. One can envisage that probiotics can only exert positive systemic effects when they reach the gastrointestinal tract alive and in sufficient numbers (27). However, the minimal dose and frequency of probiotic consumption to establish health effects are unknown. It is thought that at least 10^8–10^9 live bacteria should reach the small intestine daily and therefore, probiotics must be consumed on a regular basis.

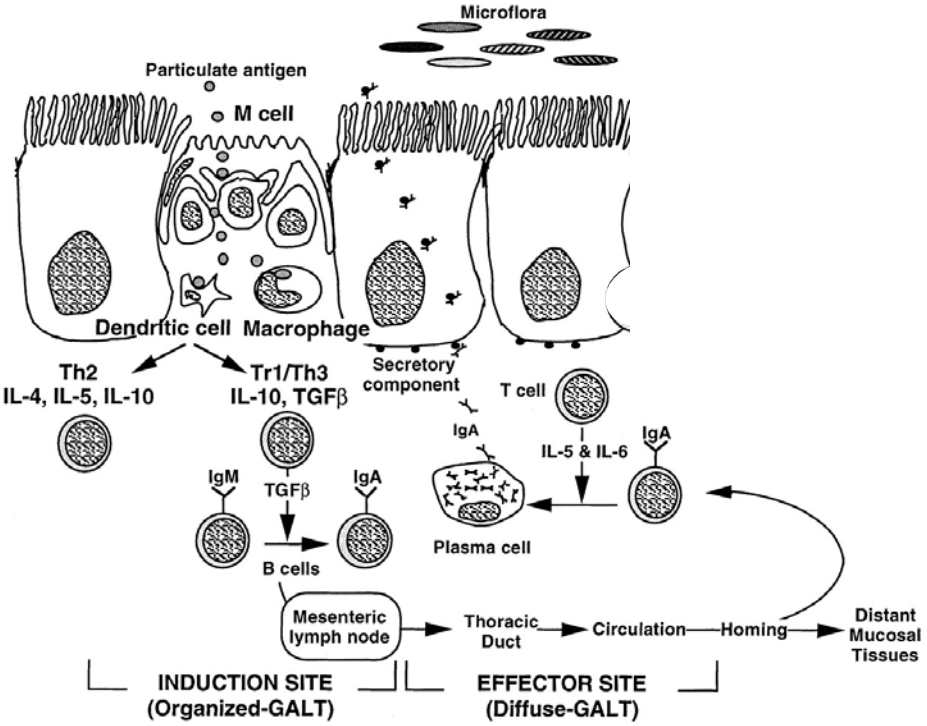

Figure 4: Representation of the mucosal immune response to luminal antigens. M cells overlying lymphoid follicles within gut-associated lymphoid tissue transport antigens to dendritic and other antigen-presenting cells (macrophages). Dendritic cells process and present antigens in context of major histocompatibility complex (MHC) and in association with costimulatory molecules to T cells. Under normal circumstances, for innocuous antigens, usual outcome is the generation of IL-10 and TGF-β, which drive the differentiation of T helper type 2 (Th2) and regulatory T cells (Th3 and Tr1), thereby promoting IgA responses and oral tolerance.

APPENDIX 2: Health effects of probiotics

Many health claims have been made concerning probiotics. These claims have been supported by data obtained in animal models. However, conclusive evidence from well-controlled clinical studies is scarce. Table 3 summarizes beneficial effects of probiotics on intestinal health in clinical trials. The effects of probiotics on the immune system have been observed in *in vitro* studies, animal models and clinical trials. Probiotics can affect the immune system via different mechanisms and an overview of the beneficial effects of probiotics on the immune system, in health and disease, will be given, together with mechanisms that are possibly involved.

Table 3: Reported health effects of probiotics in randomized, placebo, controlled clinical trials[a]

Disease	**Probiotic**
Antibiotic-associated diarrhea	*S. boulardii, L. rhamnosus* GG*, E. faecium* SF68, *L. acidophilus + L. bulgaricus*
Gastroenteritits: rotavirus diarrhea	*L. rhamnosus* GG, *B. bifidum + Streptococcus thermophilus*
Gastroenteritis: other causes	*L. rhamnosus* GG, *E. faecium* SF68, *L. reuteri, L. casei* Shirota
Traveler's diarrhea	*S. boulardii*
Lactose intolerance	Lactase-positive strains (e.g. Lactobacillus, Bifidobacterium and Streptococcus)
Pouchitis	VSL# 3 (a mix of *L. casei, L. plantarum, L. bulgaricus, L. acidophilus, B. breve, B. infantis* and *S. thermophilus*)
Ulcerative colitis	*E. coli* Nissle 1917, VSL# 3
Crohn's disease	*S. boulardii*, *E. coli* Nissle 1917, VSL# 3
Helicobacter pylori infection	*L. gasseri* OLL 2716, *L. johnsonii* La1

[a]Table is composed of information from references (28-32). Abbreviations: S.: *Saccharomyces*, L: *Lactobacillus*, E.: *Enterococcus*, B: *Bifidobacterium*

ALLERGY

Effects of probiotics have been studied in several experimental allergy models but also in human clinical trials. Allergies are Th2-mediated disorders, which are characterized by a humoral immune response with high antibody production, in particular IgE, mast cell degranulation, eosinophil activation and inhibition of Th1 responses. Th1-mediated disorders,

e.g. autoimmune diseases and contact hypersensitivity, are characterized by a cellular immune response with macrophage and Th1 activation and inhibition of Th2 responses. Probiotic bacteria seem to skew the Th1/Th2 balance towards Th1 and are in this way able to inhibit Th2 responses. *In vitro* studies have shown that different lactobacilli were able to stimulate the production of Th1 cytokines (IL-12, IL-18 and IFN-γ) in human monocytes and peripheral blood mononuclear cells (11, 33).

Experiments in animals confirm the effects of probiotics on Th1 immunity. Oral administration of LcS to BALB/c mice sensitized with ovalbumin (OVA) suppressed the elevation of total and OVA-specific IgE levels. In LcS treated mice Th1/Th2 balance was skewed to Th1. Splenocytes stimulated with OVA produced more Th1 cytokines (IL-2, IFN-γ) and less Th2 cytokines (IL-4, IL-5, IL-6 and IL-10). In a murine food allergy model *L. plantarum* administration suppressed the elevation of anti-casein IgE levels and elevated plasma IL-12 levels. After blockade of IL-12 with recombinant IL-12 the elevation of anti-casein IgE was also prevented, suggesting that IL-12 induced by *L. plantarum* may be related to suppression of IgE production (34). However, not all probiotic strains stimulate Th1 responses. In a murine model for respiratory allergy oral administration of *L. rhamnosus* HN001 stimulated a mixed Th1/Th2 cytokine pattern. *Ex vivo* restimulation of spleen cells with OVA resulted in increased production of IFN-γ, IL-4 and IL-5 (35).

Several studies have shown that probiotics positively affect gut barrier function, a feature that could also explain beneficial effects on allergies. Increased permeability of the gut will allow more antigens to pass the gut barrier and this could stimulate an allergic response. In juvenile rats increased gut permeability was induced by prolonged cow milk challenge, which prevented when simultaneously LGG was administered (36). Furthermore, probiotics have been shown to stimulate IgA production, both in the gut as systemically, and could in this way induce immunological tolerance (37).

Clinical studies have studied the effects of probiotic use on allergic diseases, such as asthma (38), birch-pollen allergy (39), food allergy (40) and atopic eczema (41-44). In patients with asthma and/or rhinitis consumption of yoghurt with *L. acidophilus* enhanced IFN-γproduction after *ex vivo* stimulation of blood lymphocytes with Concavalin A. The number of blood eosinophils was decreased after probiotic treatment, but IgE levels were not affected and clinical parameters (pulmonary function, quality of life) were not improved (38). Consumption of *L. rhamnosus* for 5 months, starting 2 months before the pollen season, did not affect allergy against birch-pollen (39). Administration of *L. rhamnosus* and *L. reuteri* to children with atopic dermatitis caused a moderate improvement of the severity of eczema (42). In another study administration of the probiotics *B. lactis* Bb-12 and LGG to infants with atopic eczema decreased the clinical score (44). In infants with cow's milk allergy and atopic eczema probiotics have been shown to decrease faecal TNF-α, indicating an alleviation

of intestinal inflammation (40). Patients with atopic dermatitis have increased intestinal permeability and consumption of *L. rhamnosus* and *L. reuteri* has been shown to decrease the intestinal permeability in patients with atopic dermatitis (41). In addition, another study in children with atopic dermatitis has shown an elevation of IL-10 after consumption of LGG (45). IL-10 is an anti-inflammatory cytokine that inhibits synthesis of IL-2, IL-4, IL-6, IL-12 TNF-α IFN-γ. and downregulates IgE synthesis. Thus, LGG consumption increased an anti-inflammatory cytokine and this might also explain the beneficial effects observed.
In conclusion, there is evidence from clinical trials that probiotic therapy has beneficial effects on atopic dermatitis, but there are hardly any studies on the effects on other allergies such as asthma. As suggested by Matricardi (2002) there is a need for well-designed clinical trials that assess the effects of probiotics on several allergic diseases. Matricardi also expressed his concerns on the prophylactic use of probiotics in infants, especially since the beneficial effects on allergy and safety are not convincingly demonstrated yet (46).

PROBIOTICS IN BABY FORMULAS

The gut microflora plays an important role in the development of the immune system and beneficial effects of probiotics intake during infancy are based on several hypotheses. The last decades the prevalence of allergies has increased in Westernized countries. One explanation is the ‘hygiene hypothesis’, which proposes that alterations in lifestyle such as better hygiene and use of antibiotics decrease microbial exposure early in life. This could influence the maturation of the immune system and increase the risk to develop hypersensitivity reactions, e.g. allergies and autoimmunity (47). In addition, several studies have shown that the microflora plays an important role in maturation of the immune system (Appendix 1). Intake of probiotics early in life might beneficially influence maturation of the immune system and reduce the risk on hypersensitivity reactions. An association between intestinal microflora and allergies has been observed in a study comparing microflora from allergic and non-allergic children in two countries with a low (Estonia) and a high (Sweden) prevalence of allergies. Children from Estonia had higher numbers of lactobacilli compared to Swedish children (48). In addition, allergic children in both countries were less often colonized with lactobacilli and bifidobacteria than non-allergic children (49). Furthermore, gut flora of formula-fed infants was different from breast-fed infants. Formula-fed infants have a complex mixture of anaerobic strains, such as *Bacteroides* and *Clostridium* while breast-fed infants were colonized with predominantly bifidobacteria and lactobacilli (43). Addition of lactobacilli and bifidobacteria to baby formulas might simulate effects of mother milk. Consumption of these baby formulas supplemented with probiotic strains might beneficially influence microbiota of breast-fed infants, leading to colonization with more bifidobacteria and lactobacilli. As

mentioned before, intake of probiotics can improve atopic eczema in infants (41-44, 50). In a clinical trial, pregnant women received LGG 4 weeks before birth and during breast-feeding until the child was 3 months of age. Breast milk from mothers who consumed probiotics had higher levels of TGF-β, an immunosuppressive cytokine. Furthermore, the incidence of atopic eczema was lower in the probiotic group. Thus, probiotics can elicit their effects by influencing breast milk and as such modulate the immune system of infants. This may be safer than direct administration of probiotics to infants (51). Until now, no reports exist on the effects of probiotic supplemented baby formulas on other allergic diseases.

AUTOIMMUNITY AND CONTACT HYPERSENSITIVITY

Autoimmunity and contact hypersensitivity are Th1-mediated diseases. Probiotics can skew the Th1/Th2 balance to Th1 and this could implicate adverse effects on Th1 disorders. However, in a murine model for contact hypersensitivity it was shown that probiotics downregulated Th1 responses. The probiotic drink Actimel (containing *L. casei*) reduced skin inflammation induced by the contact sensitizer dinitrofluorobenzene. Serum levels of predominantly hapten-specific IgG2a (Th1), but also of IgG1 (Th2) were reduced after probiotic treatment. Furthermore, hapten-specific CD8+ T cell responses and IFN-γ secretion were lower after restimulation with the hapten. Importantly, it was shown that regulatory CD4+ T cells were mandatory for the downregulation of contact hypersensitivity (52). One possibility is the involvement of Toll-like receptors (TLRs), which will be discussed in more detail in appendices 3, 4 and 5. Shortly, TLRs are pattern recognition receptors that recognize bacterial components and are present on different cell types, including regulatory T cells. Interestingly, signaling of *Candida albicans* via TLR2 resulted in activation of regulatory CD4+ T cells (53). Probiotics may also be recognized by TLRs and exert their beneficial effects in a similar way.

Beneficial effects of administration of LcS were reported in experimental models for insulin-dependent diabetes mellitus: nonobese diabetic (NOD) mice and alloxan-induced diabetes (54, 55). In both models LcS decreased the incidence of diabetes, slightly reduced plasma glucose levels and prevented the destruction of the β cells and islets of Langerhans. In mice treated with alloxan the induction of nitric oxide (NO) is thought to be responsible for the destruction of β cells. LcS reduced plasma NO levels induced by alloxan and in this way probably prevented diabetes. In NOD mice, β cell destruction is associated with CD4+ T cells, CD8+ T cells and macrophages. Mechanisms underlying the beneficial effects of LcS remain unknown. LcS skewed the Th1/Th2 balance to Th2, since spleen cells stimulated with Concavalin A produced less IFN-and more IL-2, IL-4, IL-6, IL-10. Furthermore, after LcS treatment the number of CD8+ T cells were reduced (55). Together, skewing the Th1/Th2

balance to Th2 and limiting the number of effector cells might explain improvement of this Th1-mediated autoimmune disease.

Probiotic treatment also had beneficial effects on collagen-induced arthritis, an experimental murine model for rheumatoid arthritis (56, 57). Both LcS (57) and *L. salivarius* 118 (56) reduced disease severity. *L. salivarius* 118 has been shown to reduce both IL-12 and TNF-α in an experimental model for colitis (56), cytokines that play a critical role in collagen-induced arthritis (58). After administration of LcS anti-collagen specific IgG1, IgG2a and IgG2b and delayed-type hypersensitivity reactions (DTH) were reduced. In this study the production of IFN-γ by spleen cells was suppressed, whereas IL-4 production was not affected (57). LGG has been shown to have beneficial effects on tropomyosin arthritis or adjuvant arthritis in Lewis rats (59). However, in patients with rheumatoid arthritis ingestion of LGG did not improve clinical symptoms, but in the LGG-group the number of swollen joints was reduced, although not significantly (60). A possible mechanism by which probiotics could affect rheumatoid arthritis is via an effect on the microflora. Several reports have shown that patients with rheumatoid arthritis have a disturbed intestinal microflora (60).

Probiotics have differential effects in a mouse model for multiple sclerosis, experimental autoimmune encephalomyelitis (EAE). *L. reuteri* aggravated the disease, whereas *L. casei* and *L. murines* improved the disease. *L. reuteri* also enhanced the immune response to a parenterally administered antigen and induced a Th1-like profile (TNF-α and IL-2) in the gut. In contrast, *L. casei* did not show any adjuvant activity and induced immunoregulatory cytokines (TGF-β and IL-10) in the gut. Thus, the cytokine profile induced by a probiotic strain might be predictive for the effects on ongoing immune reactions (61).

Together, the few reports that describe effects of probiotics on Th1 disorders have shown that probiotics can have beneficial, but also detrimental effects. In some experimental models the Th1/Th2 balance was skewed in the Th2 direction. For LcS these effects were the opposite of effects observed in an experimental allergy model (62). *L. reuteri* did stimulate a Th1 responses and this aggravated EAE. In summary, beneficial effects of probiotics cannot be explained solely by skewing the Th1/Th2 balance to Th1, but might involve regulatory T cells (Appendix 5).

HOST RESISTANCE

Many health claims on probiotics state that consuming the product enhances host resistance. Beneficial effects observed in some host resistance models might be the result of competition between probiotics and pathogens for binding sites and nutrients in the gut and by production of bacteriocins. Furthermore, several probiotic strains were able to stimulate the cellular immunity, illustrated by production of pro-inflammatory cytokines TNF-α, IL-1β and IL-6

(10, 63), increased phagocytosis (64) and activation of natural killer (NK) cell activity (65). Increased cellular immunity might improve resistance of the host against invading pathogens. *L. casei* enhanced the immune response in mice infected with *Pseudomonas aeruginosa* (66) and *B. breve* augmented specific IgG responses and had protective effects in a murine influenza model (67). Administration of LcS enhanced both cellular and humoral (IgG2b, Th1) immunity against *T. spiralis*, but this did not affect parasite load. Thus, enhancement of cellular immunity does not always result in increased host resistance. In addition, *B. breve* and *B. bifidum*, had no effects on *T. spiralis* infection. (68). In rats infected with *Listeria monocytogenesis*, LcS was able to reduce bacterial burden and enhanced specific
DTH reactions (69, 70). Both DTH responses and the IgG2b isotype have been associated with Th1 activity. Thus, stimulation of Th1 activity increased resistance to *Listeria monocytogenesis*.

INFLAMMATORY BOWEL DISEASE

Effects of probiotics on inflammatory bowel disease (IBD) have been studied with experimental animal models and in patients. IBD is a chronic relapsing inflammation of the gastrointestinal tract. The two main forms of IBD are ulcerative colitis and Crohn's disease (71). Disturbance of intestinal microflora appears to play an important role in IBD and probiotics may influence gut flora beneficially and as such positively influence this disease (72). In animal models of IBD the efficacy of probiotics has been confirmed. *L. reuteri* reduced intestinal inflammation in a rat model for acetic-acid colitis and improved gut permeability (73). Administration of *L. plantarum* DSM 9843, *Bifidobacterium* sp. 3B1 or *Bifidobacterium infantis* DSM 15158 significantly improved the clinical score in dextran sulfate sodium (DSS)-induced colitis (74). A probiotic cocktail, VSL#3, containing 4 strains of lactobacilli (*L. casei*, *L. plantarum*, *L. acidophilus* and *L. delbrueckii bulgaricus*), 3 strains of bifidobacteria (*B. breve*, *B. longum* and *B. infantis*) and *Streptococcus salivarius thermophilus* attenuated DSS-induced colitis. In TLR9 deficient mice the probiotic treatment could not improve colitis, indicating involvement of TLR9 in the beneficial effects of VSL#3 on experimental colitis (75). The effects of probiotics on TLRs will be discussed in more detail in Appendix 5. However, in trinitrobenzene sulfonic scid (TNBS)-induced colitis *L. plantarum* did not reduce severity of colitis nor improve gut permeability (76).
In humans, the VSL#3 cocktail has been successfully used in the treatment of ulcerative colitis, pouchitis and Crohn's disease (32). LGG consumption enhanced antigen-specific IgA immune responses in patients with Crohn's disease (77). *E. coli* Nissle 1917 prevented relapses of ulcerative colitis with the same efficacy as mesalazine, which is normally

prescribed to treat colitis (78). Thus, probiotics might be used as an alternative therapy for IBD.

EFFECTS ON THE IMMUNE SYSTEM IN HEALTHY ANIMALS/VOLUNTEERS

Effects of probiotics have also been studied in healthy volunteers and naïve animals. In mice, oral administration of LcS activated NK activity of spleen cells in both newborn and adult mice (65). Administration of *L. rhamnosus*, *L. acidophilus* or *B. lactis* resulted in a significant increase in the phagocytic activity of peripheral blood leucocytes and peritoneal macrophages in mice. Also, proliferative responses of spleen cells to T- and B cell mitogens were also significantly enhanced in these mice. Spleen cells also produced significantly higher amounts of IFN-γ but not of IL-4 in response to stimulation with ConA (79).

Several studies in healthy volunteers have shown effects on the immune system induced by consumption of probiotics. An increase in NK activity has been observed in nine healthy volunteers that consumed Yakult® (LcS) for three weeks (80) and in middle-aged people that consumed *L. casei* DN114001 fermented milk three times a day for 56 days (81). Probiotic consumption can also differentially affect the immune system, as has been shown in healthy and milk-hypersensitive volunteers. Fermented milk with LGG downregulated the inflammatory response in milk-hypersensitive subjects, but had an immunostimulatory effect in healthy individuals. This was assessed by measuring complement receptors and receptors for IgG and IgA on neutrophils and monocytes (82). In healthy elderly volunteers consumption of *B. lactis* HN019 for 6 weeks increased mitogen-induced IFN-γ production in peripheral blood mononuclear cells and enhanced phagocytosis. Since with ageing natural cellular immune functions decline, probiotics could have beneficial effects in this population (83). In healthy volunteers the effects of consuming yoghurt containing *L. bulgaricus* and *S. thermophilu*s was assessed in plasma and blood mononuclear cells. IFN-γ, IL-1γ and TNF-α were undetectable, but a enzyme induced by IFN, 2'-5'A synthetase, was increased after probiotic consumption (84). In contrast, a study performed in healthy volunteers that consumed Yakult® for 8 weeks did not detect any effects on the immune system. Immune effects were established by measuring percentages of T, NK and B cells, NK activity, humoral parameters, mitogen-induced production of IFN-γ, IL-1β and IL-2, phagocytic activity and oxidative burst (85). Thus, probiotics can affect the immune system in healthy volunteers, although another report did not detect any effects. The increase in cellular immune function could be beneficial in individuals with a compromised immune system, such as elderly. However, immunostimulation might also trigger the development of Th1 mediated diseases, such as autoimmunity and contact hypersensitivity. Exact implications of the observed immunostimulation in healthy individuals induced by probiotics are not completely clear and should be investigated more thoroughly.

APPENDIX 3: Toll-like receptors

Toll-like receptors (TLRs) are present on several innate immune cells (e.g. macrophages, NK cells, phagocytes). TLRs consist of germline encoded non-clonal receptors, called pattern recognition receptors (PRRs) that recognize conserved molecular patterns of microorganisms, the so-called pathogen-associated molecular patterns (PAMPs). (86). TLRs are type I transmembrane proteins that are evolutionarily conserved between insects and humans (87). Toll was first identified as an essential molecule for embryonic patterning and antifungal immunity in Drosophila (88). A homologous family of TLRs exists in mammals (89), with eleven members discovered until now (TLRs1-11) (90-95). These different TLRs recognize a variety of PAMPs (ref (96); Table 4). TLR4 is thought to be the most important receptor for recognition of lipopolysaccharide (LPS). Engagement of TLR with its ligand activates complex signaling pathways described in Appendix 4.

TLRs are key components of the innate immune system and essential in the recognition of microbial invaders. TLR activation induces expression of chemotactic factors and cell surface molecules and this triggers recruitment of cells to the site of infection to eliminate the invading pathogens. DCs also express several TLRs and signaling through TLRs results in maturation of DCs (97). In the initial phase of an adaptive immune response DCs are important because they capture antigens. Subsequently, DCs migrate to draining lymph nodes and present the peptides to naïve T cells. DCs play an important role in the differentiation of naïve Th cells to Th1 or Th2 cells. This is dependent on the type of costimulatory molecules that are presented and cytokines secreted by DCs (14). Pathogen-induced TLR activation on DCs results in induction of a Th1 response, although some reports also mention the induction of a Th2 response via TLR2. In conclusion, TLRs are important receptors that sense microbial infections. Additionally, DCs are key cell types that couple TLR-mediated microbial recognition to a appropriate adaptive immune response (97).

Table 4: Toll like receptors and ligands

Receptor	Ligand	Origin of ligand
TLR1	Triacyl lipopeptides	Bacteria and mycobacteria
	Soluble factors	*Neisseria meningitidis*
TLR2	Lipoprotein/lipopeptides	Various pathogens
	Peptidoglycan	Gram-positive bacteria
	Lipoteichoic acid	Gram-positive bacteria
	Lipoarabinomannan	Mycobacteria
	Phenol-soluble modulin	*Staphylococcus epidermidis*
	Glycoinositolphospholipids	*Trypanosoma cruzi*
	Glycolipids	*Treponema maltophilum*
	Porins	*Neisseria*
	Atypical lipopolysaccharide	*Leptospira interrogans*
	Atypical lipopolysaccharide	*Porphyromonas gingivalis*
	Zymosan	Fungi
	Heat-shock protein 70*	Host
TLR3	Double-stranded RNA	Viruses
TLR4	Lipopolysaccharide	Gram-negative bacteria
	Taxol	Plants
	Fusion protein	Respiratory syncytial virus
	Envelope protein	Mouse mammary-tumour virus
	Heat-shock protein 60*	*Chlamydia pneumoniae*
	Heat-shock protein 70*	Host
	Type III repeat extra domain A of fibronectin*	Host
	Oligosaccharides of hyaluronic acid*	Host
	Polysaccharide fragments of heparan sulphate*	Host
	Fibrinogen*	Host
TLR5	Flagellin	Bacteria
TLR6	Diacyl lipopeptides	*Mycoplasma*
	Lipoteichoic acid	Gram-positive bacteria
	Zymosan	Fungi
TLR7	Imidazoquinoline	Synthetic compounds
	Loxoribine	Synthetic compounds
	Bropirimine	Synthetic compounds
	Single-stranded RNA	Viruses
TLR8	Imidazoquinoline	Synthetic compounds
	Single-stranded RNA	Viruses
TLR9	CpG-containing DNA	Bacteria and viruses
TLR10	N.D.	N.D.
TLR11	N.D.	Uropathogenic bacteria

Table from Akira and Takeda, 2004 N.D.: not determined, TLR: Toll-like receptor

APPENDIX 4: TLR signaling pathways

Adapted from reviews by Akira and Takeda and Takeda and Akira (96, 98)

TLRs are type I membrane glycoproteins that have considerable homology in the cytoplasmic region with interleukin-1 receptors (IL-1R). The cytoplasmic tails of both TLRs and IL-1Rs contain a conserved region of 200 amino acids known as Toll/IL-1R (TIR) domain. The TIR domain is characterized by the presence of three homologous regions, known as box 1, 2 and 3. The extracellular region, however, is different and consists of leucine-rich repeat motifs or immunoglobulin-like domains, for TLR and IL-1R respectively. In this appendix we will only focus on signaling pathways of TLRs (Figure 5a). After engagement of ligand to TLR the TLRs undergo a conformational change to recruit downstream signaling molecules. These include the adaptor molecule myeloid differentiation primary-response protein 88 (MyD88), IL-1R-associated kinases (IRAKs), TGF-β activated kinase (TAK-1), TAK-1 binding protein 1 (TAB1), TAB2 and tumor necrosis factor (TNF)-receptor-associated factor 6 (TRAF 6). MyD88 functions as an adaptor linking TLRs with downstream signaling molecules. MyD88 is a dimer that contains two protein-interaction domains: an amino-terminal death domain and a carboxy-terminal TIR domain. Upon stimulation the TIR domain of MyD88 associates with the TIR domain of TLR, whereas the death domain interacts with the amino-terminal death domain of IRAK and recruits IRAK to the receptor complex. The IRAK family consists of four family members. IRAK-4 phosphorylates and thereby activates IRAK-1. Upon activation of TLRs, TRAF6 is recruited to the receptor complex and binds to IRAK-1. Then, the IRAK-1/TRAF6 complex dissociates from the receptor and associates with TAK1, TAB1 and TAB2 at the membrane. IRAK-1 stays in the membrane and is degraded. The remaining complex of TRAF6, TAK1, TAB1 and TAB2 translocates to the cytosol, where it associates with UEV1A (ubiquitin-conjugating enzyme E2 variant 1) and UBC13 (ubiquitin-conjugating enzyme 13). This leads to ubiquitylation of TRAF6 and induces activation of TAK1. TAK1 phosphorylates mitogen-activated protein (MAP) kinases and the IKK complex (inhibitor of nuclear factor-κB (I-κB). The IKK complex exists of IKK-α, IKK-β and IKK-γ. The IKK complex phosphorylates I-κB allowing nuclear factor-κB to translocate to the nucleus and induce expression of its target genes (Figure 5b).

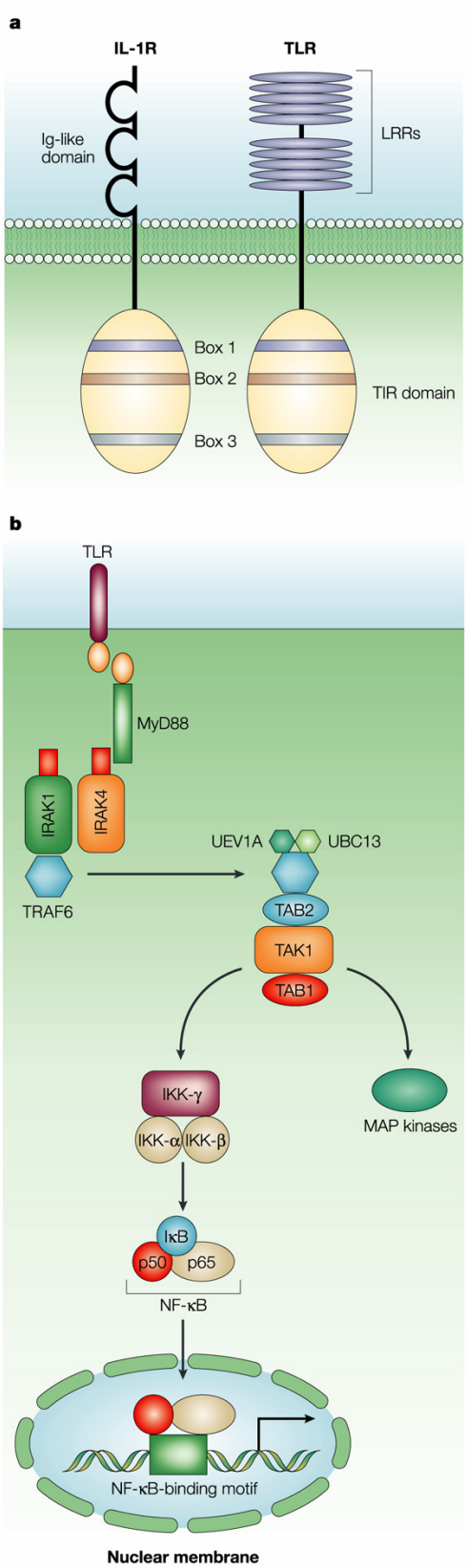

Figure 5: Toll signaling pathways (from Akira and Takeda 2004)

A: Toll-like receptors (TLRs) and interleukin-1 receptors (IL-1Rs) have a different extracellular domain but have a conserved cytoplasmic domain, known as the Toll/IL-1R (TIR) domain. **B**: Stimulation TLRs triggers a signaling cascade as described above, resulting in activation of NF-κB and MAP kinases

The significance of MyD88 has been established in MyD88 knockout mice that were unable to produce any inflammatory cytokines in response to TLR ligands. Furthermore, no activation of NF-κB and JNK in response to TLR2, TLR7 and TLR9 was observed. However, a My-D88-independent pathway exists in case of TLR4. Upon stimulation with LPS NF-κB and JNK activation was observed with delayed kinetics. The MyD88-independent pathway activates interferon (IFN)-regulatory factor (IRF3) leading to NF-κB activation and production of IFN-β and IFN-inducible genes. Recently, a new adaptor protein TIR-domain-containing adaptor protein (TIRAP), also called MyD88-adaptor-like protein (MAL) was identified and shown to function downstream of TLR4. Taken together, this indicates that TLR4 uses two adaptors with TIR domains, MyD88 and TIRAP, which control activation of distinct signal-transduction pathways. TLR2 and TLR9 use only MyD88, which accounts for differences in signaling by these receptors and TLR4 (Figure 6).

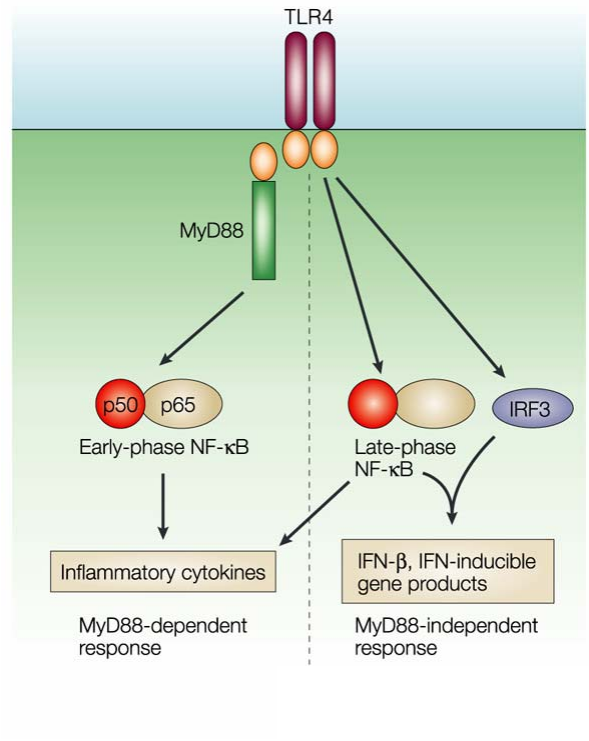

Figure 6: TLR4 signaling (from Akira and Takeda 2004) stimulation of TLR4 activates two pathways: MyD88 (myeloid differentiation primary-response protein 88)-dependent and MyD88-independent. The MyD88-dependent pathways involves the early phase of nuclear factor-κB (NF-κB) activation that leads to the production of inflammatory cytokines. The MyD88-independent pathway activates interferon (IFN)-regulatory factor (IRF3) and involves the late phase of NF-κB activation. Both lead to IFN-β production and expression of IFN-inducible genes.

APPENDIX 5: Probiotics and Toll-like receptors

PAMPs are not only expressed by pathogens but also by commensal microorganisms and probiotics. The role of TLRs in immunomodulation induced by probiotics is not completely clear. Recently, the role of TLR2 and TLR4 in pro-inflammatory activities of several *Lactobacillus* strains was investigated. Splenocytes from BALB/c, C57BL/6, C3H/HeN, C3H/HeJ (mutations in the TLR4 gene) and C57BL/6 *TLR2*$^{-/-}$ mice were cultured with several *Lactobacillus* strains or their subcellular components. The following strains were tested: *L. casei* YIT 9029, *L. fermentum* YIT 0159, *L. rhamnosus* YIT 0232, *L. acidophilus* YIT 0070, *L. plantarum* YIT 0102 and *L. reuteri* YIT 0197 (all from Yakult®, Japan). All *Lactobacillus* strains induced TNF-α production in splenocytes from BALB/c mice albeit to different degrees. In order to establish which bacterial components were responsible several fractions were tested in the macrophage cell line RAW264.7. The protoplast fraction, containing lipoteichoic acids (LTAs), induced most potently NF-κB activation and TNF-α production. These effects were not dependent on TLR4 signaling, since splenocytes of C3H/HeJ mice (mutation in TLR4 gene) produced TNF-α after stimulation with LTAs from lactobacilli. However, extracts from *L. fermentum* and *L. casei* could not induce TNF-α production from splenic cells of *TLR2*$^{-/-}$ mice. Thus, NF-κB activation by *L. fermentum* and *L. casei* appeared to be dependent on TLR2 but independent of TLR4 signaling (99). Previously, it has been shown that TLR2 and TLR4 recognize different bacterial components. TLR4 plays a role in recognition of gram-negative bacteria, whereas TLR2 is involved in the recognition of gram-positive bacteria, such as lactobacilli (100).

Other evidence for the recognition of probiotics by TLRs has been provided by a study exploring the effects of probiotics on experimental colitis. In this study a commercially available probiotic cocktail, VSL#3, containing 4 strains of lactobacilli (*L. casei*, *L. plantarum*, *L. acidophilus* and *L. delbrueckii bulgaricus*), 3 strains of bifidobacteria (*B. breve*, *B. longum* and *B. infantis*) and *Streptococcus salivarius thermophilus* was used. This cocktail has been successfully used in the treatment of ulcerative colitis, pouchitis and Crohn's disease (32). This cocktail was administrated to BALB/c mice and mice deficient in TLR2, TLR4, and TLR9 in which colitis was induced with DSS. Probiotics could attenuate the severity of colitis in wildtype mice and TLR2- and TLR4 deficient mice, but not in TLR9-deficient mice. This indicates an involvement of TLR9 in the beneficial effects of VSL#3 on experimental colitis (75).

Recently it has been shown that signaling of *Candida albicans* via TLR2 can result in activation of regulatory T cells and increased production of IL-10 (53). Thus, TLR signaling

can induce immunoregulation. Primed DCs are known to drive the immune response to either Th1, Th2 or regulatory T cell response (14). The effects of *L. reuteri, L. casei* and *L. plantarum* on DC priming and regulatory T cell development has been studied recently *in vitro*. *L. reuteri* and *L. casei*, but not *L. plantarum* were able to prime DCs to promote the development of regulatory T cells. However, the probiotic strains did not activate TLRs that were transfected in HEK 293 cells, except for *L. casei* that induced some TLR4 activation. *L. reuteri* and *L. casei* were captured by DC-SIGN, on DCs, thereby triggering the development of regulatory T cells (101). Another study focused on the effects of VSL#3 on cultured intestinal and blood derived DCs. VSL#3 could induce IL-10 in DCs and inhibit the generation of Th1 cells. Individual strains from the VSL#3 cocktail displayed distinct effects. The bifidobacteria stimulated IL-10, whereas the lactobacilli strains downregulated IL-10, except for *L. acidophilus*. All strains downregulated IL-12 production. Thus, VSL#3 can change the cytokine profile in DCs towards a more regulatory profile (12). Apparently, VSL#3 has immunoregulatory properties that involve several mechanisms, depending on the model used.