Immunomodulatory Effects of Probiotic Bacteria In Healthy Adults

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Main Abbreviations

B.	*Bifidobacterium*
Bb12	*Bifidobacterium animalis* ssp. *lactis* Bb12
Cfu	Colony-forming unit
CI	Confidence intervals
CRP	C-reactive protein
DC	Dendritic cell
E.	*Escherichia*
ELISA	Enzyme-linked immunoabsorbent assay
FACS	Fluorescent-activated cell sorting
GI	Gastrointestinal
IFN	Interferon
Ig	Immunoglobin
IL	Interleukin
IQR	Interquartile range
L.	*Lactobacillus*
Lc.	*Lactococcus*
LGG	*Lactobacillus rhamnosus* GG
Ln.	*Leuconostoc*
LPS	Lipopolysaccharide
Mo	Month
PBMC	Peripheral blood mononuclear cell
PCA	Principal component analysis
PLS/DA	Partial least squares discriminant analysis
PJS	*Propionibacterium freudenreichii* ssp. *shermanii* JS
RDBPC	Randomized, double-blind, placebo-controlled
S.	*Streptococcus*
SD	Standard deviation
sIgA	Secretory immunoglobulin A
ssp.	Subspecies
Th	T helper
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	Regulatory T cell
UPLC/MS	Ultra performance liquid chromatography/mass spectrometry
Wk	Week

List of Original Publications

I   Kekkonen RA, Kajasto E, Miettinen M, Veckman V, Korpela R, Julkunen I. Probiotic *Leuconostoc mesenteroides* ssp. *cremoris* and *Streptococcus thermophilus* induce IL-12 and IFN-γ production. World J Gastroenterol 2008;14:1192-1203.

II   Kekkonen RA, Lummela N, Karjalainen H, Latvala S, Tynkkynen S, Järvenpää S, Kautiainen H, Julkunen I, Vapaatalo H, Korpela R. Probiotic intervention has strain-specific anti-inflammatory effects in healthy adults. World J Gastroenterol 2008;14:2029-2036.

III   Kekkonen RA, Sysi-Aho M, Seppänen-Laakso T, Julkunen I, Vapaatalo H, Orešič M, Korpela R. Effect of probiotic *Lactobacillus rhamnosus* GG intervention on serum global lipidomics profiles in healthy adults. World J Gastroenterol 2008:14;3188-3194.

IV   Kekkonen RA, Vasankari TJ, Moreira A, Timonen T, Kautiainen H, Haahtela T, Julkunen I, Korpela R. Effect of probiotics on inflammatory mediators in healthy heavily-exercising adults. *Submitted.*

V   Kekkonen RA, Vasankari TJ, Vuorimaa T, Haahtela T, Julkunen I, Korpela R. The effect of probiotics on respiratory infections and gastrointestinal symptoms during training in marathon runners. Int J Sports Nutr Exerc Metab 2007;17:352-363.

Abstract

Probiotic bacteria have been mostly investigated in the prevention and treatment of different gastrointestinal diseases and allergies. Probiotic products, however, are usually consumed by the general, healthy population but not much is known on their immunomodulatory effects in healthy adults. It is not fully clear how probiotics exert their beneficial effects on health, but one of the most probable mechanisms of action is the modulation of immune responses via the mucosal immune system of the gut. The purpose of the present study was to investigate the immunomodulatory properties of probiotic strains by systematical screening in primary cell culture using human peripheral blood mononuclear cells (PBMC) and to evaluate the effects of probiotics on the immune system in healthy adults in randomized, double-blind, placebo-controlled intervention trials. In addition, novel lipidomics profiling was performed to characterize the effect of probiotics on lipid-derived mediators.

Probiotic strains from six different genera showed clear differences in their ability to induce cytokine responses in human PBMC *in vitro*. Strains from *Streptococcus* and *Leuconostoc* genera were the most potent inducers of Th1-type cytokines. Strains belonging to *Bifidobacterium* and *Propionibacterium* genera showed the best anti-inflammatory potential by inducing IL-10 production. No combinations of probiotics (probiotic multispecies) resulted in enhanced cytokine production compared with individual strains (probiotic monospecies), suggesting that different bacteria compete with each other during host cell-probiotic interactions.

The selection of strains for the clinical trials was made based on their anti-inflammatory potential. The ability to induce IL-10 (anti-inflammatory cytokine) or IL-12 (Th1-type cytokine) production was used to classify the strains as anti-inflammatory or Th1-type favoring strains, respectively. The strains possessing the best anti-inflammatory potential, namely *B. lactis* ssp. *animalis* Bb12 (Bb12) and *P. freudenreichii* ssp. *shermanii* JS (PJS), along with *L. rhamnosus* GG (LGG) as a well-documented reference probiotic, were thus selected for further clinical studies in healthy adults. The results of the *in vitro* setting did not entirely reflect the *in vivo* results as the best anti-inflammatory strain was LGG, which induced only moderate IL-10 production *in vitro* compared with Bb12 and PJS strains. During a three-week intervention in healthy adults, serum highly sensitive CRP levels showed the lowest levels in the LGG and PJS groups. In the same clinical setting, LGG was able to decrease proinflammatory TNF-α production in *ex vivo* isolated PBMC whereas Bb12 decreased IL-2 production.

As LGG seemed to demonstrate the best anti-inflammatory potential in healthy adults, the effect of LGG on lipid-derived mediators was investigated. For this purpose, global lipidomics profiles in serum were determined. A tendency towards a reduced number of inflammatory lipid-derived mediators, namely lysophosphatidylcholines and sphingomyelins, was observed in response to intervention with LGG.

The effect of LGG on the incidence of respiratory infections and gastrointestinal symptoms was assessed in a longer, three-month intervention in exercising adults participating in a marathon. In this longer intervention, LGG consumption resulted in decreased monocyte and serum IL-6 levels at the end of the study period. Serum highly sensitive CRP levels were also lower in the LGG group as compared to the controls, although the difference was not statistically significant. LGG had no effect on the incidence or duration of respiratory infections in exercising adults. However, LGG was able to reduce the duration of gastrointestinal symptoms.

In conclusion, probiotics show a strain-specific ability to modulate the release and action of inflammatory mediators in healthy adults. Immunomodulatory effects of probiotic multispecies should be studied as the effects differ from single strains. As the *in vitro* results did not entirely reflect the *in vivo* situation, PBMC *in vitro* should not be used as the only screening method for immunomodulatory effects. Instead, the *ex vivo* production of cytokines by PBMC after probiotic intervention could offer a relatively easy and quick screening method for immunomodulatory properties of probiotics. The mechanisms of specific host-probiotic interactions in the gut resulting in systemic and clinical effects warrants further investigations.

1. Introduction

Probiotics are defined as live microorganisms that confer a health benefit on the host (FAO/WHO, 2002) and they have been mostly studied in the prevention and treatment of gastrointestinal disorders (Lenoir-Wijnkoop et al., 2007). There is evidence that probiotics can prevent and reduce the duration diarrhoea in children and adults (Sazawal et al., 2006; Szajewska et al., 2001) and reduce the risk of allergy (Ouwehand, 2007). Probiotic have also been used to prevent or shorten respiratory infections (de Vrese et al., 2006; Hatakka et al., 2001). The action mechanisms of probiotics are not fully understood, but they are likely to be mediated by the gut mucosal immune system. The gut is considered to be the largest immunological organ of the body and also the gut microbiota has an important role in the maturation and maintenance of the immune system along its protective functions against pathogens (Blaut and Clavel, 2007; Guarner, 2006).

The immunomodulatory effects of probiotics have not been systematically investigated from *in vitro* screenings to short-term clinical trials and longer interventions evaluating the clinical outcome of the probiotic intervention. Although probiotics have reduced the respiratory infections in healthy children (Hatakka et al., 2001; Weizman et al., 2005) and adults (Olivares et al., 2007), not much is known about their immunomodulatory effects in healthy adults. The aim of this thesis was to systematically screen the cytokine responses induced by probiotics in primary cell culture using human peripheral blood mononuclear cells and to evaluate the impact of probiotics in the modulation of immune responses of healthy adults in randomized, double-blind, placebo controlled clinical intervention studies.

2. Review of the literature

2.1 BASIS FOR HOST-PROBIOTIC IMMUNOMODULATORY CROSS-TALK

2.1.1 Introduction to the immune system

The gastrointestinal tract constitutes a major interface with the external environment. The intestinal mucosal surface (around 300 m^2) is adapted to the main functions of the gut; the digestion of food and absorption of nutrients and the defence against microbial pathogens and other hazardous substances. The gut is considered to be the largest immunological organ of the human body and the mucosal immune system in the gut is often the first line of defence against microbial infections. Immunological reactions are divided into two parts by the specificity and speed of the reaction; innate and adaptive responses, although in practice there is extensive interaction between them and it is often hard to describe where the innate immunity ends and the adaptive immunity starts (for review, see Parkin and Cohen, 2001). The innate immunity provides the immediate but non-specific host defence and it includes physical, chemical, and microbiological barriers as well as many elements of the immune system (neutrophils, monocytes, macrophages, complement, cytokines, and acute-phase proteins). The adaptive immunity consists of antigen-specific reactions through T lymphocytes and B lymphocytes. The adaptive immune response is highly specific and it develops an immunological memory, but its development requires several days or weeks.

2.1.2 Non-immune defence mechanisms

Gut microbiota as a barrier

The human gastrointestinal tract harbours a diverse community of bacteria, and molecular analysis has revealed that the gut contains more than 1,000 bacterial species (for review, see Egert et al., 2006; Guarner, 2006). Physiological conditions, such as acidity and peristalsis, are distinct in different parts of the gut, which is reflected in the distribution of the bacteria. The concentrations of bacteria in the stomach and the duodenum is approximately 10^3 cfu/ml, increasing to 10^4-10^8 cfu/ml in the jejunum and the ileum and reaching the highest concentration of up to 10^9-10^{12} cfu/ml in the colon (Blaut and Clavel, 2007). Microbiota is established at birth and its composition and development depends on the mode of delivery (Grönlund et al., 1999; Huurre et al., 2007) and feeding (Favier et al., 2002). During the first months of life, the microbiota changes and becomes more complex, reaching a composition similar to adults by the age of one to two (Palmer et al., 2007). There is a high inter-individual variability, but

the microbiota stays relatively stable over time (Zoetendal et al., 1998) although disease (Tlaskalova-Hogenova et al., 2004), antibiotic treatments (Jernberg et al., 2007) and diet (O'Keefe, 2008) can alter it. The gut microbiota possesses several metabolic functions as it ferments non-digestible dietary residue, induces the production of short chain fatty acids to retain energy and produces vitamin K (Guarner, 2006). Furthermore, it has trophic functions in controlling epithelial cell proliferation and differentiation. The gut microbiota has an important function in the maturation and maintenance of the immune system and it also provides protection against microbial pathogens (the barrier effect) (Guarner, 2006). The commensal bacteria that have colonized the gut restrict the growth of the exogenous or opportunistic pathogenic bacteria. The barrier effect is based on the ability of the commensal bacteria to secrete antimicrobial substances, such as bacteriocins that inhibit the growth of bacteria as well as on the competition of nutrients and attachment to the epithelia.

Mucosal barrier

The mucosal surface of the gastrointestinal tract is the largest body surface that is in contact with the external environment (approximately 300 m^2). The physical and chemical barriers created by the intestinal epithelium protect the host from pathogens. The intestinal epithelial cells provide a physical barrier against pathogens, and the integrity of the epithelial layer is maintained by tight junctions, adherence junctions and desmosomes (for review, see Acheson and Luccioli, 2004). Transcellular and paracellular fluxes are tightly controlled by membrane pumps, ion channels and tight junctions, adapting the permeability according to physiological needs (Baumgart and Dignass, 2002). Furthermore, being covered by specialized mucus-producing cells, antimicrobial peptides such as defensins, and other antimicrobial molecules, such as lysozyme, which together with commensal microbiota provide defence against pathogens, the intestinal epithelium also provides a chemical barrier against pathogens (for review, see Lievin-Le Moal and Servin, 2006). Disturbance at any level, but particularly bacterial translocation due to increased permeability and breakdown of oral tolerance resulting from compromised epithelial and T cell interaction, can cause inflammation and tissue damage (Baumgart and Dignass, 2002).

2.1.3 Intestinal epithelium and recognition of microorganisms

The intestinal epithelium is rapidly and constantly renewed by epithelial stem cell population. The stem cell population differentiates into multiple intestinal epithelial cell types specialized in different functions (goblet cells, enteroendocrine cells, enterocytes, Paneth cells and M cells). These epithelial cells serve as a physical barrier between the luminal contents (food antigens and commensal microbiota) and the complex mucosal

immune system (for review, see McCracken and Lorenz, 2001). They also play a crucial role in signalling and mediating host innate and adaptive immune responses. Activation of the host defence mechanisms is based on the rapid recognition of specific structural components of microorganisms (both commensal and pathogenic microorganisms), known as pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), lipoprotein, peptidoglycan, lipoteichoic acid, flagelline and CpG-containing (unmethylated) DNA (for review, see Alexopoulou and Kontoyiannis, 2005; Sansonetti, 2004; Winkler et al., 2007). These components are recognized by pattern-recognition receptors, of which the best known are the Toll-like receptors (TLRs) and nucleotide-binding oligomerisation domain proteins (NODs) (for review, see Uematsu and Akira, 2008; Underhill, 2007). TLR family includes at least 12 proteins that recognizes microbe-derived ligands. For example, TLR2 recognizes lipoproteins and lipid-modified sugars, TLR4 recognizes LPS whereas TLR9 recognizes CpG motifs in bacterial DNA (for review, see Uematsu and Akira, 2008). NOD receptors are cytoplasmic receptors that recognize peptidoglycan from bacterial cell walls (for review, see Underhill, 2007). In general, the stimulation of TLRs or NODs by PAMPs results to the activation of adaptor proteins, which initiates a signalling cascade involving several kinases. Eventually, the signals are transmitted via transcription factors such as NF-κB, which then initiate the transcription of genes leading to the synthesis of immunomodulatory molecules such as cytokines, chemokines and other inflammatory effector molecules.

2.1.4 Communication within the immune system

In order for immune cells to work effectively, they need to be recruited to the site of inflammation and become appropriately activated (for review, see Parkin and Cohen, 2001). This is achieved by the interaction of cellular receptors signalling internally to the nucleus as described above, and external factors, such as cytokines, which are able to bind the receptors, and also with other adhesion molecules. A summary of the mediators within the immune system is presented in Table 1.

Adhesion molecules
Adhesion molecules are cell surface molecules involved in cell-to-cell interactions (Muzio et al., 2000). Their main function is in facilitating cellular functions, such as directing cell migration, phagocytosis, and cellular cytotoxicity. The main adhesion molecules include the intercellular adhesion molecules (ICAMs), integrins, selectins, and cadherins (calcium-dependent adherins). In addition to the molecules on leukocytes, and vascular endothelium, there are also tissue-specific adhesion molecules called addresins.

Cytokines

Cytokines are small proteins that are released by immune cells as well as by many other cell types (Vilcek, 2003). Cytokines alter the behaviour of the cell in an autocrine or paracrine fashion, i.e. by affecting the same cell that produced the cytokine or another cell that is close the cytokine releasing cell. Each cytokine may have multiple activities on different cell types. Cytokines act by binding to their specific receptors on the cell surface and by inducing changes in the growth, development, or activity of the target cell. Cytokines produced by leukocytes and having effects mainly on other white cells are termed interleukins (ILs). Cytokines that have chemoattractant activity are called chemokines. Colony-stimulating factors (CSFs) function as important differentiation and proliferation factors for stem cells. Interferons (IFNs) inhibit the replication of viruses and regulate the activation of the immune system.

Cytokine production patterns are used to divide T helper cell (Th) responses to different classes. Th1-type cells produce IL-2 and IFN-γ and promote cell-mediated immunity (Kidd, 2003). Th2-type cells produce IL-4, IL-5, IL-6 and IL-13 and activate the antibody-mediated immune response as well as eosinophils and mast cells (Kidd, 2003). Th3-type cells downregulate the inflammatory response through TGF-β production and Tr1 cells balance the immune responses through IL-10 production (Izcue and Powrie, 2008; Taylor et al., 2006; Wu et al., 2007). Cytokines can also be classified according to their pro- or anti-inflammatory properties. Inflammatory cytokines include TNF-α (Bertazza and Mocellin, 2008), IL-1α/β (Barksby et al., 2007), IL-6 (Gabay, 2006) and IFN-γ (Schoenborn and Wilson, 2007) whereas IL-10 is considered to be an anti-inflammatory or regulatory cytokine (Couper et al., 2008). TNF-α, IL-1 and IL-6 also mediate the systemic effects of inflammation, such as fever, weight loss, and hepatic acute-phase protein synthesis. The production of appropriate amounts of these cytokines is important in response to inflammation. However, inappropriate or excessive production of cytokines can be dangerous, and these cytokines, particularly TNF-α, are implicated in causing some of the pathological responses that occur in acute and chronic inflammatory conditions.

TNF-α is a proinflammatory cytokine that activates immune system and is involveld in the local inflammation (Bertazza and Mocellin, 2008). It is produced by professional immune cells like macrophages, NK cells and T cells, but also non-immune cells like epithelial cells are able to induce TNF-α. IL-1β is mainly produced by macrophages and epithelial cells and it is involved in T cell and macrophage activation (Barksby et al., 2007). IL-6 is produced by T cells, macrophages and endothelial cells and is involved in T- and B-cell growth and and differentiation, acute phase response and fever (Gabay, 2006).

IL-2, produced by T cells, is called as a T cell growth factor and it induces T cell proliferation. IL-12 induces the production of IFN-γ and differentiates Th cells to

Th1-type cells and forms a link between innate and adaptive immunity (Trinchieri, 2003). Dendritic cells and phagocytes produce IL-12 during infection in response to pathogens and it also activates NK cells (Trinchieri, 2003). IFN-γ, produced by T cells and NK cells, possesses antiviral activity and it activates macrophages and suppresses Th2 responses (Schoenborn and Wilson, 2007).

IL-4 and IL-5 are produced by T cells and mast cells. IL-4 activates B cells, is involved in IgE switch and suppresses Th1 cells. IL-5 is induces the growth and differentiation of eosinophils. IL-10 is an anti-inflammatory cytokine and it can be produced by many types of cells (Couper et al., 2008). During infection it inhibits the activity of Th1 cells, NK cells, and macrophages, all of which are required for optimal pathogen clearance but also contribute to tissue damage (Couper et al., 2008).

Chemokines

Chemokines are particular members of the cytokine family that have a key role in regulating leukocyte migration (Ono et al., 2003). They have substantial chemotactic function (inducing the directional movement of cells). Chemokines are produced by most cells on stimulation with proinflammatory cytokines or bacterial products and chemokine receptors are found on all leukocytes. Inflammatory chemokines control leukocyte traffic in acute inflammation or infection (Bono et al., 2007). Homeostatic chemokines in turn regulate leukocyte haematopoiesis and leukocyte migration to spleen and lymph nodes (Bono et al., 2007). CXCL8, interleukin 8 (IL-8), has a chemotactic role in attracting neutrophils into the site of inflammation or infection (Kobayashi, 2008). CXCL10, interferon-inducible protein 10 (IP-10), as well CCL2, monocyte chemotactic protein-1 (MCP-1), play a role in the recruitment of monocytes to sites of injury and infection (Ono et al., 2003).

Acute phase proteins

A systemic acute-phase response may develop during inflammation or infection (for review, see Beutler, 2004; Gabay and Kushner, 1999; Volanakis, 2001). Most infections trigger several secondary effects throughout the host. These include the development of fever, a fall in plasma iron levels, and the synthesis of acute-phase proteins. Although most APRs are synthesized by hepatocytes in response to the action of peripheral cytokines (IL-6, TNF-α and IL-1β), some are produced by other cell types, including monocytes, endothelial cells, fibroblasts and adipocytes. The major acute-phase proteins in humans are serum amyloid A (SAA) and C-reactive protein (CRP). Acute-phase proteins have a wide range of activities that contribute to host defence: they can bind and neutralize inflammatory agents, such as pathogenic microbes, help to minimize the extent of local tissue damage, as well as participate in tissue repair and regeneration. Erythrocyte sedimentation rate (ESR) is used as a non-specific measure

of inflammation and as a "sickness index", but it is limited by its low sensitivity and specificity (Brigden, 1998). The high-sensitivity CRP is considered to be a very sensitive marker of inflammation. Low-grade inflammation, characterised by increased plasma CRP concentrations and increased systemic levels of certain cytokines are nowadays considered to be risk factors for various chronic diseases, such as atherosclerosis and metabolic syndrome (Chen, 2006).

Lipid-derived mediators

The inflammatory response consists of immunological and non-immunological reactions. Non-immunological reactions are induced by the release of lipid-derived compounds from injured tissues and migrating cells. Many of the changes in lipid-derived compounds during infection/inflammation help to protect the host from harmful effects of inflammatory substances (for review, see Khovidhunkit et al., 2004).

Eicosanoids are derived from cell membrane phospholipids by the activation of phospholipase A2 and the release of arachidonic acid (Khanapure et al., 2007). The arachidonic acid is subsequently transformed by cyclooxygenase and lipoxygenase pathways to prostaglandins, thromboxane and leukotrienes collectively termed eicosanoids. Eicosanoid production is considerably increased during inflammation. The main eicosanoids are prostaglandins (PGD_2, PGE_2, $PGF_{2\alpha}$), prostacyclin (PGI_2), thromboxane A_2 (TXA_2) and leukotrienes (LTs). Prostaglandins are involved in the regulation of circulation. In addition to its proinflammatory effects, PGE_2 has cytoprotective functions e.g. in the gastric mucosa. Prostaglandins and leukotrienes also contribute to the generation of the signs and symptoms of inflammation.

Also lipid-derived compounds have been related to different diseases. For example phospholipids, such as lysophosphatidylcholine, have been associated with atherosclerosis (Kougias et al., 2006) as well as inflammatory bowel disease (Haapamäki et al., 1999; Minami et al., 1994), impaired mucosal barrier function and increased gut permeability (Karlqvist et al., 1986; Otamiri et al., 1986; Sawai et al., 2002; Tagesson et al., 1985). Sphingolipids and lipids of the sphingomyelin/ceramide pathway in high concentrations have been associated with inflammatory processes in the development of atherosclerosis (Bismuth et al., 2008) and inflammatory bowel disease (Homaidan et al., 2002; Sakata et al., 2007).

Reactive oxygen species, reactive nitrogen species and degradative enzymes

Major phagocytic cells (neutrophils, macrophages, monocytes) engulf pathogenic microbes and use different metabolites for killing microbes (for review, see Beutler, 2004; Chaplin, 2006; Parkin and Cohen, 2001; Won and Singh, 2006). The oxygen-dependent response or respiratory burst involves the sequential reduction of oxygen by an NADPH oxidase leading to production of oxygen metabolites, such as hydrogen

peroxide, hydroxyl radicals, superoxide and singlet oxygen. The oxygen-independent response uses the degradative enzymes, myeloperoxidase and lysozyme, on microbe destruction. Nitric oxide takes part in many physiological processes; besides its role in the regulation of circulation and inflammation, it also acts as an effector molecule in innate immune responses and contributes to host defence (for review, see Tripathi et al., 2007).

Amines

Histamine is an essential biological amine in inflammation and allergy (Xie and He, 2005). In addition to its role in acute inflammatory and immediate hypersensitivity responses, it has been demonstrated to affect chronic inflammation and regulate several essential events in the immune response (Jutel et al., 2006). Histamine is mostly found in the lungs, skin and the gastrointestinal tract. It is stored in mast cells and basophils from which it is released by the action of complement or IgE. Serotonin (5-HT) is found in the gastrointestinal tract, platelets and central nervous system. 5-HT is known to increase gastrointestinal motility, to contract bronchi, uterus and arteries, and its role in inflammatory and immunological responses has recently been of interest (Cloez-Tayarani and Changeux, 2007).

Table 1. Summary of main mediators within the immune system.

Mediator		Major functions
Adhesion molecules	Integrins, selectins, cadherins, addresins	Facilitation of cell-to-cell interactions
Cytokines		
Tumour necrosis factors	TNF-α, TNF-β	Activation of many cells, induction of apoptosis, proinflammatory function
Interleukins	IL-1α/β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13	Regulation of immune cell function and activation
Transforming growth factor	TGF-β	Inhibition of cell growth, anti-inflammatory, induction of IgA secretion
Interferons	IFN-α, -β, -λ, -γ	Antiviral activity, inhibition of tumour cell growth, activation of cells
Chemokines	CCL2, -3, -4, -5 CXCL8, -10 etc.	Chemotaxis and activation of immune cells
Colony-stimulating factors	GM-CSF, G-CSF, IL-3	Growth and differentiation of haematopoietic cells
Growth factors	EPO, TPO, EGF, FGF, PDGF	Growth and differentiation of cells
Acute-phase proteins	CRP, serum amyloid A, fibrinogen	Participation in host defence, neutralisation of inflammatory agents, inhibition of local tissue damage
Lipid-derived mediators		
Eicosanoids	Prostaglandins, prostacyclin, thromboxane, leukotrienes	Inflammatory mediators, some members have cytoprotective functions
Phospholipids	Phosphatidylcholine, lysophosphatidylcholine	Regulation of cell function, involved in oxidative metabolism, inflammatory mediators
Sphingolipids	Sphingomyelin, ceramide	Regulation of many biological processes, part of cell membranes, immunomodulation
Reactive oxygen and nitrogen species	Hydrogen peroxide, hydroxyl radicals, superoxide, singlet oxygen, nitric oxide	Participation in innate immune response, killing of microbes
Enzymes	Lysozyme, myeloperoxidase	Participation in innate immune response, killing of microbes
Amines	Histamine, serotonine	Inflammatory reactions, immunomodulation

2.1.5 Mucosal immunity

The mucosal immunity serves as the first line of defence that reduces the need for systemic immunity, which is principally "proinflammatory". The mucosal immune system can be functionally and physically divided into inductive sites (the Peyer's patches) and effector sites (the lamina propria) (for review, see Cummings et al., 2004; McCracken and Lorenz, 2001). In the inductive sites, the mucosa-associated lymphoid tissue (MALT) is covered with follicle-associated epithelium (FAE) which contains M cells. These specialized cells effectively transport microorganism as well as foreign antigens from the gut lumen into the underlying organized lymphoid tissue containing antigen-presenting cells (APC) (macrophages and dendritic cells) and mucosal T and B cells. Microorganisms/antigens are presented to T and B cells after intracellular processing. In addition, antigens may be taken up and presented by intra- or subepithelial dendritic cells directly as they may penetrate epithelium by their cellular extensions or processes. Activated T cells release cytokines which may differentiate B cells to antibody (IgA, IgM) producing plasma cells. Most activated B cells migrate to mesenteric lymph nodes where they are further stimulated, followed by homing into distant mucosal effector sites, particularly the intestinal lamina propria where they finally develop to antibody producing plasma cells. Subsequently, antibody-mediated mucosal defence inhibits the surface colonization of the pathogens.

Another important role of the mucosal immune system in addition to preventing the entry of pathogens through the gastrointestinal tract is to discriminate pathogenic microorganisms from harmless food antigens and commensal bacteria (Dubois et al., 2005). Every year, approximately 1,000 kilograms of food pass the human gut containing roughly two kilograms of commensal bacteria. To this end, to avoid local and peripheral hypersensitivity, immunosuppressive mechanisms inducing "oral tolerance" have developed. (Dubois et al., 2005).

2.1.6 Innate immune response

The innate immune response provides fast and unspecific host defence (for review, see Parkin and Cohen, 2001; Tosi, 2005). Inflammation is the body's immediate response to different inflammatory stimuli or injury and is an integral part of the innate immune response. Inflammation protects the host from threatening agents or events and their ultimate purpose is to neutralize the original cause (pathogen, injury). Recognition of a threatening agent or event initiates an inflammatory response in the epithelial surfaces. During the very early stages of inflammation or tissue damage, a release of cytokines from the activated macrophages occurs. These cytokines recruit leukocytes to the inflammation site. Cytokines released by phagocytes (macrophages, monocytes, neutrophils) also activate the acute-phase response. Chemokines released by phagocytes

recruit more cells, such as leukocytes, to the inflammatory site. After entering tissues, many pathogens are killed by phagocytes or natural killer (NK) cells. The ingestion and killing of pathogens is more effective if the microbe is first opsonised with specific antibody or complement which is a system of plasma proteins that interacts with pathogens to mark them for destruction by phagocytes.

2.1.7 Adaptive immune response

Non-specific responses of innate immunity are necessary for the adaptive immunity to be initiated, and several lymphocyte subpopulations and antibodies take part in regulating innate and adaptive immunity. The adaptive immune response is specific with immunological memory, but takes several days or weeks to develop (for review, see Parkin and Cohen, 2001). T and B cell responses are initiated in peripheral lymphoid organs by activated antigen-presenting cells, after which the effector responses take place. Cytokines produced in the early phases of an inflammation influence the functional differentiation of T cells into T helper (Th1, Th2, Th3), cytotoxic T cells or regulatory T (Tr1) cells (Izcue and Powrie, 2008; Kidd, 2003). Activated T cells leave the lymphoid tissue and home in the inflammatory site. Th1 and cytotoxic T cells promote cell-mediated immunity. Th1 cells coordinate the host response to intracellular pathogens and have a central role in phagocyte activation by promoting microbial killing. They must be tightly regulated to avoid tissue damage. Cytotoxic T cells are selective killers of microbe-infected target cells expressing a specific antigen. Th2 cells promote humoral immunity leading to activation of B cells (plasma cells) and the release of antibodies (immunoglobulins, Ig) into blood and tissue fluids. IgG and IgA neutralize bacteria, bacterial toxins and the infectivity of viruses whereas IgE-mediated activation of accessory cells (mast cells, basophils, eosinophils) has an important role in parasite infections and also in allergy. Regulatory T cells (Th3 and Tr1) balance the immune responses and limit the inflammatory response. Resolution of inflammation is accompanied by the death of most of the effector cells, generation of memory cells as well as repair of the local tissue damage.

2.1.8 Importance of regulated immune and inflammatory responses

Inflammation is a general term for the local accumulation of fluid, plasma proteins and white blood cells which is initiated by a threatening agent or event. Classical signs of acute inflammation include redness (rubor), heat (calor), swelling (tumor) and pain (dolor), as well as loss of function (functio laesa). Acute inflammation is a limited beneficial response, especially during infectious challenges, whereas chronic inflammation is a persistent phenomenon that can lead to tissue damage. Chronic

inflammation is not characterized by the classic signs of acute inflammation listed above. Instead, chronically inflamed tissue is characterized by the infiltration of mononuclear immune cells (monocytes, macrophages, lymphocytes and plasma cells), tissue destruction, and attempts of healing, which include angiogenesis and fibrosis. Regulated inflammatory responses are therefore essential for the overall health and maintenance of homeostasis (Figure 1).

Immune responses may also be misdirected or overreactive as in the case of autoimmune and atopic diseases (Chaplin, 2006). In autoimmune diseases, such as rheumatoid arthritis and type 1 diabetes, the body overreacts to self-antigens with Th1-type immune response patterns. In atopic diseases, the immune response is skewed towards a Th2-type response leading to hypersensitivity to normally innocuous environmental antigens. The body can also remain nonresponsive in certain situations, giving for instance tumours an opportunity to develop.
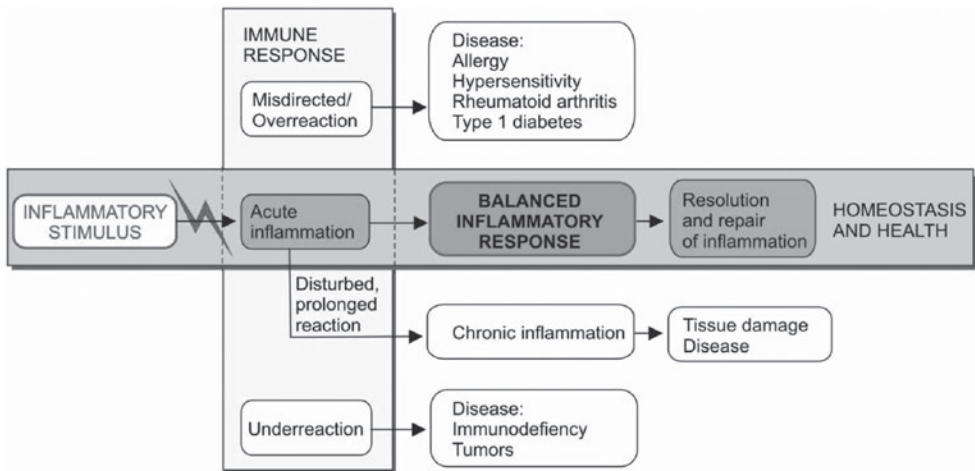

Figure 1. Balanced and sufficient immune response is essential for the resolution and repair of inflammation as well as maintenance of homeostasis and health. Both excessive and inadequate immune responses can lead to the development of a disease.

2.1.9 Factors affecting the immune system

The immune system is influenced by genetic and environmental factors (for review, see Albers et al., 2005; Calder and Kew, 2002; Cummings et al., 2004). Factors contributing to the immune function include genes, age, gender, acute and chronic exercise, smoking, alcohol consumption, obesity, pregnancy, hormonal status and commensal microbiota in the gut. Due to the diversity of these factors, there is wide inter-individual variation in immune responses (Yaqoob et al., 1999).

Age

Immune function changes with age. The immune system of a newborn child is still developing, and the adaptive immune system is immature due to low antigen exposure. Postnatally, intestinal microbes provide the developing gut with stimuli that are necessary for healthy maturation of the intestinal immune system (Rautava and Walker, 2007). Nutrition plays a role in the maturation of the immune system as well (Calder et al., 2006). In normal healthy adults, the nature of the immune response varies according to the inflammatory or infectious agent. There may be small increases or decreases in different markers of immune function over time, but a change in one or two immune functions is probably not clinically important because other components may compensate for the change. Aging is associated with changes in immune function (for review, see Pawelec, 2006); Th1 responses, lymphocyte proliferation and immunoglobulin levels decrease with age and the increase in Th2 responses and prostaglandin E2 levels result in decreased infection resistance.

Exercise

Heavy exercise causes a temporary depression of immune function and periods of intense training lasting over one week as well as exercise competitions may lead to a longer lasting immune dysfunction (for review, see Gleeson, 2007). This can lead to an increased risk of upper respiratory tract infections in heavily exercising athletes (Gleeson, 2000). Strenuous exercise is also known to cause many gastrointestinal symptoms, such as nausea, vomiting, diarrhoea and heartburn (Peters et al., 2001; Simons and Kennedy, 2004). Moderate regular training is associated with reduced incidence of infections compared with a sedentary state. Furthermore, exercise mediates several other health benefits (for review, see Gleeson, 2007).

2.2 Immunomodulatory effects of probiotics

2.2.1 Overview of probiotics

Probiotics are defined as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2002). The most commonly used probiotics are lactobacilli and bifidobacteria, but also other strains have been used as a probiotics (Table 2). Reported possible health effects in human intervention trials include amelioration of acute diarrhoea in children, reduction of the risk of antibiotic-associated gastrointestinal symptoms, relief of milk allergy/atopic dermatitis in infants, reduction in the risk of atopic diseases and respiratory infections, relief of irritable bowel syndrome and rheumatoid arthritis symptoms, suppression of *Helicobacter pylori* and modulation of the immune response (for review, see Lenoir-Wijnkoop et al., 2007). The ways in which probiotic bacteria elicit their health effects are not fully understood. One of the most probable means is the immunomodulatory effect mediated by the gut mucosal immune system which is reflected on the systemic level. Immunomodulative effects of probiotics have been investigated in allergy (for review, see Boyle and Tang, 2006; Ouwehand, 2007) and inflammatory diseases, such as inflammatory bowel disease (for review, see Ewaschuk and Dieleman, 2006; Limdi et al., 2006). Probiotics are, however, usually consumed by the general, healthy population but not much is known about the nature of the effects, if any, they have on the immune function in normal healthy subjects. The need to study the health effects of probiotics also in the healthy population is evident, and only a few randomised, double-blind, placebo-controlled studied have thus far addressed this question.

Table 2. The most commonly used probiotics.

Lactobacillus	Bifidobacterium	Other
L. rhamnosus	B. bifidum	Streptococcus thermophilus
L. casei	B. lactis	Lactococcus
L. acidophilus	B. longum	Propionibacterium
L. plantarum	B. breve	Saccharomyces
L. johnsonii	B. animalis	Escherichia coli ssp.
L. reuteri		Enterococci
L. salivarius		Bacillus
L. helveticus		
L. fermentum		
L. bulgaricus		

2.2.2 Possible mechanisms for host-probiotic immunomodulatory cross-talk

It is known that probiotics are able to modulate many aspects of the non-immune defence. The possible mechanisms for host-probiotic interaction have mainly been studied in animal and *in vitro* models. *Lactobacillus* strains have been shown to modulate the composition and metabolism of commensal microbiota (Kuisma et al., 2003; Siigur et al., 1996) and to compete with the pathogens. They can inhibit pathogen adhesion by competitive exclusion, as shown by different *Lactobacillus*, *Bifidobacterium* and *Propionibacterium* strains in *in vitro* models (Collado et al., 2006; Collado et al., 2007) and by inducing intestinal mucin production, as shown by *Lactobacillus*, *Bifidobacterium* and *Streptococcus* strains in rat and *in vitro* models (Caballero-Franco et al., 2007; Mack et al., 2003; Mack et al., 1999). In addition, lactobacilli and bifidobacteria are able to produce antimicrobial agents, such as organic acids, hydrogen peroxide, diacetyl, short chain fatty acids and bacteriocins, against pathogens (for review, see Servin, 2004). Probiotics, such as VSL#3 probiotic mixture, *E. coli* Nissle and *L. rhamnosus* GG, can also strengthen the mucosal barrier function in mice and *in vitro* models (Johnson-Henry et al., 2008; Madsen et al., 2001; Ukena et al., 2007). Strengthening of the mucosal barrier function may be due to normalization of gut permeability (Isolauri et al., 1993a; Isolauri et al., 1993b), regeneration of epithelial cells (Banasaz et al., 2002) and strengthening of tight junctions (Zyrek et al., 2007), as shown for *L. rhamnosus* GG in rat model and for *E. coli* Nissle in *in vitro* model.

Probiotics are likely to mediate their health effects through the gut mucosal immune system and thereby modulate innate and adaptive immune responses (Schiffrin and Blum, 2002). Probiotics are recognised by TLRs (Foligne et al., 2007; Miettinen et al., 1998; Vinderola et al., 2005) in the gut epithelial cells and/or antigen-presenting cells and they thus induce a cascade of immunological events. Probiotics, such as *Lactobacillus* and *Bifidobacterium* strains, may therefore modulate inflammatory processes through epithelial cells as shown in an *in vitro* model (O'Hara et al., 2006) and through underlying professional antigen-presenting cells, such as macrophages and dendritic cells. It has been shown that macrophages in the Peyer's patches of mice can ingest lactobacilli in a strain dependent manner (Sun et al., 2007). Probiotics may then further modulate the cytokine expression patterns of dendritic cells and macrophages, as shown by *L. rhamnosus* GG strain in an *in vitro* model (Miettinen et al., 2000; Veckman et al., 2003; Veckman et al., 2004). These probiotic-primed antigen-presenting cells can in turn modulate the differentiation of naïve T cells into different T cell populations depending on the types of cytokines produced, as in the case of different *Lactobacillus* strains (Braat et al., 2004; Smits et al., 2005). Cytotoxic T cells and Th1 cells promote the cell-mediated immune response and induce phagocytosis. *Lactobacillus* and *Bifidobacterium* strains

have increased phagocytosis (Arunachalam et al., 2000; Olivares et al., 2006a; Roessler et al., 2008) as well as the number of cytotoxic T cells in clinical studies in adults (de Vrese et al., 2005b; Meyer et al., 2006). In allergic children, the induction of Th1-type immune response and low-grade inflammation by *L. rhamnosus* GG has been proposed as an action mechanism for the prevention of atopic diseases (Marschan et al., 2008; Viljanen et al., 2005b). Th2 cells promote antibody-mediated immune response and probiotics have been shown to modulate this response. *Bifidobacterium* and *Lactobacillus* strains have regulated the synthesis of IgA by mucosal lymphoid cells (Leblanc et al., 2004; Park et al., 2002). *L. rhamnosus* GG has increased antibody-secreting cells in patients with Crohn's disease (Malin et al., 1996) and in children (Kaila et al., 1992; Majamaa et al., 1995) and also faecal IgA in allergic (Viljanen et al., 2005a) and Bifidobacteria in healthy children (Fukushima et al., 1998). Moreover, a combination of *L. rhamnosus* GG and *B. animalis* ssp. *lactis* Bb12 has increased the IgA-secreting cells in infants (Rautava et al., 2006). *L. rhamnosus* GG and *B. animalis* ssp. *lactis* Bb12 have also modulated the formation of regulatory T cells in a murine model (Feleszko et al., 2007) which in turn control inflammatory and immune responses. Probiotics can also modulate the production of inflammatory mediators other than cytokines. Induction of low-level synthesis of NO may be involved in the protective actions of *Lactobacillus* strains in the gastrointestinal tract as shown in rat and *in vitro* models (Korhonen et al., 2001; Sobko et al., 2006). Additionally, probiotics may modulate the functional responses of mast cells from degranulation and histamine production to cytokine production. Supplementation with *L. paracasei* has attenuated the onset of atopic dermatitis-like symptoms, accompanied by less mast cell infiltration and lower plasma IgE levels in a mice model (Wakabayashi et al., 2008). Milk fermentation products of *L. helveticus* have increased the number of mucosal mast cells and goblet cells in a mice model, which could mean an improved state of mucosal surveillance at sites of infection (Vinderola et al., 2007). Events regulating host-probiotic cross-talk in the gut and intestinal mucosa is presented in Figure 2.

> *In summary, probiotics are likely to mediate their health effects through the gut mucosal immune system and thereby modulate innate and adaptive immune responses. Probiotics can also strengthen non-immune defence systems. The mechanisms of action of probiotics have been mainly investigated in experimental studies. Some studies performed in different disease groups, such as in allergy, have addressed the action mechanisms of probiotics.*
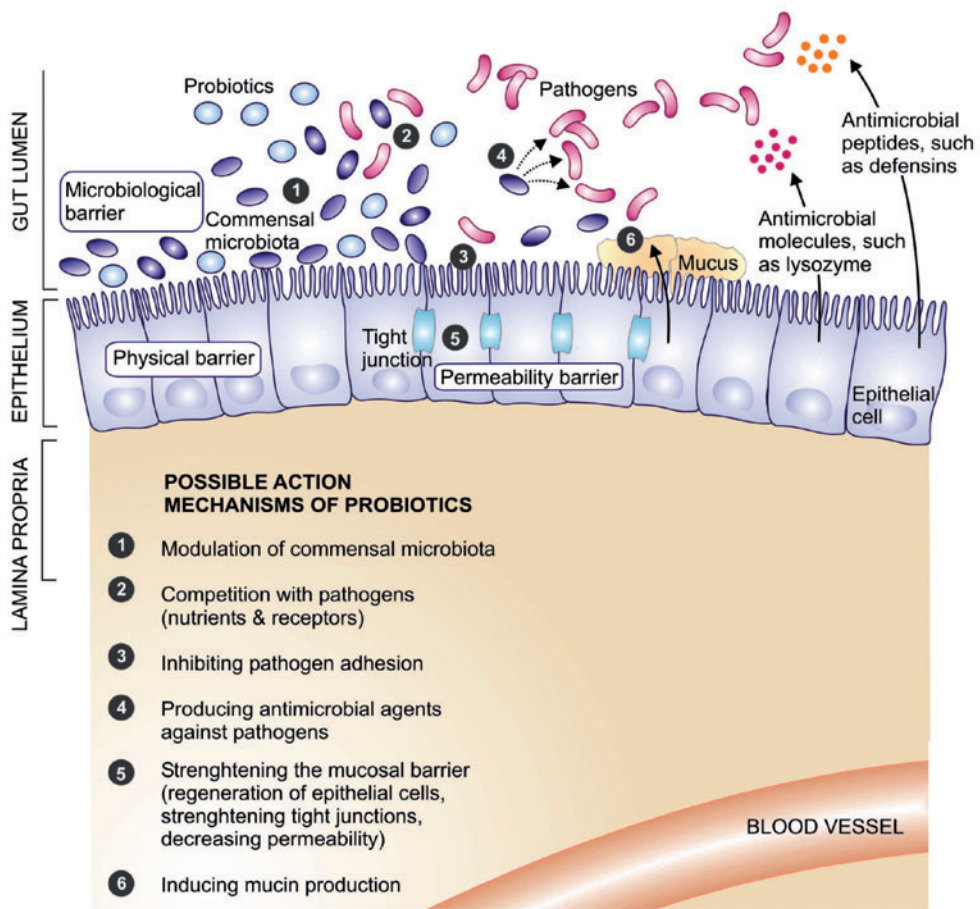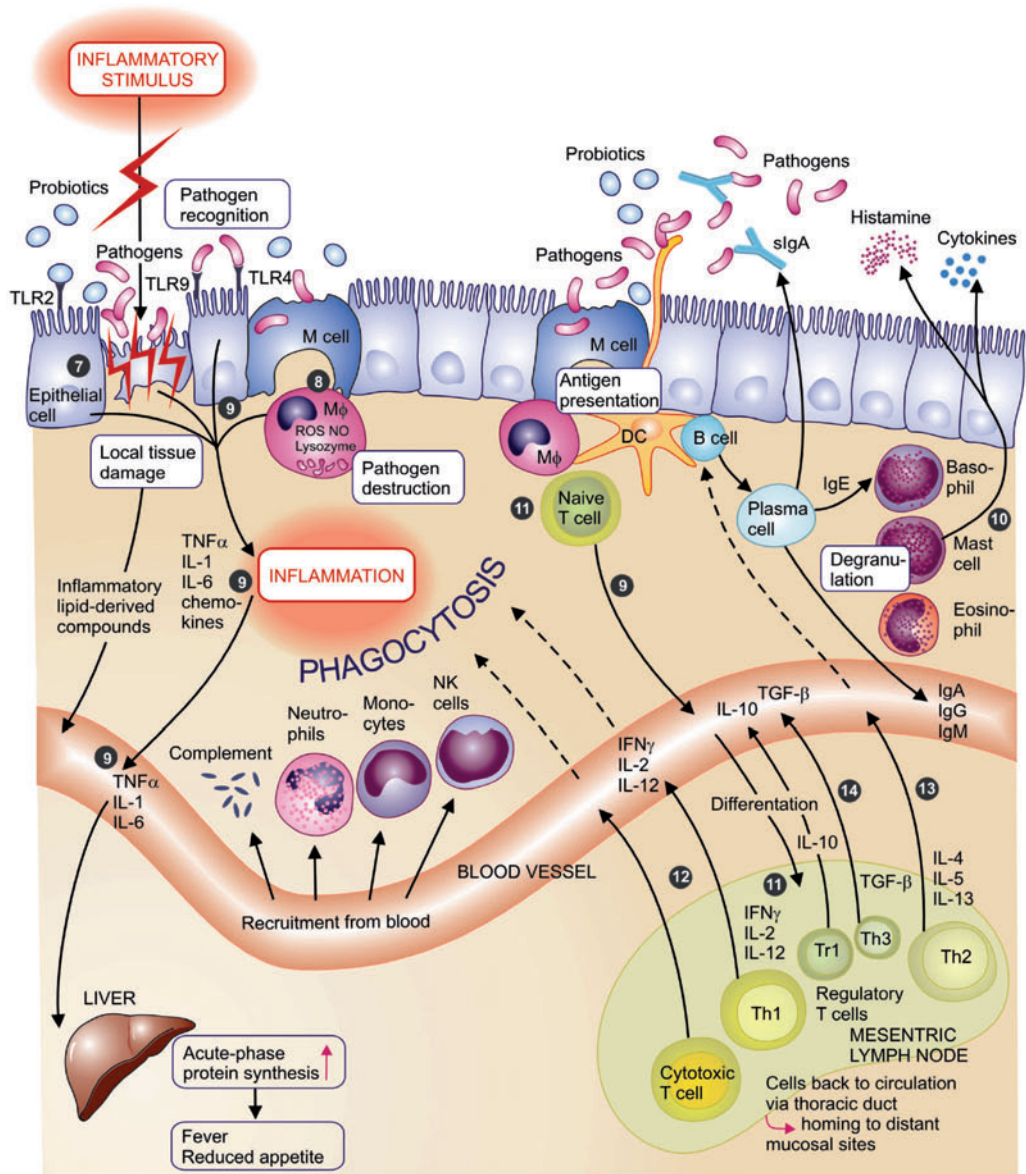
Figure 2. A schematic presentation of the host-probiotic cross-talk in the intestinal mucosa and the possible action mechanisms of probiotics.

INFLAMMATORY STIMULUS

Probiotics

Pathogen recognition

Pathogens

TLR2   TLR9   TLR4

M cell

Epithelial cell

Local tissue damage

Mφ ROS NO Lysozyme

Pathogen destruction

Probiotics   Pathogens

Pathogens   sIgA   Histamine   Cytokines

M cell

Antigen presentation

Mφ   DC   B cell

IgE

Plasma cell

Degranu-lation

Baso-phil

Mast cell

Eosino-phil

Inflammatory lipid-derived compounds

TNFα IL-1 IL-6 chemo-kines

INFLAMMATION

Naive T cell

PHAGOCYTOSIS

TNFα IL-1 IL-6

Complement

Neutro-phils   Mono-cytes   NK cells

IL-10   TGF-β

IgA IgG IgM

IFNγ IL-2 IL-12

Differentiation

BLOOD VESSEL

Recruitment from blood

IL-10

IFNγ IL-2 IL-12

TGF-β

IL-4 IL-5 IL-13

Th1   Tr1   Th3   Th2

Regulatory T cells

Cytotoxic T cell

MESENTRIC LYMPH NODE

LIVER

Acute-phase protein synthesis ↑

Fever Reduced appetite

Cells back to circulation via thoracic duct homing to distant mucosal sites

7 Modulating cytokine response from epithelial cells

8 Inducing NO production

9 Modulating inflammatory processes through epithelial & immune cells and decreasing proinflammatory cytokine production

10 Modulating the degranulation of mast cells (from histamine to cytokine production)

11 Modulating the differentiation of CD4+ naive T cells into

12 Cytotoxic and Th1 cells and promoting cell-mediated immune response

13 Th2 cells and promoting antibody-mediated immune response

14 Regulatory T cells and balancing immune function by inducing IL-10 and TGF-β production

2.2.3 Immunomodulatory properties in human primary cell cultures

In vitro studies are studies in which isolated cells are exposed directly to different stimulants, such as probiotics or pathogens, in a culture. This allows the investigation of immunomodulatory properties of different agents in highly controlled experimental conditions. Peripheral blood mononuclear cells (PBMC) have been used widely for screening of the ability of probiotics to induce cytokine production. A summary of the studies of probiotic-induced cytokine production in PBMC is presented in Table 3. Probiotics in PBMC cultures are generally characterized by the induction of TNF-α; practically all strains are able to induce TNF-α (see Table 3). Most studied strains are *Lactobacillus* and *Bifidobacterium* strains.

In general, *Bifidobacterium* strains have been very good inducers of anti-inflammatory IL-10 (Drouault-Holowacz et al., 2006; Foligne et al., 2007; Gackowska et al., 2006; Helwig et al., 2006; Lammers et al., 2003; Medina et al., 2007; Niers et al., 2005; O'Mahony et al., 2006; Timmerman et al., 2007), although *B. breve* have also induced high concentrations of proinflammatory cytokines (Timmerman et al., 2007). Many *Lactobacillus* strains, *L. casei* (Niers et al., 2005; Shida et al., 2006b; Timmerman et al., 2007), *L. plantarum* (Helwig et al., 2006; Niers et al., 2005) and *L. rhamnosus* (Foligne et al., 2007; Hessle et al., 1999; Miettinen et al., 1998), have been good inducers of IL-10 as well. It has been found that *L. rhamnosus* strains are weaker IL-10 inducers than *Bifidobacterium* strains (Helwig et al., 2006; Timmerman et al., 2007). *L. delbrueckii* (Castanheira et al., 2007) and *L. acidophilus* (Drouault-Holowacz et al., 2006; Foligne et al., 2007) strains have been weak inducers of anti-inflammatory IL-10.

On the whole, *Lactobacillus* strains have been potent inducers of Th1-type cytokines. *L. rhamnosus* (Miettinen et al., 1998; Pochard et al., 2002), *L. plantarum* (Niers et al., 2005; Pochard et al., 2002) and *L. acidophilus* (Drouault-Holowacz et al., 2006; Foligne et al., 2007; Gackowska et al., 2006) strains have induced IFN-γ. *L. rhamnosus* (Miettinen et al., 1998; Pochard et al., 2002), *L. paracasei* (Castellazzi et al., 2007; Hessle et al., 1999), *L. plantarum* (Foligne et al., 2007) and *L. acidophilus* (Drouault-Holowacz et al., 2006; Foligne et al., 2007; Gackowska et al., 2006) strains have been good inducers of IL-12. On the contrary, *L. delbrueckii* strains have decreased IL-12 and IFN-γ production (Castanheira et al., 2007) and induced IL-1β production (Lammers et al., 2003). In addition, *L. plantarum* strains have induced proinflammatory IL-6 (Niers et al., 2005) and IL-1β (Helwig et al., 2006; Lammers et al., 2003; Niers et al., 2005). The data on *L. paracasei* (Drouault-Holowacz et al., 2006; Timmerman et al., 2007), *L. plantarum* (Drouault-Holowacz et al., 2006; Timmerman et al., 2007), *L. acidophilus* (Helwig et al., 2006; Lammers et al., 2003; Timmerman et al., 2007) and *L. delbrueckii* (Helwig et al., 2006) strains is conflicting as some studies have found them as weak inducers of any cytokines.

Other strains are much less studied. *E. coli* has induced IL-10 and IL-1β production (Helwig et al., 2006). *Lc. lactis* strains have been investigated only in five studies (Foligne et al., 2007; Miettinen et al., 1998; Niers et al., 2005; Pochard et al., 2002; Timmerman et al., 2007). In these studies, *Lc. lactis* induced IL-10 production (Timmerman et al., 2007) whereas in the other studies *Lc. lactis* strains were found potent inducers of IL-12 (Foligne et al., 2007; Niers et al., 2005) and IFN-γ (Pochard et al., 2002). *Streptococcus* strains have only been examined in two studies; it has induced IL-10 and IL-1β (Lammers et al., 2003) or remained quite inert (Timmerman et al., 2007). Only two studies have addressed the effect of probiotic combinations on cytokine production, and these studies have indicated similar or lower induction of cytokines compared to single strains (Castellazzi et al., 2007; Drouault-Holowacz et al., 2006).

Human monocytes, macrophages and dendritic cells have also been used for screening of cytokine production, although less extensively than PBMC. Virtually no data exists on human primary NK cells. In general terms, Gram-positive bacteria have been shown to be better inducers of IL-12 than Gram-negative bacteria in human monocytes (Hessle et al., 2000). *L. rhamnosus* GG has mainly induced proinflammatory cytokines in macrophages (Miettinen et al., 2000; Veckman et al., 2003). Cytokine responses in human dendritic cells have remained quite weak in *Lactobacillus*, *Bifidobacterium* and *Lactococcus* strains (Niers et al., 2007; Veckman et al., 2004).

> *Taken together, PBMC cultures have been used extensively to screen the induction of cytokines by probiotics. Probiotics in PBMC cultures are generally characterized by the induction of TNF-α, which may be due to the inflammatory response of PBMC to any stimulus. The discrimination between strains is therefore better realized by measuring other cytokines. Lactobacillus and Bifidobacterium are the most studied strains and, in general, Lactobacillus strains are able to induce Th1 cytokines as well as IL-10 whereas Bifidobacterium induce mainly anti-inflammatory IL-10. Other probiotic strains, such as Lactococcus and Streptococcus, have only been investigated in few studies and practically no data exists on the effect of probiotic combinations (probiotic multispecies) compared with single strains (probiotic monostrain).*

Table 3. Effect of probiotics on cytokine production in human peripheral blood mononuclear cells.

Probiotic strains	Main findings	Reference
L. delbrueckii UFV H2b29	-IL-12, IFN-γ, TNF-α ↓ -IL-10 ←→	(Castanheira et al., 2007)
L. paracasei I 1688, *L. salivarius* I 1794 and combination of them	-IL-12, IFN-γ ↑ with single strains and their combination	(Castellazzi et al., 2007)
L. salivarius Ls33, *L. rhamnosus* Lr32, *L. casei* B123, *L. acidophilus* NCFM, *L. acidophilus* IPL908, *L. plantarum* NCIMB8826, *L. plantarum* Lp115, *B. animalis* ssp. *lactis* BL04, *B. animalis* ssp. *lactis* BI07, *B. bifidum* BB02, *Lc. lactis* MG1363, *E. coli* TG1	-all strains TNF-α ↑ -*L. acidophilus* IPL908, *L. salivarius* Ls33, *L. rhamnosus* Lr32, *B. animalis* ssp. *lactis* BL04 and *B. bifidum* BB02 the most potent inducers of IL-10 -*L. plantarum* Lp115, *L. plantarum* NCIMB8826, *B. animalis* ssp. *lactis* BI07, *L. acidophilus* NCFM and *Lc. lactis* MG1363 the most potent inducers of IL-12 -IL-10/IL-12 ratio ↑ in *L. acidophilus* IPL908 and *L. salivarius* Ls33	(Foligne et al., 2007)
B. longum BB536, *B. longum* NCC 2705, *B. longum* W 11, *B. longum* ATCC 15707, *B. longum* BIR 324, *B. longum* BIF 53	-all strains TNF-α ↑ -IL-2 and IFN-γ ↑ by *B. longum* W11 than by other strains -IL-10 ↑ by *B. longum* NCIMB 8809 and BIF53 than by other strains -IL-4 ←→	(Medina et al., 2007)
L. acidophilus, *E. coli* Nissle	-intracellular IFN-γ/IL-4 ratio ↑ in lymphocytes in allergic patients but not in healthy	(Rasche et al., 2007)
L. acidophilus, *L. brevis*, *L. bulgaricus*, *L. casei*, *L. helveticus*, *L. paracasei*, *L. plantarum*, *L. rhamnosus*, *L. salivarius*, *B. bifidum*, *B. breve*, *B. infantis*, *B. lactis*, *B. longum*, *E. faecium*, *Lc. lactis*, *S. thermophilus*	-all strains TNF-α ↑ -*B. bifidum*, *B. infantis*, *L. casei* and *Lc. lactis* the most potent inducers of IL-10 -*B. breve*, *L. brevis*, *L. rhamnosus* and *L. salivarius* did not induce IL-10 -*B. breve*, *L. brevis* and *L. rhamnosus* induced high concentrations of proinflammatory cytokines	(Timmerman et al., 2007)
L. acidophilus LA 201, *L. plantarum* LA 301, *L. salivarius* LA 302, *B. lactis* LA 303, and combination of them	-all strains IL-12 and IL-10 ↑ -*L. salivarius* and *B. lactis* IL-10/IL-12 ratio ↑ -cytokine production of the combination was an average of the single strains	(Drouault-Holowacz et al., 2006)
L. salivarius UCC118, *B. infantis* 35624	-both TNF-α and IL-12 ↑ -IL-10 ↑ by *Bifidobacterium* than by *Lactobacillus*	(O'Mahony et al., 2006)
L. casei Shirota	-TNF-α, IFN-γ, IL-12, IL-10 ↑	(Shida et al., 2006b)

Continued

Table 3. Continued

Probiotic strains	Main findings	Reference
L. acidophilus La-5R, *L. delbrueckii* ssp. *bulgaricus* LbY-27, *B. bifidum* Bb12 and combination of them	-all strains and combination TNF-α and IL-10 ↑ -*L. acidophilus* the strongest IFN-γ and IL-12 inducer -cytokine production by bacterial combinations lower than in single strains	(Gackowska et al., 2006)
B. breve Y8, *B. infantis* Y 1, *B. longum* Y 10, *E. coli* Nissle 1917, *L. acidophilus* MB 443, *L. rhamnosus* GG, *L. casei* MB 451, *L. delbrueckii* ssp. *bulgaricus* MB 453, *L. plantarum* MB 452	-IL-10, IL-1β, TNF-α ↑ by *Bifidobacteria* and *E. coli* more than by *Lactobacillus* -from lactobacilli IL-10, IL-1β, TNF-α ↑ by *L. bulgaricus* and *L. plantarum* more than by *L. casei*,*L. acidophilus* and *L. rhamnosus*	(Helwig et al., 2006)
Genomic DNAs of *L. acidophilus* LA14, *L. delbrueckii* ssp. *bulgaricus* LB31, *L. casei* LC10, *L. plantarum* LPT, *B. infantis* BI07, *B. breve* BSF, *B. longum* BL04, *S. salivarius* ssp. *thermophilus* TA061	- IL-10 ↑ by *Bifidobacterium* and *Streptococcus* more than by *Lactobacillus* - IL-6 ↓ by *Lactobacillus* more than by *Bifidobacterium* and *Streptococcus* -IL-1β ↑ *B. breve*, *Streptococcus*, *L. plantarum* and *L. delbrueckii*	(Lammers et al., 2003)
L. rhamnosus GG, *L. casei* ATCC393, *L. plantarum* NCIMB8826, *Lc. lactis* MG1363	-IL-4 and IL-5 ←→ -all strains IFN-γ ↑ -all strains IL-4 and IL-5 ↓ when stimulated with allergen	(Pochard et al., 2002)
L. johnsonii La 1, *L. sakei* LTH 681	-both TNF-α, IL-12, IFN-γ ↑ -IL-10 ←→	(Haller et al., 2000)
L. rhamnosus 50A, *L. rhamnosus* 7D, *L. plantarum* 52A, *L. plantarum* 67B, *L. paracasei* ssp. *paracasei* 02A, *L. paracasei* ssp. *paracasei* 34D	-IL-12 ↑ by *L. paracasei* more than by *L. plantarum* or *L. rhamnosus* -IL-10 ↑ by *L. rhamnosus* more than by *L. plantarum* or *L. paracasei*	(Hessle et al., 1999)
L. rhamnosus GG, *L. rhamnosus* E509, *L. bulgaricus* E585	-all strains TNF-α, IL-1β, IL-6, IL-18 and IL-10 ↑ -IFN-γ and IL-12 ↑ by *L. rhamnosus* more than by other strains -IL-4 ←→	(Miettinen et al., 1998)
L. rhamnosus GG, *L. rhamnosus* E509, *L. plantarum* E98, *L. acidophilus* E507, *L. paracasei* ssp. *paracasei* E506, *L. paracasei* ssp. *paracasei* E510, *B. longum* E505, *B. animalis* E508, *Lc. lactis* ssp. *cremoris* ARH74, *Lc. lactis* ssp. *lactis* E414	-all strains TNF-α and IL-6 ↑ - *L. rhamnosus* E509, *L. rhamnosus* GG, *B. animalis* E508, *L. acidophilus* E507 were the best inducers of TNF-α -*L. rhamnosus* E509, *L. rhamnosus* GG, *B. animalis* E508 were the best inducers of IL-6 -live bacteria induced cytokine production better than glutaraldehyde-fixed bacteria	(Miettinen et al., 1996)

↑ = increase; ←→ = no induction; ↓ = decrease

2.2.4 Immunomodulatory properties in healthy adults

2.2.4.1 Assessment of the immunomodulatory effects in probiotic interventions

Balanced and regulated immune and inflammatory responses are crucial for health, and diet is one of the major exogenous factors modulating immune responses. Assessing the effect of probiotic intervention on the immune system requires a thorough methodological approach targeting a large spectrum of immunological parameters (for review, see Albers et al., 2005; Calder and Kew, 2002; Cummings et al., 2004). Currently, no single marker is available to predict the outcome of a probiotic intervention on immune function and resistance to infection or to other immune system-related diseases. Since nutrition influences various components of inflammation, it may, together with several bioactive substances, such as probiotics, have a role in alleviating inflammatory conditions. Moreover, it may be used as a preventive therapy against different infections.

It is possible to assess the basal status of the immune system by measuring number of circulating leukocytes, lymphocyte subtypes, as well as the levels of immunoglobulins, cytokines and acute-phase proteins. However, it must be kept in mind that for example only 2% of lymphocytes at a given time are found in the circulation. In animal studies, it is also possible to asses the size of lymphoid organs and to collect and measure the function of immune cells from various organs. In humans, it is usually only possible to take blood, saliva, urine and faecal samples and in some cases biopsies of the gut.

Especially in the case of probiotics, gut-associated markers of immune function are important. The integrity of the mucosal barrier can be assessed by the sugar permeability test. Secretory IgA in saliva can reflect the mucosal immune function. Measuring different inflammation markers, such as cytokines or calprotectin, from faeces may reflect possible inflammation in the gut. Mucosal histology and for example gene expression can be studied from biopsy specimens obtained from the gut.

The activity and functional capacity of several immune system components can be studied in controlled conditions outside the body (*ex vivo*). *Ex vivo* markers of immune function provide information on the activity of specific immune cells reflecting the capacity of either innate or adaptive immune responses. Examples of *ex vivo* measures include phagocytosis of neutrophils and monocytes, oxidative burst i.e. superoxide generation of neutrophils and monocytes, NK cell activity, lymphocyte proliferation and production of cytokines by peripheral blood mononuclear cells, monocytes or lymphocytes. The clinical relevance of changes in *ex vivo* markers is not clear, but they can provide important mechanistic information.

In vivo markers of immune function are best measured under controlled challenge conditions, such as vaccination (vaccine-specific antibodies and vaccine-specific antibody-forming cells) or intradermal application of an antigen (delayed-type hypersensitivity). With all of the different measures of immune function, it is worth remembering that normal ranges have only been established for a few of them, such as circulating cell numbers, immunoglobulins and CRP, but there are no normal ranges for instance for immune cell *ex vivo* functional responses.

Finally, the clinical outcome i.e. the incidence, severity and duration of infection can be measured by questionnaires or diagnoses made by physician. The symptoms of infection, number of antibiotic prescriptions and absence from work or day care can also be measured as additional factors. The summary of markers of immunomodulation is presented in Table 4.

> *In conclusion, to gain a comprehensive understanding of the immune function, it would be advisable to use a combination of measures of basal status of the immune system, gut-associated and ex vivo markers reflecting the functional capacity of cells of innate and adaptive immunity as well as clinical endpoints.*

Table 4. Summary of markers of immunomodulation (adapted from Albers et al., 2005; Calder and Kew, 2002; Cummings et al., 2004).

| Basal markers | Gut-associated | Ex vivo | | In vivo | Clinical outcome/ questionnaires |
		Innate immune response	Adaptive immune response		
Cell counts (differential leukocyte count, lymphocyte subpopulations)*	Secretory IgA in saliva/ faeces	Oxidative burst activity of neutrophils/ monocytes	Lymphocyte proliferation	Response to vaccination (vaccine-specific antibodies in serum, vaccine-specific antibody-forming cells)	Incidence, severity and duration of the infection
Circulating immunoglobulins	Mucosal histology (biopsy)	NK cell activity	Lymphocyte antibody production following stimulation	Delayed-type hypersensitivity	Symptoms of the infection
Circulating cytokines**	Integrity of the mucosal barrier (sugar permeability test, bacterial permeability, serum endotoxins)	Cytokine production by PBMC, whole blood or monocytes following stimulation	Lymphocyte cytokine production following stimulation		Absence from work/school/ daycare
Circulating acute-phase proteins such as CRP***	Intestinal inflammation (faecal calprotectin, cytokines)	Surface expression of activation markers involved in antigen presentation by antigen-presenting cells	Lymphocyte activation (surface expression of activation markers)		Days with use of antibiotics
Complement activity		Chemotactic response of neutrophils/ monocytes	Cytotoxic T cell activity		
Size of lymphoid organs		Eicosanoid production by neutrophils/ monocytes			
		Phagocytosis of neutrophils/ monocytes			

* Facilitates interpretation of functional *ex vivo* markers
** Might be useful to measure under specific stress situations, such as exercise and fat-rich meals, known to cause an increase in proinflammatory cytokines
*** Not a marker of immune function but reflects the presence of inflammation or infection

2.2.4.2 Immunomodulatory effects of probiotics

The immunomodulatory effects of probiotics in healthy adults have not been extensively studied. The problem in the performed clinical studies is that in many cases they were not properly randomized and controlled. Only 15 studies in adults have been conducted in randomized, double-blind and placebo-controlled (RDBPC) clinical settings. Two RDBPC trials have been done in healthy children and one in the elderly. A summary of the RDBPC clinical studies on the effect of probiotics on different aspects of immunomodulation (basal status of the immune system, gut-associated and *ex vivo* markers of immune function) is presented in Table 5 and a summary of the clinical studies performed in other clinical settings is presented in Table 6. A summary of the studies on the effect of probiotics on *in vivo* markers of immune function is presented in Table 7.

Basal status of the immune system

In healthy adults, probiotics (different *Lactobacillus* and *Bifidobacterium* strains, for details see Table 5 and 6) have usually had no effect on the number of leukocytes or lymphocyte subpopulations (de Vrese et al., 2005b; de Vrese et al., 2006; Klein et al., 2007; Lorea Baroja et al., 2007; Meyer et al., 2006; Olivares et al., 2006a; Olivares et al., 2006b; Parra et al., 2004a; Parra et al., 2004b; Roessler et al., 2008; Schiffrin et al., 1997; Spanhaak et al., 1998; Takeda and Okumura, 2007; Tiollier et al., 2007). However, a combination of *L. gasseri* PA16/8, *B. longum* SP07/3 and *B. bifidum* MF20/5 (de Vrese et al., 2005b; de Vrese et al., 2006) and *L. casei* DN114001 (Meyer et al., 2006) has been reported to increase the number of cytotoxic T cells and a combination of *L. gasseri* CECT 5714 and *L. coryniformis* CECT 5711 has increased the number of NK cells (Olivares et al., 2006a; Olivares et al., 2006b). A combination of *L. gasseri* CECT 5714 and *L. coryniformis* CECT 5711 has also increased serum IgA (Olivares et al., 2006a), whereas others reported no effects on serum immunoglobulins by interventions with *L. casei* Shirota, *L. casei* DN114001 or a combination of *B. animalis* ssp. *lactis* Bb12 and *L. paracasei* ssp. *paracasei* CRL-431 (Christensen et al., 2006; Marcos et al., 2004; Pujol et al., 2000; Spanhaak et al., 1998). *L. casei* Shirota has had no effects on complement factors (Spanhaak et al., 1998), but *L. rhamnosus* GG has had differential effects on complement receptors in healthy and allergic adults; in healthy adults, the expression of complement receptors increased whereas it was down-regulated in allergic adults (Pelto et al., 1998). *L. casei* DN114001, *L. casei* Shirota, *L. fermentum* VRI003 or a combination of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 have had no effects on serum cytokine levels (Cox et al., 2008; Lorea Baroja et al., 2007; Pujol et al., 2000; Takeda and Okumura, 2007), but a probiotic combination of *L. gasseri* and *L. coryniformis* has increased the IL-4 and IL-10 levels (Olivares et al., 2006a) and *L. acidophilus* has increased the IFN-γ level in saliva (Clancy et al., 2006).

The effect of probiotics on acute-phase proteins has not been studied in healthy adults. Previously, the effect of probiotics on CRP has only been examined in immunocompromised patients (Anderson et al., 2004; McNaught et al., 2005; McNaught et al., 2002; Reddy et al., 2007; Sugawara et al., 2006), allergic children (Viljanen et al., 2005b) and patients suffering from rheumatoid arthritis (Hatakka et al., 2003) and ulcerative colitis (Furrie et al., 2005). In immunocompromised patients, a combination of *L. casei* Shirota, *B. breve* Yakult and prebiotic galactooligosaccharides (Sugawara et al., 2006) and in patients with ulcerative colitis *B. longum* (Furrie et al., 2005) have reduced serum CRP levels and also resulted in improvement in the overall clinical appearance of chronic inflammation (Furrie et al., 2005). In contrast to the above-mentioned studies, *L. rhamnosus* GG has increased serum CRP levels compared with placebo in infants with IgE-associated atopic eczema dermatitis syndrome (Viljanen et al., 2005b). However, *L. rhamnosus* GG had no effect on serum CRP levels in patients with rheumatoid arthritis (Hatakka et al., 2003). It is of interest that a combination of four probiotic bacteria (*L. rhamnosus* GG, *L. rhamnosus* Lc705, *B. breve* Bb99 and *P. freudenreichii* ssp. *shermanii* JS) had no effect on sensitive CRP levels in blood (Viljanen et al., 2005b) in the same clinical setting with the allergic children. Furthermore, neither *L. plantarum* 299V (McNaught et al., 2005; McNaught et al., 2002) nor a combination of *L. acidophilus* La5, *B. animalis* ssp. *lactis* Bb12, *S. thermophilus* and *L. bulgaricus* (Anderson et al., 2004; Reddy et al., 2007) changed serum CRP concentrations in immunocompromised patients undergoing surgical procedures. It would seem that the effect of probiotics on CRP as an example of the acute-phase proteins is controversial in different diseases and no data on the effects in healthy adult population is available.

Gut-associated markers of immune function

Not many studies assessing the effect on gut-associated markers, such as salivary IgA or gut permeability, have been performed in healthy adults, and the results are quite conflicting. There are indications that *L. casei* DN114001 might prevent the decrease of salivary IgA in heavily training men (Tiollier et al., 2007), while *L. fermentum* VRI003 had no effect on salivary IgA in another study in elite male distance runners (Cox et al., 2008). *L. rhamnosus* GG has been reported to decrease the non-steroidal anti-inflammatory drug (NSAID)-induced gastric permeability (Gotteland et al., 2001). Only two studies have examined the effects of probiotic intervention on gut mucosa from biopsies. In duodenal mucosa biopsies, *L. rhamnosus* GG affected mainly genes involved in immune response and inflammation (TGF-β, TNF family members, cytokines, nitric oxide synthase 1, defensin alpha1), apoptosis, cell growth and cell differentiation (cyclins, caspases, oncogenes), cell signalling (ICAMs and integrins), cell adhesion (cadherins), signal transcription and transduction (Di Caro et al., 2005).

L. reuteri has induced the numbers of duodenal B lymphocytes and ileal CD4+ T lymphocytes and decreased the number of mucosal histiocytes in gastric mucosa (Valeur et al., 2004).

Ex vivo markers of immune function

Quite many studies have investigated the effects of probiotics on the *ex vivo* functional responses of specific immune cells, especially the activity of the cells of innate immunity, in healthy adults. It seems that probiotics (different *Lactobacillus* and *Bifidobacterium* strains, for details see Table 5 and 6) are able to increase the phagocytic activity of macrophages and granulocytes (Arunachalam et al., 2000; Chiang et al., 2000; Donnet-Hughes et al., 1999; Klein et al., 2007; Olivares et al., 2006a; Olivares et al., 2006b; Roessler et al., 2008; Schiffrin et al., 1997; Sheih et al., 2001; Takeda and Okumura, 2007), although there are also studies in which no effects have been found (Christensen et al., 2006; de Vrese et al., 2005b; de Vrese et al., 2006). *L. casei* DN114001 intervention has been reported to increase the oxidative burst activity (Parra et al., 2004a; Parra et al., 2004b) and NK cell activity (Parra et al., 2004b). A combination of *L. gasseri* PA16/8, *B. longum* SP07/3 and *B. bifidum* MF20/5 has had no effects on T cell activation (de Vrese et al., 2005b; de Vrese et al., 2006).

The effect of probiotics on the *ex vivo* cytokine production by immune cells has been investigated only in a few studies. A combination of *B. animalis* ssp. *lactis* Bb12 and *L. paracasei* ssp. *paracasei* CRL-431 has had no effects on *ex vivo* cytokine production by whole blood cells in young healthy adults (Christensen et al., 2006), while B. *lactis* HN019 has increased *ex vivo* IFN-α production by PBMC in healthy elderly (Arunachalam et al., 2000). *L. casei* DN114001 has had no effect on *ex vivo* cytokine production by PBMC (Marcos et al., 2004), but *L. rhamnosus* GG has decreased the production of proinflammatory cytokines and increased anti-inflammatory cytokines production by PBMC (Schultz et al., 2003). In another trial, *L. rhamnosus* GG decreased the *ex vivo* cytokine production by PBMC both in healthy adults and patients with Crohn's disease (Braat et al., 2004).

Table 5. Effect of probiotics on basal status, gut-associated and *ex vivo* markers of immune function in randomized, double-blind, placebo-controlled clinical settings in healthy adults, elderly and children. Statistically significant difference is indicated with bold font.

Subjects	Design and duration	Probiotic strains	Main findings	Reference
Healthy adults, n=122	R, DB, PC 14 d	Combination of *L. gasseri* PA16/8, *B. longum* SP07/3, *B. bifidum* MF20/5 and vitamins +minerals or placebo	**-cytotoxic T cells ↑** -lymphocytes, T lymphocytes, B lymphocytes, T helper cells, NK cells ←→ -phagocytic activity of macrophages and granulocytes ←→ -T cell activation ←→	(de Vrese et al., 2005b)
Healthy adults, n=122	R, DB, PC 14 d	Combination of *L. gasseri* PA16/8, *B. longum* SP07/3, *B. bifidum* MF20/5 and vitamins +minerals or placebo	**-cytotoxic T cells ↑** -lymphocytes, T lymphocytes, B lymphocytes, T helper cells, NK cells ←→ -phagocytic activity of macrophages and granulocytes ←→ -T cell activation ←→	(de Vrese et al., 2006)
Healthy adults, n=30	R, DB, PC 1 mo	Combination of *L. gasseri* CECT 5714 and *L. coryniformis* CECT 5711 or placebo	**-NK cells ↑** -lymphocytes, T cells, cytotoxic T cells, T helper cells, B cells, memory T cells, suppressor T cells ←→ **-serum IgA ↑** -serum IgG, IgE ←→ **-serum IL-10, IL-4 ↑** -serum TNF-α, IL-12 ←→ **- phagocytic activity of macrophages and granulocytes ↑ (both groups)**	(Olivares et al., 2006a)
Healthy adults, n=26	R, DB, PC, C-O 1 mo	Combination of *L. acidophilus* 74-2 and *B. animalis* ssp. *lactis* DGCC 420 or placebo	-lymphocytes, monocytes, granulocytes, T cells, B cells, T helper cells, cytotoxic T cells, NK cells, activated T cells ←→ **-phagocytic activity of granulocytes and monocytes ↑** -oxidative burst activity of monocytes and granulocytes ←→	(Klein et al., 2007)
Healthy adults, n=15 Adults with atopic dermatitis, n=15	R, DB, PC 2 mo	Combination of *L. paracasei* Lpc-37, *L.acidophilus* 74-2 and *B. animalis* ssp. *lactis* DGCC 420	**-natural killer cell marker CD57+ ↑ (healthy)** **- CD4+ T lymphocytes expressing CD54+ (ICAM-1) ↓ (atopic)** -major leukocyte subsets, lymphocytes, T cells, B cells, cytotoxic T cells, NK cells, activated T cells, suppressor/cytotoxic T cells bearing CD57+ and CD54 (ICAM-1) ←→ **- phagocytic activity of monocytes and granulocytes ↑ (healthy)**	(Roessler et al., 2008)
Healthy middle aged adults, n=45	R, DB, PC 2 mo	*L. casei* DN114001 or placebo	-white blood cell count, T cells, activated T cells, B cells, T helper cells, cytotoxic T cells, NK cells ←→ **-oxidative burst capacity of monocytes↑** **-NK cells tumoricidal activity ↑**	(Parra et al., 2004b)

Table 5. Continued

Subjects	Design and duration	Probiotic strains	Main findings	Reference
Healthy middle-aged adults, n=45	R, DB, PC 2 mo	*L. casei* DN114001 or placebo	-white blood cell count, T cells, activated T cells, B cells, T helper cells, cytotoxic T cells, NK cells ←→ **-oxidative burst capacity of monocytes and granulocytes ↑**	(Parra et al., 2004a)
Healthy men in French Commando training, n=47	R, DB, PC 1 mo	*L. casei* DN114001 or placebo	-leukocytes, leukocyte subsets, T cells, T helper cells, cytotoxic T cells, NK cells ←→ **-salivary IgA ↓ in placebo but not in probiotic group**	(Tiollier et al., 2007)
Healthy elite male distance runners, n=20	R, DB, PC, C-O 1 mo	*L. fermentum* VRI003 or placebo	-serum IL-4, IL-12 ←→ -tendency for ↑ IFN-γ in whole blood culture -salivary IgA ←→	(Cox et al., 2008)
Adults (heavy smokers), n=36	R, DB, PC 1,5 mo	*L. plantarum* 299v or placebo	-production of intracellular reactive oxygen species by monocytes ←→ **-plasma F_2-isoprostane ↓** **-plasma IL-6 ↓**	(Naruszewicz et al., 2002)
Young healthy adults, n=71	R, DB, PC 3 wk (dose–response)	A mixture of *B. animalis* ssp. *lactis* Bb-12 and *L. paracasei* ssp. *paracasei* CRL-431	-faecal IgA concentrations ←→ -serum IgA, IgM, IgG ←→ -phagocytic activity in blood leukocytes ←→ -IFN-γ and IL-10 production from *in vitro* stimulated whole blood ←→	(Christensen et al., 2006)
Healthy milk-tolerant adults n=9 Milk- hypersensitive adults n=8	R, DB, PC, C-O 2 wk (1 wk wash-out)	*L rhamnosus* GG or placebo	**-in healthy expression of complement receptors on neutrophils and monocytes ↑** **-in milk-hypersensitive prevented ↑ of complement receptor expression on neutrophils and monocytes**	(Pelto et al., 1998)
Healthy children, n=154	R, DB, PC 1 mo	*L. rhamnosus* GG or placebo	-intestinal integrity (sugar permeability test) ←→	(Galpin et al., 2005)
Healthy elderly, n=25	R, DB, PC 1,5 mo	*B. lactis* HN019 or placebo	**-phagocytic activity ↑** **-*ex vivo* production of IFN-α ↑ by PBMC**	(Arunachalam et al., 2000)

R = randomized; DB = double-blind; PC = placebo-controlled; C-O = cross-over
↑ = increase; ←→ = no induction; ↓ = decrease

Continued

Table 6. Effects of probiotics on basal status, gut-associated and *ex vivo* markers of immune function in other clinical settings in healthy adults and children. Statistically significant difference is indicated with bold font.

Subjects	Design and duration	Probiotic strains	Main findings	Reference
Healthy males, n=6	R, PC 1 mo	*L. rhamnosus* GG or placebo	-in biopsies from duodenal mucosa LGG affected mainly **genes involved in immune response and inflammation** (TGF-β, TNF family members, cytokines, nitric oxide synthase 1, defensin alpha1), apoptosis, cell growth and cell differentiation (cyclins, caspases, oncogenes), cell signaling (ICAMs and integrins), cell adhesion (cadherins) signal transcription and transduction	(Di Caro et al., 2005)
Healthy adults, n=10	O 1 mo	*L. rhamnosus* GG	**-TNF-α, IL-6, IFN-γ secretion by peripheral blood cells ↓** **-IL-4, IL-10 secretion by peripheral blood cells ↑** **-helper T cell activation ↑**	(Schultz et al., 2003)
Healthy adults, n=6 Patients with Crohn's disease, n=6	O 2 wk	*L. rhamnosus* GG	**-In healthy adults *ex vivo* production of IL-2, IL-4 and IL-10 by T cells ↓** **-In Crohn's disease patients production of IL-2, IFN-γ, IL-10 by T cells ↓** -TGF-β production ←→	(Braat et al., 2004)
Healthy adults, n=16	R 5 d	NSAID or NSAID with *L. rhamnosus* GG	**-strengthening of gastric mucosal barrier (sugar permeability test) ↑** -strengthening of intestinal mucosal barrier (sugar permeability test) ←→	Gotteland et al., 2001)
Healthy adults, n=20 Patients with inflammatory bowel disease (IBD), n=20	O 1 mo	Combination of *L. rhamnosus* GR-1 and *L. reuteri* RC-14	**-Treg cells (CD4+ CD25^{high}) ↑ (IBD)** **-TNF-α and IL-12-producing monocytes and dendritic cells ↓ (IBD)** **-serum IL-12 ↓ (IBD)** -T cell surface activation markers -*ex vivo* proliferative responses of PBMC ←→	(Lorea Baroja et al., 2007)
Healthy, n=10 Ileostomy patients, n=9	O 1 mo (biopsy before and after supplementation)	*L. reuteri*	**-gastric mucosal histiocyte numbers ↓** **-duodenal B lymphocytes ↑** **-ileal CD4+ T lymphocytes ↑**	(Valeur et al., 2004)

Continued

Table 6. Continued

Subjects	Design and duration	Probiotic strains	Main findings	Reference
Healthy students, n=155	R, C 1,5 mo (under academic examination stress)	*L. casei* DN114001 milk or milk	**-NK cells ↑** -T cells, T helper cells, cytotoxic T cells, B cells ←→ -leukocytes, granulocytes, monocytes, lymphocytes ←→ -serum IgA, IgM, IgG ←→ -phagocytosis and oxidative burst of leukocytes ←→ -IL-2, IFN-γ, TNF-α, IL-4, IL-5, IL-10 cytokine production by PBMC ←→	(Marcos et al., 2004)
Young healthy women, n=33	R, C 2 wk 100g + 2 wk 200g	*L. casei* DN114001 yoghurt or conventional yoghurt	**-cytotoxic T cells ↑** -leukocytes, granulocytes, monocytes, lymphocytes, T lymphocytes, regulatory T cells, T helper cells, NK cells ←→	(Meyer et al., 2006)
Healthy athletes having low NK cell numbers selected (from 99 athletes), n=25	O, C 1 mo, ergometer stress test in the end of study periods	*L. casei* DN114001 or milk	-numbers of T cells, T helper cells, B cells, NK cells ←→ -serum IgA, IgM, IgG ←→ -serum IL-1β, sIL-2r, IL-6, IL-2, IFN-γ ←→ -NK cell cytotoxic activity ←→	(Pujol et al., 2000)
Healthy males, n=20	R, PC 1 mo	*L. casei* Shirota or placebo	-T cells, T helper cells, cytotoxic T cells, NK cells and B cells ←→ -serum IgM, IgG, IgA, IgD and IgE ←→ -complement factors C3, C4 and factor B ←→ -NK cell activity ←→ -phagocytosis and oxidative burst of neutrophils ←→ -IL-1β, IL-2, IFN-γ production by PBMC ←→	(Spanhaak et al., 1998)
Healthy adults, n=41 Healthy children, n=36	SB, C-O 1 mo	*L. casei* or combination of *L. casei*, *L. acidophilus* LA8F, *B. animalis* ssp. *lactis* BLi8F, *B. breve* BBr8F and *B. breve* BBl8F	**-secretory IgA in saliva ↑ (children) (ns between groups)** **-NK cell activity ↑ (adults) (ns between groups)**	(Zanini et al., 2007)
Healthy adults, n=50	R 3 wk	*B. lactis* HN019 in milk or *B. lactis* HN019 in lactose-hydrolyzed milk	**-polymorphonuclear cells phagocytosis ↑ (lactose-hydrolyzed milk)** **-NK cell tumour killing activity ↑ (lactose-hydrolyzed milk)**	(Chiang et al., 2000)
Healthy elderly, n=27	R 3 wk	*L. rhamnosus* HN001 or *B. lactis* HN019	**-NK cell activity ↑ both probiotics**	(Gill et al., 2001a)

Continued

Table 6. Continued

Subjects	Design and duration	Probiotic strains	Main findings	Reference
Healthy elderly, n=30	R 3 wk	*B. lactis* HN019 5×10^{10} or *B. lactis* HN019 5×10^{9}	**-NK cell activity ↑ (both doses)** **-T cells, T helper cells, activated T lymphocytes, NK cells ↑ (both doses)** -cytotoxic T cells, B cells ←→ **-phagocytic activity of leukocytes ↑ (both doses)**	(Gill et al., 2001b)
Healthy adults, n=28	R 3 wk	*L. acidophilus* La1 or *B. bifidum* Bb12	-lymphocytes, T cells, activated T cells, B cells, T helper cells, cytotoxic T cells, NK cells ←→ **-leukocyte phagocytic activity ↑ (both probiotics)**	(Schiffrin et al., 1997)
Healthy adults, n=42	R, O 3 wk	*S. thermophilus*, *L. johnsonii* La1 10^{6} cfu/ml or *L. johnsonii* La1 10^{7} cfu/ml	**-phagocytic activity of peripheral blood cells and of monocytes and granulocytes ↑ (*L. johnsonii* La1 10^{7} cfu/ml)** **-leukocyte respiratory burst activity ↑ (*L. johnsonii* La1 10^{7} cfu/ml)**	(Donnet-Hughes et al., 1999)
Healthy athletes, n=18 Fatigued athletes, n=9	O 1 mo	*L. acidophilus* LAFTI®L10	**-salivary IFN-γ ↑ (healthy)** **-secretion of IFN-γ by helper T cells from whole blood culture ↑ (fatigued)** -secretion of IL-4, IL-12 by helper T cells from whole blood culture ←→ -salivary IgA ←→	(Clancy et al., 2006)
Healthy adults, n=30	R, PC 2 wk supplementation after 2 wk deprivation diet of fermented food	Combination of *L. gasseri* CECT 5714 and *L. coryniformis* CECT 5711 or placebo	**-NK cells ↑** -lymphocytes, T cells, cytotoxic T lymphocytes, T helper cells, B cells, memory T cells, activated T cells ←→ **- phagocytic activity of monocytes and granulocytes ↑ (both groups)**	(Olivares et al., 2006b)

R = randomized; PC = placebo-controlled; SB = single-blind; C = controlled; O = open C-O = cross-over

↑ = increase; ←→ = no change; ↓ = decrease

In vivo markers of immune function

The effect of probiotics on the *in vivo* markers of immune function (response to vaccines, delayed type hypersensitivity and response to attenuated pathogens) in healthy population has not been extensively studied (Table 7). There are indications that probiotics may increase the vaccine-specific antibodies after vaccinations. *L. rhamnosus* GG has been reported to increase vaccine-specific antibodies after oral polio vaccine in adults (de Vrese et al., 2005a) and after oral rotavirus vaccine in children (Isolauri et al., 1995), whereas *L. fermentum* ECT5716 has increased vaccine-specific antibodies after intramuscular anti-influenza vaccine in adults (Olivares et al., 2007). *L. rhamnosus* GG or *Lc. lactis* intervention had no effects on *Salmonella typhi* specific antibodies after oral *S. typhi* vaccination (He et al., 2000), but a combination of *B. animalis* ssp. *lactis* Bb12 and *L. acidophilus* LA-1 increased *S. typhi* specific IgA after administration of attenuated *S. typhi* bacteria (Link-Amster et al., 1994).

> *Taken together, not many randomized, double-blind, placebo-controlled studies have addressed the immunomodulatory effects of probiotics in healthy adults. There are indications that probiotics could affect the functional responses of immune cells, especially the activity of the cells of innate immunity. There are also indications that probiotics could be used as adjuvants for vaccines. The effect of probiotics on CRP in healthy adults has not been studied previously. It is clear that immunomodulatory effects are strain-specific and therefore each probiotic monostrain as well as probiotic multispecies should be evaluated individually.*

Table 7. Effect of probiotics on *in vivo* markers of immune function in healthy adults and children. Statistically significant difference is indicated with bold font.

Subjects	Design and duration	Probiotic strains	Main findings	Reference
Healthy males, n=43	R, DB, PC 5 wk (oral polio vaccine)	*L. rhamnosus* GG or placebo	**-poliovirus neutralizing antibody titer ↑** **-serum poliovirus-specific IgA ↑**	(de Vrese et al., 2005a)
Healthy adults, n=30	R, DB, PC 1 wk (oral *Salmonella typhi* vaccine)	*L. rhamnosus* GG, *Lc. lactis* or placebo	-IgA, IgG, IgM-secreting cells ↔ **-complement receptor CR3 expression ↑ (*L. lactis*)** -IgG receptors on neutrophils and monocytes ↔	(He et al., 2000)
Healthy infants, n=57	R, DB, PC 5 d (oral rotavirus vaccine)	*L. rhamnosus* GG or placebo	**-number of rotavirus-specific IgM-secreting cells ↑** **-trend towards ↑ IgA**	(Isolauri et al., 1995)
Healthy adults, n=50	R, DB, PC 1 mo + 5 mo follow-up (intramuscular anti-influenza vaccine)	*L. fermentum* ECT5716 or placebo	-T cells, cytotoxic T cells, T helper cells, B cells, memory T cells ↔ **-NK cells ↑** **-serum TNF-α ↑** -serum IFN-γ, IL-12, IL-10 ↔ **-serum anti-influenza–specific IgA ↑** -serum anti-influenza–specific IgG, IgM ↔ -serum IgA, IgG ↔ **-serum IgM ↑**	(Olivares et al., 2007)
Healthy adults, n=30	R, C 3 wk (attenuated *Salmonella typhi* administration)	Combination of *B. lactis* ssp. *animalis* Bb12 and *L. acidophilus* LA-1 or control without fermented foods	**-serum *S. typhi* specific IgA ↑** **-serum IgA ↑**	(Link-Amster et al., 1994)
Healthy males, n=20	R, PC 1 mo	*L. casei* Shirota or placebo	-delayed type hypersensitivity ↔	(Spanhaak et al., 1998)

2.2.4.3 Effect of probiotics on clinical outcome

Respiratory infections

There is increasing evidence that probiotics may provide protection against respiratory tract infections and common colds. The effect of probiotics in prevention of respiratory infections has been investigated in seven studies in healthy adults, two in the elderly and in four studies in children. In adults, *L. fermentum* CETC5716 have reduced the incidence of respiratory infection (Olivares et al., 2007), and *L. fermentum* VRI003 the number of days with respiratory symptoms (Cox et al., 2008), while in other studies there was no reduction in the number of respiratory infections by a combination of *L. gasseri* PA16/8, *B. longum* SP07/3 and *B. bifidum* MF20/5 or by *L. casei* DN114001 (de Vrese et al., 2005b; de Vrese et al., 2006; Tiollier et al., 2007; Winkler et al., 2005). However, a combination of *L. gasseri* PA16/8, *B. longum* SP07/3 and *B. bifidum* MF20/5 has reduced the duration (de Vrese et al., 2005b; de Vrese et al., 2006) or symptoms (de Vrese et al., 2005b; Winkler et al., 2005) of respiratory infection and *L. reuteri* has reduced the absence from work in adults (Tubelius et al., 2005). In the elderly, *L. casei* DN114001 intervention decreased the incidence of infection but had no effect on the duration of infection (Turchet et al., 2003) while a combination of *L. rhamnosus* GG, *L. rhamnosus* Lc705, *B. breve* Bb99 and *P. freudenreichii* ssp. *shermanii* JS was ineffective in prevention of respiratory infections in the elderly (Hatakka, 2007). In children, *L. rhamnosus* GG, *L. casei* DN114001 and a combination of *L. rhamnosus* GG, *L. rhamnosus* Lc705, *B. breve* Bb99 and *P. freudenreichii* ssp. *shermanii* JS reduced the incidence of respiratory infections (Cobo Sanz et al., 2006; Hatakka, 2007; Hatakka et al., 2001). Treatment with *L. rhamnosus* GG or a combination of *L. reuteri* SD112 and B. *lactis* Bb12 has resulted in fewer days of absence from day care due to illness (Hatakka et al., 2001; Weizman et al., 2005). A summary of the studies on probiotics in preventing respiratory infections is presented in Table 8.

Table 8. Effects of probiotics on prevention of respiratory infections in clinical settings in healthy adults, elderly and children. Statistically significant difference is indicated with bold font.

Subjects	Design and duration	Probiotic strains	Main findings	Reference
Adults				
Healthy adults, n=262	R, DB, PC 2,5 mo	*L. reuteri* or placebo	**-sick-leave ↓** -duration of sick-leave ←→	(Tubelius et al., 2005)
Healthy adults, n=477	R, DB, PC 3-5,5 mo	Combination of *L. gasseri* PA16/8, *B. longum* SP07/3, *B. bifidum* MF20/5 and vitamins+minerals or placebo	-incidence and duration of respiratory infection ←→ **-days with fever ↓**	(Winkler et al., 2005)
Healthy adults, n=479	R, DB, PC 3 mo	Combination of *L. gasseri* PA16/8, *B. longum* SP07/3, *B. bifidum* MF20/5 and vitamins+minerals or placebo	-incidence of respiratory infection ←→ **-duration of respiratory infection ↓** -symptom score ←→ **-days with fewer ↓** **-bronchial symptoms ↓**	(de Vrese et al., 2005b)
Healthy adults, n=479	R, DB, PC 3-5 mo	Combination of *L. gasseri* PA16/8, *B. longum* SP07/3, *B. bifidum* MF20/5 and vitamins+minerals or placebo	-incidence of respiratory infection ←→ **-duration of respiratory infection ↓** -symptom score ←→ **-bronchial symptoms ↓**	(de Vrese et al., 2006)
Healthy adults, n=50	R, DB, PC 1 mo + 5 mo follow-up (intramuscular anti-influenza vaccine)	*L. fermentum* CECT5716 or placebo	**- number of respiratory infections ↓**	(Olivares et al., 2007)
Healthy elite male distance runners, n=20	R, DB, PC, C-O 1 mo	*L. fermentum* VRI 003 or placebo	-incidence of respiratory infections ←→ **-number of days with respiratory symptoms ↓** -severity of symptoms ←→	(Cox et al., 2008)
Healthy men in French Commando training, n=47	R, DB, PC 1 mo	*L. casei* DN114001 or placebo	-incidence of respiratory infection ←→ **-proportion of rhinopharyngitis ↑** -symptoms of infection ←→	(Tiollier et al., 2007)
Elderly				
Healthy elderly, n=260	R, C 3 wk	*L. casei* DN114001 and control group	-incidence of respiratory infection ←→ **-duration of respiratory infection ↓**	(Turchet et al., 2003)
Institutionalized elderly, n=265	R, DB, PC 5 mo	Combination of *L. rhamnosus* GG, *L. rhamnosus* Lc705, *B. breve* Bb99, *P. freudenreichii* ssp. *shermanii* JS or placebo	-incidence of respiratory infection ←→ **-duration of respiratory infection ↑**	(Hatakka, 2007)

Table 8. Continued

Subjects	Design and duration	Probiotic strains	Main findings	Reference
Children				
Healthy day care children, n=571	R, DB, PC 7 mo	*L. rhamnosus* GG or placebo	**-respiratory infections ↓** **-absence from day care due to illness ↓** **-antibiotic treatments ↓**	(Hatakka et al., 2001)
Healthy, otitis-prone children, n=309	R, DB, PC 6 mo	Combination of *L. rhamnosus* GG, *L. rhamnosus* Lc705, *B. breve* Bb99, *P. freudenreichii* ssp. *shermanii* JS or placebo	-occurrence or duration of acute otitis media ←→ **-occurrence of recurrent respiratory infections ↓**	(Hatakka, 2007)
Healthy infants, n=201	R, DB, PC 3 mo	*L. reuteri* SD 112 or *B. lactis* Bb12 or placebo	-respiratory infections ←→ **-absence from day care due to illness ↓ (*L. reuteri*)** **-antibiotic treatments ↓ (*L. reuteri*)**	(Weizman et al., 2005)
Healthy school children, n=251	R, DB, PC 5 mo	*L. casei* DN114001 or placebo	-incidence and duration of respiratory infection or GI infection ←→ **-incidence of lower respiratory infection and fatigue ↓**	(Cobo Sanz et al., 2006)

R = randomized; DB = double-blind; PC = placebo-controlled; C-O = cross-over; C = controlled
↑ = increase; ←→ = no change; ↑ = decrease

Continued

Gastrointestinal symptoms and infections

Probiotics have been most extensively studied in the treatment of diarrhoeal diseases, where their efficacy can be considered well established. Probiotics, especially *Lactobacillus* and *Bifidobacterium* strains, have prevented and reduced the duration of infectious, hospital-acquired diarrhoea (for review, see Guandalini, 2006; Sazawal et al., 2006; Szajewska et al., 2001), traveller's diarrhoea (for review, see McFarland, 2007; Sazawal et al., 2006) and antibiotic-associated diarrhoea (for review, see Sazawal et al., 2006). The effect of probiotics on community-acquired diarrhoea has not been extensively studied. Only one study in healthy adults, two in the elderly and six studies in children have been conducted. Of these, only two interventions, one with *L. casei* DN114001 and the other with a combination of *L. reuteri* SD112 and B. *lactis* Bb12 were effective in decreasing the number of diarrhoea in children (Pedone et al., 2000; Weizman et al., 2005). There is more evidence that probiotics could decrease the duration of community-acquired diarrhoea. In children, *L. casei* DN114001, B. *lactis* Bb12 and a combination of *L. reuteri* SD112 and B. *lactis* Bb12 (Chouraqui et al., 2004; Pedone et al., 1999; Weizman et al., 2005) and *L. casei* DN114001 in the elderly (Turchet et al., 2003) have decreased the duration of diarrhoea. A summary of the studies in the prevention of community-acquired diarrhoea by probiotics is presented in Table 9.

> *To summarize, mainly Lactobacillus and Bifidobacterium strains or combinations thereof have been used for studying the preventive effects of probiotics on respiratory infections. There are indications that probiotics could reduce especially the duration of respiratory infections in healthy population. Virtually no data on the prevention of community-acquired diarrhoea or gastrointestinal symptoms in healthy adults is available, but probiotics have strain-specifically reduced the duration or incidence of community-acquired diarrhoea in healthy children.*

Table 9. Effect of probiotics on prevention of community-acquired diarrhea and gastrointestinal infections in clinical settings in healthy adults, the elderly and children. Statistically significant difference is indicated with bold font.

Subjects	Design and duration	Probiotic strains	Main findings	Reference
Adults				
Men in military camps, n=502	R, PC 2 mo	*L. casei* DN114001 or placebo	-incidence and duration of diarrhoea ←→	(Pereg et al., 2005)
Elderly				
Healthy elderly, n=260	R, C 3 wk	*L. casei* DN114001 and control group	-incidence of GI infection ←→ **-duration of GI infection ↓**	(Turchet et al., 2003)
Institutionalized elderly, n=265	R, DB, PC 5 mo	Combination of *L. rhamnosus* GG, *L. rhamnosus* Lc705, *B. breve* Bb99, *P. freudenreichii* ssp. *shermanii* JS or placebo	-incidence of GI infection ←→ -duration of GI infection ←→	(Hatakka, 2007)
Children				
Healthy infants and children, n=928	R, DB, PC 4 mo	*L. casei* DN114001 or placebo	**-incidence of diarrhoea ↓**	(Pedone et al., 2000)
Healthy infants and children, n=287	R, PC 19 mo	*L. casei* DN-114001 or placebo	-incidence of diarrhoea ←→ **-duration of diarrhoea ↓**	(Pedone et al., 1999)
Healthy children, n=154	R, DB, PC 1 mo	*L. rhamnosus* GG or placebo	-incidence of diarrhoea ←→	(Galpin et al., 2005)
Healthy infants, n=201	R, DB, PC 3 mo	*L. reuteri* SD 112 or *B. lactis* Bb12 or placebo	**-episodes and duration of diarrhea in probiotic groups ↓**	(Weizman et al., 2005)
Healthy infants, n=90	R, DB, PC 4,5 mo	*B. lactis* Bb12 or placebo	-incidence of diarrhoea ←→ **-duration of diarrhoea ↓**	(Chouraqui et al., 2004)
Healthy infants, n=971	R, DB, PC 5 mo	Combination of *B. breve* and *S. thermophilus* or placebo	-incidence and duration of diarrhoea ←→	(Thibault et al., 2004)

2.2.5 Safety of probiotics

The possible concerns regarding the safety of probiotics relate to the occurrence of disease, such as bacteraemia or endocarditis, toxic or metabolic effects on the gastrointestinal tract and the transfer of antibiotic resistance in the gastrointestinal flora (for review, see Snydman, 2008). Although there are rare cases of bacteraemia or fungemia related to the use of probiotics, epidemiologic evidence suggests no population increase in risk on the basis of usage data (for review, see Snydman, 2008). For example, increased use of probiotic *L. rhamnosus* GG in Finland has not lead to increase in *Lactobacillus* bacteraemia (Salminen et al., 2002). *L. rhamnosus* GG has also been given to immunocompromised patients in several clinical studies (e.g. preterm and newborn infants, patients with IBD, HIV, cancer, organ transplants) without reported adverse effects or any case of bacteraemia. Therefore, *Lactobacillus*, *Bifidobacterium* and *Lactococcus* strains have generally been regarded as safe (for review, see Hammerman et al., 2006; Snydman, 2008). Also the European Food Safety Authority (EFSA) has concluded that there are no specific safety concerns regarding *Lactobacilllus* and *Bifidobacterium* or *Propionibacterium* strains as they have a long history of safe use in food. However, it should be taken into consideration that the safety of probiotics has not been as systematically investigated as in drugs and the safety evaluation is partly based on long term experience only.

3. Aims of the study

Probiotics have been mostly examined in the prevention and treatment of different gastrointestinal diseases and allergies. The ways in which probiotic bacteria elicit their health effects are not fully understood. One of the most probable mechanisms is the immunomodulatory effect mediated by the gut mucosal immune system on the systemic level. Probiotic products are mainly consumed by healthy people but their effects on the immune system are poorly understood. The aim of this series of studies was to evaluate the ability of probiotics to modulate the immune system in healthy adults in randomized, double-blind, placebo-controlled clinical studies as well as in *in vitro* studies.

The specific aims were:

1. To compare the ability of potentially probiotic bacteria to induce cytokine responses in primary cell culture using human peripheral blood mononuclear cells (I).
2. To evaluate whether probiotic intervention has immunomodulatory effects in healthy adults (II, IV).
3. To characterize the effect of probiotics on global serum lipidomics profiles in healthy adults (III).
4. To investigate whether probiotics are able to reduce the incidence of respiratory infections and gastrointestinal symptoms in healthy adults (V).

4. Materials and methods

4.1 BACTERIAL STRAINS (I)

Eleven potentially probiotic strains or known probiotics used in the study were obtained from Valio Research Centre (Helsinki, Finland). Two pathogenic bacterial strains, used as controls in the study, were obtained from the collection of the National Public Health Institute (Helsinki, Finland). The bacteria were stored in skimmed milk at -70°C and passaged three times (except *Bifibobacterium* strains which were passaged four times) prior to use in stimulation experiments. For the stimulation experiments, bacteria were grown to logarithmic growth phase, and the number of bacteria was determined by counting in a Petroff-Hauser counting chamber. Bacterial strains used in this study are presented in Table 10.

4.2 PRIMARY CELL CULTURE USING PERIPHERAL BLOOD MONONU- CLEAR CELLS

4.2.1 *In vitro* (I)

In the study I, PBMC were isolated by a Ficoll-Paque density gradient (Amersham-Pharmacia Biotech, Uppsala, Sweden) from freshly collected, leukocyte-rich buffy coats obtained from healthy blood donors (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland) (Pirhonen et al., 1999). After washing, the cells were resuspended in RPMI-1640 medium (Sigma, St. Louis, Mo., USA) containing 10% heat-inactivated foetal calf serum (FCS) with low-endotoxin concentration of 0.7 EU/ml (Integro, Zaandam, Holland) and supplemented with 2 mmol/l L-glutamine (Sigma), 100k U/ml penicillin and 100 mg/ml streptomycin (Gibco BRL, Paisley, Scotland). Previously, FCS from different producers has been tested to evaluate their endotoxin level on cytokine induction and Integro's FCS was selected as it causes the lowest background in cytokine analyses. In bacterial stimulation experiments, purified leukocytes (2×10^6 cells/ml) were incubated with bacteria in a final volume of 1 ml in 24-well plates (Nunc, Roskilde, Denmark) in 5% CO_2 at 37°C in RPMI-1640 medium containing 100 ml/l FCS. Live bacteria were added into the cell culture to obtain a 10:1 bacteria:host cell ratio. RPMI was used as a negative control. When PBMC were stimulated with a combination of two bacteria or more, equal numbers of different bacteria were used, and the sum bacterial

Table 10. Bacteria used in the study.

Strain	Abbreviation used in the study	ATCC or DSM number	Culture media	Growth conditions	Use
Probiotic					
Streptococcus thermophilus THS	THS	-	M17 agar[1] with lactose[2] and M17 broth[3]	37°C, aerobic	Yoghurt
Lactobacillus rhamnosus GG	LGG	ATCC 53103	de Man, Rogosa and Sharpe (MRS) medium[1]	37°C, aerobic	Probiotic
Lactobacillus rhamnosus Lc705	Lc705	DSM 7061	MRS medium[1]	37°C, aerobic	Probiotic
Lactobacillus helveticus 1129	1129	DSM 13137	MRS medium[1]	42°C, aerobic	Cheese, fermented milk
Lactobacillus helveticus Lb 161	161	-	MRS medium[1]	42°C, aerobic	Cheese
Bifidobacterium longum 1/10	1/10	-	MRS medium[1] with cysteine[4]	37°C, anaerobic	None
Bifidobacterium animalis ssp. lactis Bb12	Bb12	DSM 15954	MRS medium[1] with cysteine[4]	37°C, anaerobic	Probiotic
Bifidobacterium breve Bb99	Bb99	DSM 13692	MRS medium[1] with cysteine[4]	37°C, anaerobic	Probiotic
Lactococcus lactis ssp. cremoris ARH74	ARH74	DSM 18891	Calciumcitrate agar[5] and M17-broth[3] with lactose[2]	22°C, aerobic	Finnish viili
Leuconostoc mesenteroides ssp. cremoris PIA2	PIA2	DSM 18892	MRS medium[1]	22°C , aerobic	Finnish viili
Propionibacterium freudenreichii ssp. shermanii JS	PJS	DSM 7067	Propioni-medium[5]	30°C, aerobic	Probiotic, cheese
Pathogens					
Streptococcus pyogenes serotype T1M1	GAS	-	Sheep blood agar6 and tryptone-yeast broth with glucose[7]	37°C, aerobic	-
Escherichia coli (DH5α)	DH5α	-	Luria-medium[8]	37°C, aerobic	-

[1]Lab M, Topley House, Lancashire, UK; [2]J.T. Baker B.V., Deventer, Holland; [3]Difco, Beckton Dickinson, MD, USA; [4]Merck, Darmstadt, Germany; [5]Valio Ltd, Helsinki, Finland; [6]Oxoid, Ogdensburg, NY, USA; [7](Holm and Falsen, 1967); [8]National Public Health Institute, Helsinki, Finland

dose of the combinations was a 10:1 bacteria:host cell ratio. Cell culture supernatants were collected from individual donor cell cultures and stored at 20°C before analysis. A schematic illustration of the cell culture is presented in Figure 3.

4.2.2 *Ex vivo* (II)

In study II, PBMCs were isolated from healthy adults before and after the probiotic intervention as described in the previous chapter. In stimulation experiments, purified leukocytes (2×10^6 cells/ml) were incubated with or without stimulants for 24 h. PBMC were left unstimulated or were stimulated with one of three different stimulants; a Gram-positive bacterium, a chemical stimulant or a virus. Live Group A streptococci *S. pyogenes* serotype T1M1 was used as a Gram-positive bacterium at 1:1 bacteria:host-cell ratio; lipopolysaccharide (LPS) from *E. coli* serotype 0111:B4 (L-3024, Sigma) was used as a chemical stimulant at a concentration of 100 ng/ml; and Influenza A H_3N_2 virus (A/Beijing/353/89) was used to infect cells at a multiplicity of infection of 5. Cell culture supernatants were collected individually at the 24-hour time point and stored at -20°C before analysis.
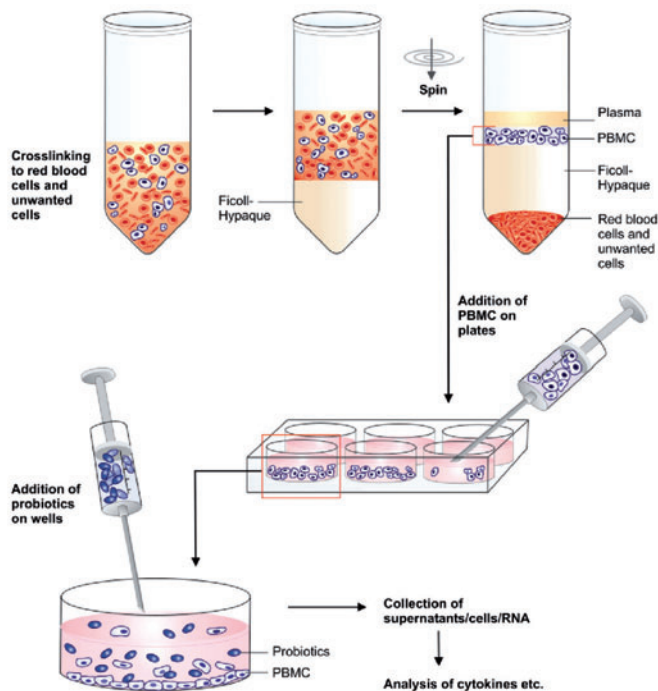
Figure 3. Schematic illustration of the primary cell culture using peripheral blood mononucealr cells (PBMC).

4.3 Subjects and study designs (II, III, IV, V)

Studies II and III
The subjects of studies II and III were healthy adults (n=62). Only the LGG and placebo groups of study II were used in study III. The study was a randomised, double-blind, placebo-controlled parallel group intervention study carried out in September-January. The intervention period was preceded by a three-week run-in period, after which the subjects were randomized to receive either a LGG, Bb12, PJS or placebo drink for three weeks (Figure 4). After the intervention period, the subjects were followed up for another three weeks. Venous blood samples and saliva samples were taken at baseline, day 1, 7 and 21 and three weeks after the intervention. Faecal samples were collected at home at baseline and at the end of the three-week intervention period. The subjects were asked to fill in a structured symptom diary throughout the whole study.

Studies IV and V
The subjects of the studies IV and V were healthy, exercising adults who participated in the Helsinki City Marathon at the end of the intervention. Studies IV and V were conducted as a randomized, double-blind, placebo-controlled parallel group intervention studies. The studies were carried out in spring and summer. Prior to the intervention period there was a four-week run-in period in April, after which the subjects were randomized and stratified according to their personal best marathon time to receive either LGG or placebo (Figure 4). The subjects received LGG or placebo for three months until the day of the Helsinki City Marathon. The marathon was followed by a four-week follow-up period. Venous blood samples were taken at the baseline, after the three-month intervention period and on the day of the marathon immediately before and after the race. The subjects were asked to fill in a structured symptom diary and exercise diary throughout the entire study.

A summary of the subjects and interventions is presented in Table 11.

STUDY II and III

LGG group
Bb12 group
PJS group
Placebo group

Run-in period 3 wk

Follow-up period 3 wk

= blood sample
= saliva sample
= fecal sample
X = study diary

0 d 1 d   7 d   21 d   42 d
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

STUDY IV and V

Marathon race

LGG group

Placebo group

Run-in period 1 mo

Follow-up period 1 mo

0 mo   3 mo   Before race After race
                              (1 wk before
                              marathon race)

XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

= blood sample
X = study diary

Figure 4. Schematic presentation of the study designs.

Table 11. A summary of the subjects and interventions.

Study	Subjects	Number of subjects	Gender Female/ Male	Age, y Mean (Range)	BMI, kg/m² Mean (Range)	Intervention [1]	Duration of intervention	Main endpoints
II	Healthy adults	62	45/17	44 (23-58)	24 (18-30)	Milk-based fruit drink (2.5 dl) with LGG (1.6 x 10¹¹cfu) or Bb12 (3.5 x 10¹¹cfu) or PJS (3.3 x 10¹¹ cfu) or without probiotics	3 wk	Serum CRP, cytokines produced by cultured PBMC, serum cytokines
III	Healthy adults	28	15/11	42 (23-55)	24 (18-30)	Milk-based fruit drink (2.5 dl) with LGG (1.6 x 10¹¹cfu) or without probiotics	3 wk	Serum global lipidomics platform
IV	Exercising adults	141	16/125	40 (23-69)	22 (18-26)	Milk-based fruit drink (2 x 65 ml) with LGG (3.9 x 10¹⁰ cfu) or without probiotics	3 mo	Serum CRP, serum cytokines, lymphocyte subtypes
V	Exercising adults	141	16/125	40 (23-69)	22 (18-26)	Milk-based fruit drink (2 x 65 ml) with LGG (3.9 x 10¹⁰ cfu) or without probiotics	3 mo	Number of healthy days, occurrence and duration of respiratory infections and GI-symptoms

[1] Study products were obtained from Valio Research Centre, Helsinki, Finland. The appearance and taste of the study products were identical.

wk = week, mo = month; LGG = *Lactobacillus rhamnosus* GG; Bb12 = *Bifidobacterium animalis* ssp. *lactis* Bb12; PJS = *Propionibacterium freudenreichii* ssp. *shermanii* JS

4.4 Immunological and biochemical measurements

The immunological and other biochemical parameters determined in the present study are summarized in Tables 12 and 13. Detailed descriptions of the measurements are available in the original publications. The classification of cytokines into pro/anti-inflammatory, Th1/Th2/Treg and chemokines is presented in Table 14.

Table 12. Immunological measurements carried out in the study.

Variable	Method	Manufacturer/Reference	Study
Acute-phase proteins			
Serum highly sensitive CRP	Immunoturbidimetric assay by Tina-quant CRP high-sensitive assay reagent (Roche Hitachi 912 analyzer)	Roche Diagnostics GmbH, Mannheim, Germany	II, IV
Immune cells			
White blood cell counts (leukocytes, monocytes, neutrophils, basophils and lymphocytes)	Electronic counter (Coulter MAXM Hematology Analyzer)	Beckman Coulter, Fullerton, CA, USA	II, IV
Lymphocyte subpopulations: T cells, Th cells, cytotoxic T cells, B lymphocytes, NK cells	Flow cytometry (FACStar flow cytometer)	BD Biosciences, San Jose, CA, USA	IV
Immunoglobulins			
Serum immunoglobulins (IgA, IgG and IgM)	Immunoturbidimetric method with Tina-quant Roche/Hitachi System reagent (Roche Hitachi 912 analyzer)	Roche Diagnostics GmbH, Mannheim, Germany	II
Saliva secretory IgA	ELISA	Gentaur, Brussels, Belgium	II
Cytokines			
Gene expression of TNF-α, IL-12p40, IL-12p35, IFN-γ and IL-10	Northern Blotting	(Miettinen et al., 1998)	I
In vitro production of cytokines in PBMC culture (TNF-α, IL-10, IFN-γ, IL-12p70)	ELISA	BD Pharmingen, San Diego, CA, USA (TNF-α, IL-10) BioSite, Täby, Sweden (IFN-γ, IL-12p70)	I
Ex vivo production of cytokines in PBMC culture (TNF-α, IFN-γ, IL-1β, Il-2, IL-4, IL-5, IL-6, IL-8, IL-10 and IL-12p70)	Multiplex Fluorescent Bead Immunoassay (FlowCytomix human Th1/Th2 10plex kit II)	Bender MedSystems, Vienna, Austria	II
Serum cytokine levels (TNF-α, IL-6, IFN-γ and IL-10)	ELISA (Quantikine high sensitive immunoassays)	R&D Systems, Minneapolis, MN, USA	II, IV
Serum chemokine levels MCP-1 (CCL2) and IP-10 (CXCL10)	ELISA	BD Pharmingen, San Diego, CA, USA	IV

Table 13. Biochemical measurements carried out in the study.

Variable	Method	Manufacturer/Reference	Study
Haematology			
Erythrocytes, haemoglobin, haematocrit, MCV, MCH, MCHC, thrombocytes	Electronic counter (Coulter MAXM hematology analyzer)	Beckman Coulter, Fullerton, CA, USA	V
Serum lipids			
Total cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides	Autoanalyser (Roche/Hitachi 912 Automatic Analyzer)	Roche Diagnostics GmbH, Mannheim, Germany	III
Serum low-density lipoprotein (LDL) cholesterol	Calculation using Friedewald's equation	(Friedewald et al., 1972)	III
Hormones			
Serum cortisol	Luminoimmunoassay (VITROS ECi analyser)	Ortho-Clinical Diagnostics, Amersham, UK	IV
Serum prolactin	Immunometric assay	Diagnostic Products Corporation, Los Angeles, CA, USA	IV
Serum testosterone	Electrochemiluminescence immunoassay	Testosterone, Roche Diagnostics GmbH, Mannheim, Germany	IV

Table 14. Classification of the cytokines measured in the study.

Cytokine	Pro-inflammatory	Anti-inflammatory	Th1	Th2	Treg	Chemokine
TNF-α	x					
IL-1β	x					
IL-6	x			x		
IFN-γ	x		x			
IL-2			x			
IL-12			x			
IL-4				x		
IL-5				x		
IL-10		x			x	
CCL2	x					x
CXCL8	x					x
CXCL10	x					x

4.5 Global serum lipidomics profiling (III)

In the study III in healthy adults the global lipidomics platform based on Ultra Performance Liquid Chromatography (UPLC) coupled to high resolution mass spectrometry (MS) was utilized. The applied platform affords broad screening of multiple lipid classes from total lipid extracts within a single sample run (major monoacylglycerols and phospholipids, diacylglycerols and diacylglycero-phospholipids, sphingolipids, triacylglycerols and cholesterol esters). The lipid profiling was carried out on Waters Q-Tof Premier mass spectrometer. Data were processed using MZmine software version 0.60 (Katajamaa et al., 2006). Lipids were identified using internal spectral library or with tandem mass spectrometry (Yetukuri et al., 2007). The normalization of lipidomics data was performed as follows: all monoacyl lipids except cholesterol esters, such as monoacylglycerols and monoacylglycero-phospholipids were normalized with GPCho(17:0/0:0), all diacyl lipids except ethanolamine phospholipids were normalized with GPCho(17:0/17:0), ceramides with Cer(d18:1/17:0), the diacyl ethanolamine phospholipids were normalized with GPEtn(17:0/17:0), and the triacylglycerols and cholesterol esters with TG(17:0/17:0/17:0). Other (unidentified) molecular species were calibrated with GPCho(17:0/0:0) for retention time < 300 seconds, GPCho(17:0/17:0) for retention time between 300s and 410s, and TG(17:0/17:0/17:0) for higher retention times. Lipids from the lipidomic analysis were named according to Lipid Maps (http://www.lipidmaps.org).

4.6 Questionnaires (II, V)

Symptom diary
The subjects of studies II and V were asked to fill in a symptom diary with ready-made questions throughout the study. Each day the subjects rated their health status (healthy or sick) and recorded whether they had had any upper respiratory tract infection symptoms (fever, rhinitis, sore throat, cough, wheezing, earache), gastrointestinal symptoms (diarrhoea, vomiting, stomach ache) or any other symptoms or medication during the day. Subjects were considered to have a respiratory tract infection if they suffered from any respiratory tract infection symptoms for at least two consecutive days and if the next respiratory tract infection symptoms appeared only after three or more days; otherwise they were considered to be suffering from the same respiratory tract infection. The criterion for an episode of gastrointestinal symptoms was a duration of at least one day and at least three days before the next gastrointestinal symptom appeared. In the study V in exercising adults, the incidence and duration of respiratory tract

infections and gastrointestinal symptom episodes were the main outcome measures while the purpose of the study diary in the study II in healthy adults was to monitor that the health status of the subjects remained the same throughout the study and that falling ill would not affect the immunological measurements. The symptom diary was not systematically validated, but it has been used in previous studies by our group.

Exercise diary
In the study II in healthy adults, the study diary included a question about the amount of exercise daily. In studies IV and V, the exercising adults were asked to keep a separate daily exercise diary in which they reported their running distance in kilometres and minutes and other exercise in minutes.

4.7 Compliance to the probiotic interventions

Consumption of probiotic products (II, III, IV, V)
In studies II, III, IV and V, the subjects recorded the consumption of the study products in the study diary daily.

Measuring probiotics from faecal samples (II)
In study II, faecal samples were taken to examine the recovery of probiotics in faeces in order to confirm compliance. Samples were collected at home at baseline and at the end of the three-week intervention period. The subjects were asked to put the samples into the deep-freeze (-20°C) at home immediately after collection. The samples were subsequently transported to the study centre on the morning of the study day where they were immediately refrigerated and stored at -70°C until analysis. The amounts of the probiotic strains LGG, Bb12 and PJS in the faecal samples were analysed with real-time quantitative PRC as previously described (Myllyluoma et al., 2007).

4.8 Statistical analysis

The statistical analyses in studies II, IV and V were performed using SPSS version 14.0 software (SPSS Inc, Chicago, IL, USA) or STATA version 9.1 software (StataCorp, Texas, USA). All outcome analyses were performed by intention to treat (ITT). The results are expressed as mean with standard deviation (SD) or a 95 percent confidence interval (95% CI) or as medians with interquartile range (IQR).

In study II, the responses for the outcomes were calculated as the area under the curve from days 0, 1, 7 and 21, subtracted by the baseline value (AUC_{0-21} minus baseline). The differences between the groups were compared using either the Kruskal-Wallis test or median regression analysis with Holm's adjustment for pairwise comparisons. These nonparametric methods were adopted because of skewed distributions. The 95% confidence intervals for Spearman's correlation coefficients were obtained by bias-corrected bootstrapping (5000 replications).

In study IV, the change to the end of intervention was estimated using either mean change or Hodges-Lehman estimate for median shift. The confidence intervals for changes were obtained by bootstrapping. Statistical comparisons between groups were performed using bootstrapped-type analysis of covariance (ANCOVA) with the baseline value as the covariate. These methods were used due to the violations of parametric assumptions. Analysis of covariance for cytokines was done by median regression with baseline value as a covariate.

In study V, a continuous statistical comparison between the groups was made using the t-test, ANCOVA or Mann-Whitney test (with Monte Carlo p-value). Measures with a discrete distribution were analyzed using the Chi-Square or Fischer's exact test and are expressed as counts (%).

In study III, principal components analysis (PCA) and partial least squares discriminant analysis (PLS/DA) with log-transformed values were utilized as modelling methods for clustering and discrimination (Barker and Rayens, 2003). The VIP (variable importance in the projection) values (Wold, 1987) were calculated to identify the most important molecular species for the clustering of specific groups. Comparisons between the levels of selected molecular species were performed using the paired Wilcoxon test. A change detection plot was used to account for multiple hypothesis testing in univariate comparisons. In order to asses whether any of the clinical variables are explained by lipidomics profile data, the lipidomics profile data was regressed on selected clinical variables using the elastic net method (Zou, 2005). PLS/DA and PCA analyses were performed using Matlab version 7.2 (Mathworks, Natick, MA, USA), PLS Toolbox version 4.0 Matlab package (Eigenvector Research, Wenatchee, WA, USA). All other analyses were performed using the R statistical language (http://www.r-project.org/).

4.9 Ethics

The ethical permission to use freshly collected, leukocyte-rich buffy coats obtained from healthy blood donors (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland) in the study I was given by the ethics committee of the Finnish Red Cross Blood Transfusion Service in Helsinki. All clinical studies were carried out according to the ethical principles of the Declaration of Helsinki and Good Clinical Practice. The study protocols of the clinical interventions were approved by the Ethics Committee of the Hospital District of Helsinki and Uusimaa. Before entering the study, the subjects gave their informed consent.

5. Results

5.1 Cytokine responses in peripheral blood mononuclear cells in vitro (I)

The ability of known and potentially probiotic bacterial strains and their combinations to induce cytokine responses was studied in *in vitro* cultured human PBMC. The optimal dose (10:1 bacteria:host cell) and stimulation time were selected based on dose-response and kinetics experiments. Probiotics were found able to direct immune responses to either cell-mediated (Th1) or anti-inflammatory (Treg) direction in a genera-specific manner. All tested bacteria induced TNF-α production. The best inducers of Th1-type cytokines IL-12 and IFN-γ were THS and PIA2, being also more potent inducers of these cytokines than the probiotic LGG and Lc705 strains presently in use. All *Bifidobacterium* strains (Bb12, Bb99 and 1/10) as well as PJS induced the highest anti-inflammatory IL-10 production.

The probiotic bacteria and their combinations which have been used in previous clinical trials, namely combinations of LGG, Lc705, PJS and Bb99 or Bb12 and a combination of LGG, Lc705 and PJS, were used to study whether the effect of probiotic combinations differs from an individual strain. In addition, the representatives of the best cytokine inducers, namely THS, Bb12, Bb99, 1/10, PIA2 and PJS as well as LGG as a reference bacterium, were analysed for their possible additive and synergistic effects on cytokine production. Stimulation of PBMC with any bacterial combinations did not result in enhanced cytokine production. Instead, the responses induced by the combinations were closer to an average of responses induced by the individual bacteria suggesting that different bacteria compete with each other during host cell interactions.

The probiotic bacteria were selected to the clinical studies based on their anti-inflammatory potential. The ability to induce high IL-10 (anti-inflammatory cytokine) and low IL-12 (Th1-type cytokine) production was used for the classification of strains to "anti-inflammatory and Th1-type strains" (Figure 5.) The highest producers of IL-10, being also the lowest producers of IL-12, were thus selected for further clinical studies. The strains selected for further studies were Bb12 and PJS due to their anti-inflammatory capacity and LGG as a well-documented reference probiotic.
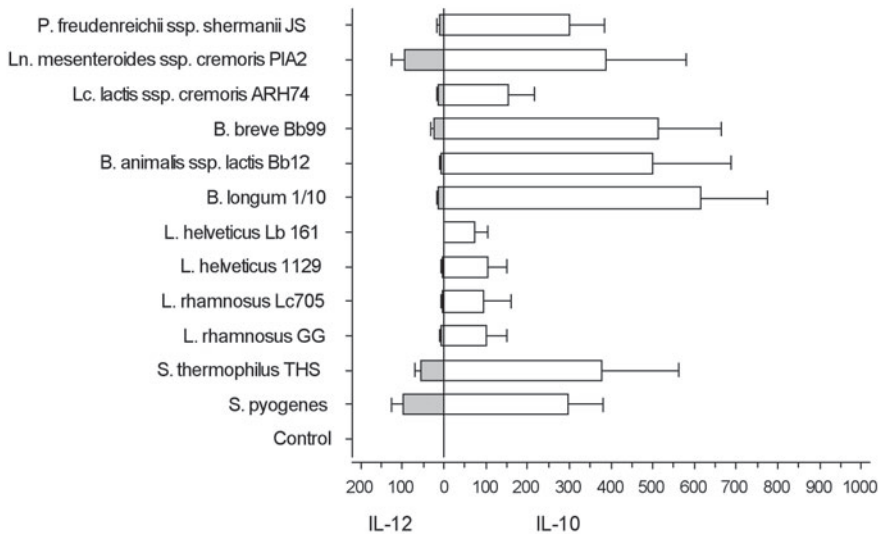
Figure 5. Production of IL-10 and IL-12 by peripheral blood mononuclear cells after 24h stimulation by different bacteria (10:1 bacteria:host-cell ratio). The bars represent the mean and SEM (pg/ml) of 16 blood donors.

5.2 IMMUNOMODULATORY EFFECTS OF PROBIOTICS IN HEALTHY ADULTS IN CLINICAL STUDIES (II, IV)

5.2.1 Serum CRP (II, IV)

In order to study the effect of probiotics on the inflammatory status of the subjects, the serum highly sensitive CRP levels were measured in studies II and IV. In study II carried out in healthy adults, CRP (median AUC_{0-21} minus baseline) was -0.240 mg/l in LGG, -0.085 mg/l in PJS, 0.090 mg/l in Bb12 and 0.018 mg/l in the placebo group (p=0.014 between groups); a statistically significant difference was observed between the LGG and Bb12 groups by pairwise comparisons. CRP appeared to be at a lower level in the LGG and PJS groups during the intervention (Figure 6). In study IV in exercising adults, the mean change in serum CRP levels after the three-month intervention period was -0.48 mg/l in the LGG group and 0.52 mg/l in the placebo group (p=0.20).
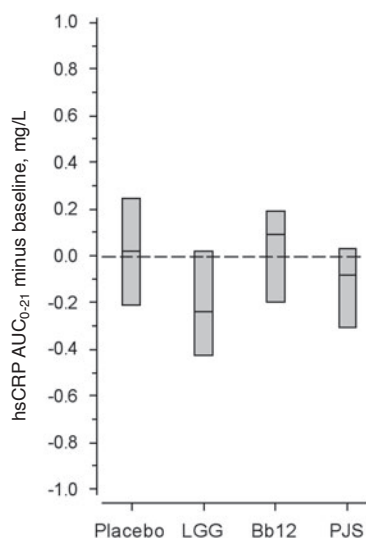
Figure 6. The median AUC_{0-21} (minus baseline) with IQR for serum highly sensitive CRP (hsCRP) levels during the three-week intervention period in healthy adults in study II (n=16 in placebo, n=13 in LGG, n=16 in Bb12 and n=17 in PJS group). LGG = *Lactobacillus rhamnosus* GG; Bb12 = *Bifidobacterium animalis* ssp. *lactis* Bb12; PJS = *Propionibacterium freudenreichii* ssp. *shermanii* JS

5.2.2 Serum cytokines (II, IV)

The serum IL-6 concentration was lower in the LGG group versus the placebo group after the three-month intervention in study IV in exercising adults (p=0.041). No differences between the groups were observed in the serum TNF-α or chemokine (MCP-1, IP-10) levels. In study II in healthy adults, there were no differences between the study groups in serum cytokines during the three-week intervention. Nearly all samples of serum IFN-γ and IL-10 were under the detection limit in both studies and therefore no further analysis was made.

5.2.3 Cytokine responses in peripheral blood mononuclear cells *ex vivo* (II)

In study II in healthy adults, the cytokine production by *ex vivo* isolated, stimulated (*Streptococcus pyogenes*, Influenza A virus, LPS) PBMC was determined as a measurement reflecting the functional responses of cells of both innate and adaptive immunity (PBMC consists of monocytes, NK cells, T and B cells). *S. pyogenes*-stimulated production of proinflammatory cytokine TNF-α differed between the groups (p=0.025), reaching the lowest level in subjects receiving LGG during the three-week intervention (Figure 7.).

Statistically significant difference was observed between LGG and placebo group by pairwise comparisons. Also Influenza A virus-stimulated production of Th1 associated, proinflammatory cytokine IL-2 was significantly different between the groups (p<0.001), being at its lowest in the Bb12 group during the intervention (Figure 7.). There were no significant differences between the study groups with respect to the other measured cytokines (IL-1β, IL-6, IFN-γ, IL-12p70, IL-8, IL-10). IL-4 and IL-5 were not analysed since less than 80% of the samples exceeded the detection limit.
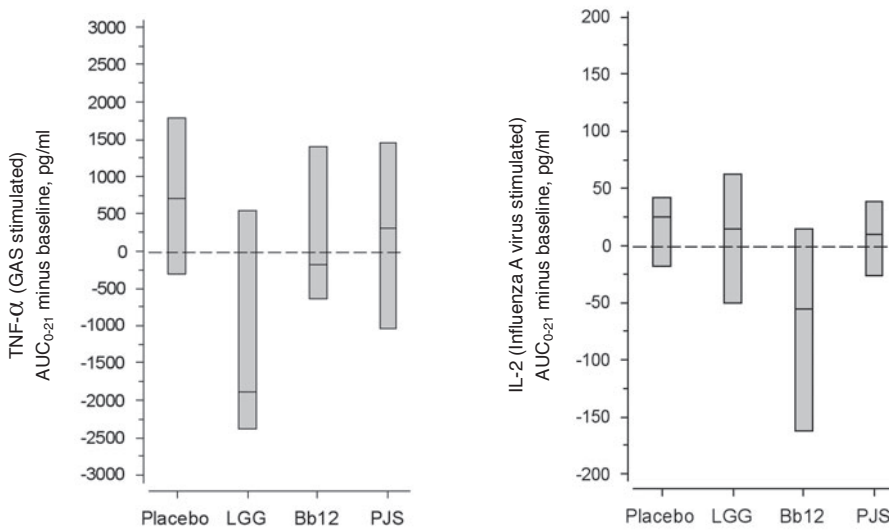
Figure 7. The median of the area under curve (AUC_{0-21} minus baseline) with interquartile range for *Streptococcus pyogenes* (GAS) -stimulated TNF-α production (left) and Influenza A virus-stimulated IL-2 production (right) after 24h stimulation by peripheral blood mononuclear cells during the three-week intervention period in healthy adults (n=16 in placebo, n=13 in LGG, n=16 in Bb12 and n=17 in PJS group). LGG = *Lactobacillus rhamnosus* GG; Bb12 = *Bifidobacterium animalis* ssp. *lactis* Bb12; PJS = *Propionibacterium freudenreichii* ssp. *shermanii* JS

5.2.4 Ratio between IL-10 and IL-12 produced by peripheral blood mononuclear cells *ex vivo* (unpublished)

As the classification of the probiotic strains to "anti-inflammatory and Th1-type strains" in study I *in vitro* was based on the IL-10 and IL-12 production and the selection of strains to study II in healthy adults was premised on this assumption, the ratio between IL-10 and IL-12 was calculated from the cytokines produced by the *ex vivo* isolated PBMC in study II in healthy adults. Here the high versus low IL-10/IL-12 ratio exhibits an "anti-inflammatory" versus "Th1-type" profile, respectively. The mean value of IL-10/IL-12 ratio was four-fold after the LGG intervention when PBMC were stimulated with *S. pyogenes* (p=0.16 between groups at day 21) (Figure 8.). The ratios between IL-10 and IL-12 in influenza A virus and LPS-stimulated PBMC before and after the intervention are presented in Figure 8 (p=0.80 and p=0.85 between groups at day 21, respectively).
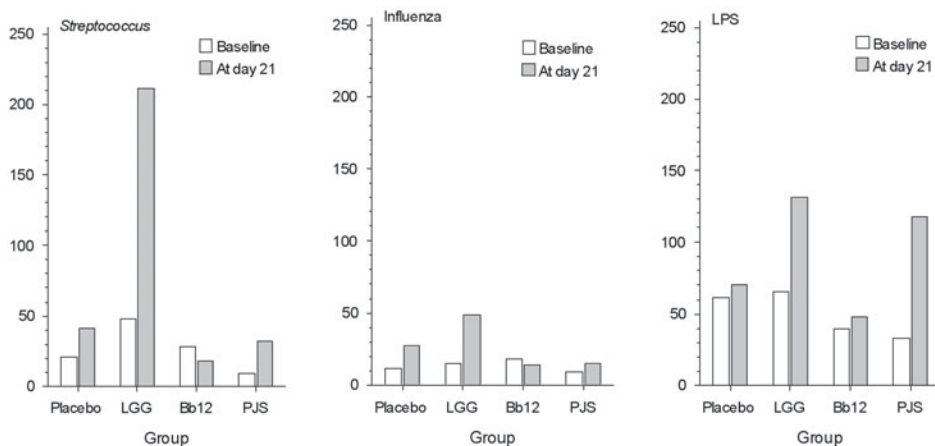
Figure 8. The ratio between IL-10 and IL-12 measured from the supernatants of *ex vivo* isolated, stimulated (*Streptococcus pyogenes*, Influenza A virus, LPS) peripheral blood mononuclear cells at baseline (open bars) and at the end of the three-week intervention (filled bars) represented as mean. LGG = *Lactobacillus rhamnosus* GG; Bb12 = *Bifidobacterium animalis* ssp. *lactis* Bb12; PJS = *Propionibacterium freudenreichii* ssp. *shermanii* JS

5.2.5 Correlation between serum cytokine levels and cytokines produced by peripheral blood mononuclear cells *ex vivo* (unpublished)

In order to evaluate whether serum cytokine levels and cytokine production by unstimulated and stimulated PBMC *ex vivo* correlate with each other, Spearman's correlation coefficient was determined from the baseline samples of study II in healthy adults. There was no statistically significant correlation between serum cytokine levels and cytokines produced in stimulated PBMCs. The correlation between serum TNF-α and TNF-α produced by unstimulated and LPS-stimulated PBMC is presented in Figure 9 and for IL-6 in Figure 10. Correlation coefficients between cytokines induced by *S. pyogenes* and influenza A virus -stimulated PBMC and serum cytokines varied from 0.02 to 0.04.
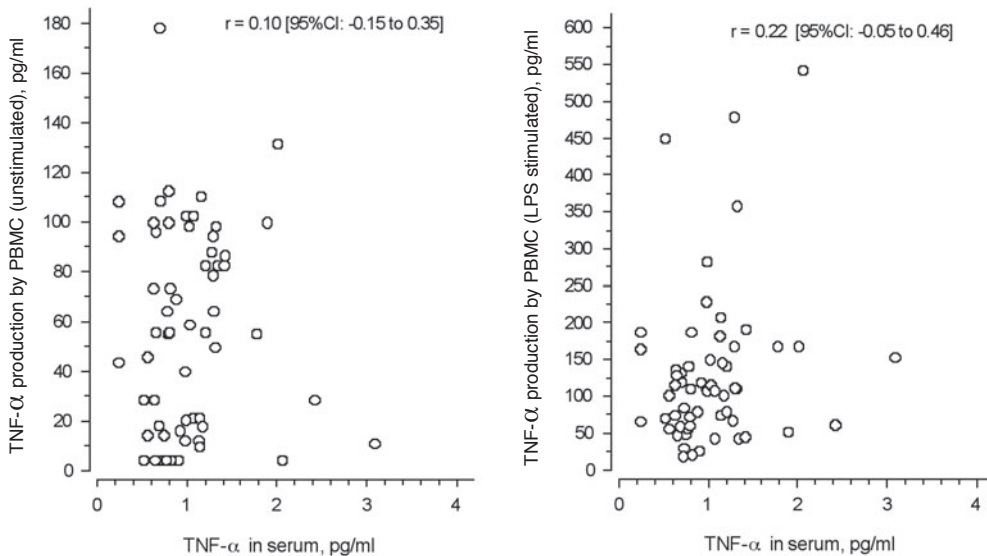
Figure 9. Scatter plots with Spearman's correlation coefficient between serum TNF-α and TNF-α produced by unstimulated (left) and LPS-stimulated (right) PBMC *ex vivo* at baseline in healthy adults (n=62).
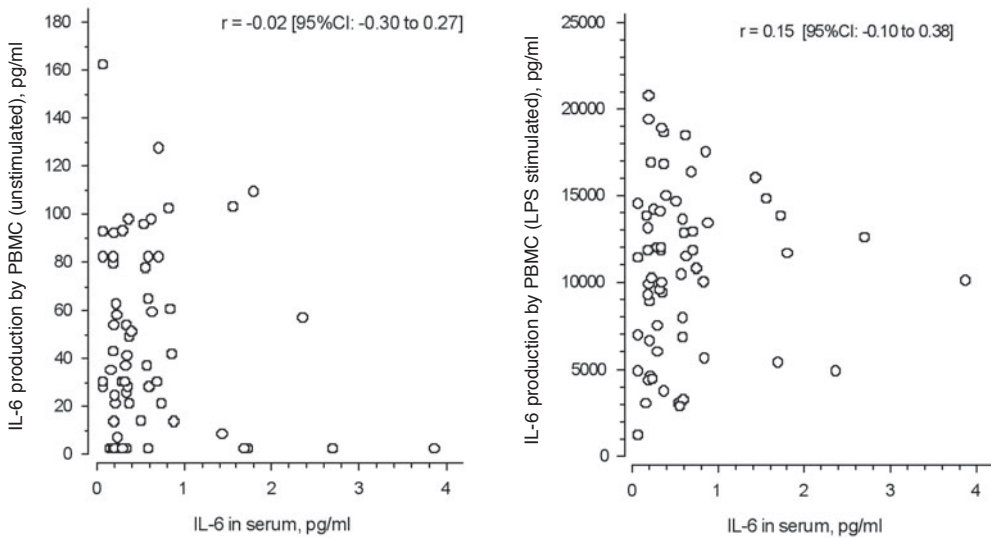
Figure 10. Scatter plots with Spearman's correlation coefficient between serum IL-6 and IL-6 produced by unstimulated (left) and LPS-stimulated (right) PBMC *ex vivo* at baseline in healthy adults (n=62).

5.2.6 Leukocytes, lymphocyte subtypes and immunoglobulins (II, IV)

In study IV in exercising adults, the number of monocytes in serum was lower in the LGG group versus placebo after the three-month intervention (p=0.041). However, in the shorter three-week intervention in healthy adults (study II), no differences in the number of monocytes were observed. In studies II and IV, there were no differences in the total leukocyte counts or in the major leukocyte subtype distribution (neutrophils, basophils, eosinophils, lymphocytes) between the study groups. In study IV, the major lymphocyte subsets (CD3+ T cells, CD4+ helper T cells, CD8+ cytotoxic T cells, CD19+ B cells, CD16+CD56+ NK cells) remained unchanged after the intervention. Also the serum immunoglobulin levels (IgA, IgG, IgM) remained unchanged during the three-week intervention in study II. The secretory IgA in saliva was measured in study II in healthy adults but no differences between the study groups were detected during the three-week intervention.

5.3 Global serum lipidomics profiles (III)

As LGG seemed to exhibit the best anti-inflammatory potential in study II in healthy adults, the LGG and placebo groups of study II were used for investigating the effect of probiotics on lipid-derived mediators in study III. For this purpose, global lipidomics profiles in serum were determined. A total of 407 lipids falling into 13 different lipid classes were identified. In the principal component analysis, no major outliers exist in the data. Partial least squares discriminant analysis on the log-transformed data indicated that the groups are separable (Figure 11a.) The analysis also revealed that the LGG and placebo groups differed at the baseline and therefore only within-person changes were used in further analyses. As the within-person changes before and after intervention were compared using the paired Wilcoxon test, six lipids [triacylglycerol TAG(55:3); sphingomyelin SM(d18:1/24:2); lysoglycerophosphatidylcholine LysoGPCho(18:2); glycerophosphoserine GPSer(36:1); glycerophosphoethanolamine GPEtn(44:6e); glycerophosphocholine GPCho(38:7)] were found to change significantly (Figure 11b). Based on multiple hypothesis testing using the change detection plot, no lipid changes were found significant within the 95% confidence interval. One thus cannot exclude the possibility of some of the significant changes being detected by chance. However, some common trends were observed; triacylglycerols and glycerophosphocholines increased mainly in the LGG intervention group, while several of the proinflammatory lysophosphatidylcholines and sphingomyelins decreased.
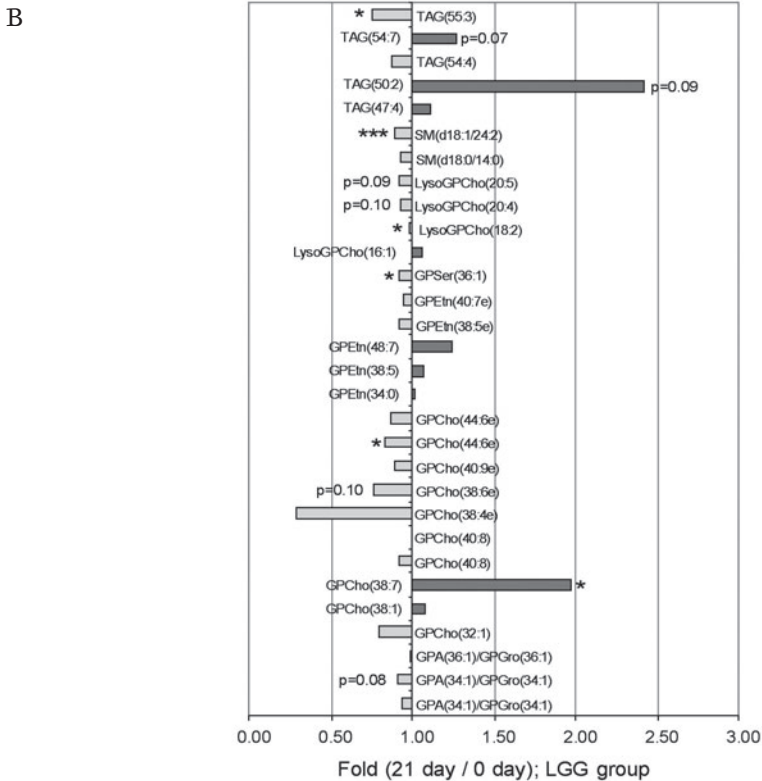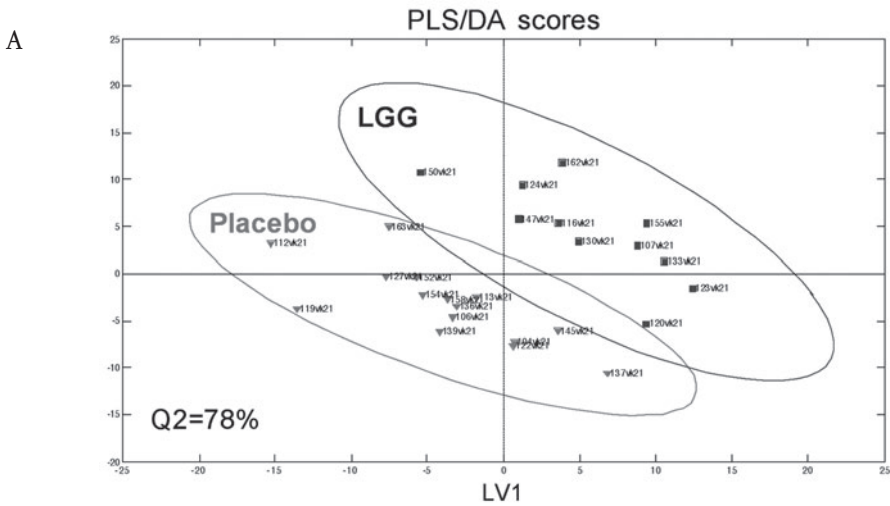
Figure 11. A. Partial least squares discriminant analysis (PLS/DA) of serum lipidomic data. The labels are subject ID numbers. **B.** Fold changes for the top 30 ranking lipids contributing to the PLS/DA model based on variable important in the projection analysis (VIP) (* p<0.05, *** p<0.001). LGG = *Lactobacillus rhamnosus* GG

5.4 CLINICAL OUTCOME (V)

In addition to different immunological measurements, the incidence and duration of respiratory infections and gastrointestinal symptom episodes were measured by a daily symptom diary in study V in exercising adults. The mean number of healthy days, i.e. days without any symptoms of respiratory infection or gastrointestinal symptoms was 79 in the LGG group and 73 in the placebo group during the three-month training period before the marathon (p=0.82).

Respiratory infections
There were no differences between the LGG and placebo groups in the number and duration of respiratory infections during the three-month probiotic intervention period before the marathon or two weeks immediately after the marathon.

Gastrointestinal symptom episodes
During the three-month probiotic intervention period before the marathon and two weeks after the marathon, no differences between groups in the number of gastrointestinal symptom episodes were detected. However, during the two-week follow-up after the marathon, the duration of the gastrointestinal symptom episodes was shorter in the LGG group than in the placebo group (1.0 vs. 2.3 days, p=0.046). During the three-month probiotic intervention period before the marathon, the duration of the gastrointestinal symptom episodes was 2.9 days in the LGG group and 4.3 days in the placebo group (p=0.35).

Exercise habits and serum hormones reflecting exercise-induced stress
In study V, subjects in the LGG group exercised (running and other forms of exercise) more often than those in the placebo group during the training period (4.8 vs. 4.4 times weekly, p=0.024). The higher exercise rate of the LGG group during the intervention may also have been reflected as a higher serum cortisol level at the end of the intervention compared to the placebo group (p=0.011). However, the levels of the other measured stress hormones, testosterone and prolactin, remained the same at the end of the intervention (p=0.25 and p=0.22, respectively) (unpublished) (Table 15).

Table 15. The mean change in hormones (nmol/l) reflecting exercise-induced stress during a three-month intervention in study V in exercising adults.

| Hormone | Baseline | | Change to the end of intervention | | P-value between groups [1] |
	Placebo Mean (SD)	LGG Mean (SD)	Placebo Mean (95 % CI)	LGG Mean (95 % CI)	
Cortisol	378 (89)	404 (107)	-7 (-31 to 16)	24 (-2 to 49)	0.011
Testosterone	18 (7)	16 (8)	-0.2 (-1.4 to 0.9)	0.7 (-0.4 to 1.8)	0.25
Prolactin	183 (113)	185 (118)	1.3 (-20.7 to 23.3)	19.6 (-1.7 to 40.8)	0.22

[1]Bootstrap type analysis of covariance.

5.5 Compliance and recovery of probiotic strains in faeces (II-V)

According to the self-reported consumption of study products based on daily diaries, compliance was good in all studies. Indicated by the received study diariesin studies II and III in healthy adults, the self-reported mean consumption (SD) of LGG was 20.9 (0.3) days, of Bb12 20.4 (1.8) days, of PJS 20.1 (2.0) days and of placebo 20.6 (1.0) days (p=0.60). In studies IV and V in exercising adults, the self-reported mean consumption of LGG was 84/89 days and of placebo 76/89 days (p=0.93).

In study II in healthy adults, the recovery of probiotic strains in faeces was measured in order to confirm the compliance. The baseline bacterial DNA levels for all three studied probiotics were low in the faecal samples. Despite the three-week run-in period with probiotic restriction, a detectable level of the probiotic strains, especially LGG, was harboured in some of the subjects at baseline before the probiotic intervention period. The amount of studied probiotic strains in faeces in a given probiotic intervention group increased significantly from the baseline values during the intervention (p<0.001). In the placebo group, the levels of probiotics in faeces remained low during the whole intervention period.

6. Discussion

It is not fully understood how probiotics exert their beneficial effects on health, but one of the most probable mechanisms of action is the modulation of immune responses via the mucosal immune system of the gut. Most of the research on probiotics has concentrated on the prevention and treatment of gastrointestinal diseases and allergy. Probiotic products, however, are usually consumed by the healthy population but not much is known on their immunomodulatory effects in healthy adults. This study investigated the immunomodulatory properties of probiotics by systematical screening in primary cell culture using human PBMC and evaluated the effects of the most effective probiotic strains on the immune system of healthy adults in randomized, double-blind, placebo-controlled clinical trials. In addition, novel lipidomics profiling was performed to characterize the effect of probiotics on lipid-derived mediators.

6.1 METHODOLOGICAL ASPECTS

6.1.1 Selection of strains

Bacteria from different genera (*Lactobacillus*, *Bifidobacterium*, *Propionibacterium*, *Streptococcus*, *Lactococcus*, *Leuconostoc*) and different species from a given genera were selected for the screening of immunomodulatory effects. *L. rhamnosus* GG (LGG) (Saxelin et al., 2005) and *B. animalis* ssp. *lactis* Bb12 (Bb12) (Salminen et al., 2006) are widely used and studied probiotics in Europe. However, not much is known on how these strains affect the immune system of a healthy individual. The combination of LGG, *L. rhamnosus* Lc705 (Lc705), *P. freudenreichii* ssp. *shermanii* JS (PJS) and *B. breve* Bb99 (Bb99) or Bb12 has ameliorated the symptoms of irritable bowel syndrome (Kajander et al., 2005; Kajander et al., 2008), reduced the side-effects of *Helicobacter pylori* treatment (Myllyluoma et al., 2005) and the probiotic combination with and without prebiotic galacto-oligosaccharides has prevented atopic eczema (Kukkonen et al., 2007; Viljanen et al., 2005c). The possible immunomodulatory effects of these individual strains or a combination thereof have not been fully elucidated, although there is some data suggesting that the probiotic combination modulates the immune system differently in allergic children compared to LGG alone (Pohjavuori et al., 2004; Viljanen et al., 2005b). *L. helveticus* Lb 161 (161) (Staaf et al., 2000), *L. helveticus* 1129 (1129) (Yang et al., 2000), *Lc. lactis* ssp. *cremoris* ARH74 (ARH74) (Yang et al., 1999) and *S. thermophilus* THS (THS) (Nordmark et al., 2005) have been studied due to their exopolysaccharide producing characteristics, but no data on the immunomodulatory effects of these

strains is available. The health effects of *B. longum* 1/10 (1/10) isolated from faeces of healthy infants and *Ln. mesenteroides* ssp. *cremoris* PIA2 (PIA2) used in the production of Finnish viili have not been previously studied.

The anti-inflammatory potential of the strains in the primary cell culture using PBMC was used as a basis for the selection of strains for clinical studies in healthy adults. The anti-inflammatory potential was determined as high IL-10 and low IL-12 production. The IL-10/IL-12 ratio has been used to characterize the anti-inflammatory effects in previously conducted probiotic (Foligne et al., 2007; O'Mahony et al., 2005), virological (Arvå and Andersson, 1999; Feng et al., 2003; Gurney et al., 2005; Legg et al., 2003) and other immunological studies (Emmer et al., 2006; Itoh et al., 2002; Koopman et al., 2003; Townsend et al., 2007). In addition to the most effective anti-inflammatory strains Bb12 and PJS, LGG was selected for further studies as a well-documented reference probiotic (for review, see Saxelin et al., 2005).

6.1.2 Primary cell culture model using human peripheral blood mononuclear cells

Human PBMC were used as a screening model for the immunomodulatory effects of probiotic strains. The protein production and mRNA expression of representative examples of proinflammatory (TNF-α), Th1-type (IFN-γ, IL-12) and anti-inflammatory (IL-10) cytokines were analyzed. Mononuclear cells consist of monocytes, NK cells, T cells and B cells, which are most likely not in direct contact with probiotics in the actual *in vivo* situation in the human gut. The probiotic-host interaction takes place on the gut epithelial cells. However, probiotics may be taken up for example by macrophages (Sun et al., 2007) and presented to T and B lymphocytes, leading to their activation in Peyer's patches (for review, see McCracken and Lorenz, 2001). As *in vitro* cell models are very artificial in the sense that they lack interaction with other cells, PBMC may be a better model compared to single cell cultures, such as macrophages or dendritic cells, because PBMC comprise many types of cells. Indeed, PBMC have been extensively used for studying and screening the effects of probiotics on cytokine production (Castanheira et al., 2007; Castellazzi et al., 2007; Drouault-Holowacz et al., 2006; Foligne et al., 2007; Gackowska et al., 2006; Haller et al., 2000; Helwig et al., 2006; Hessle et al., 1999; Lammers et al., 2003; Medina et al., 2007; Miettinen et al., 1998; Miettinen et al., 1996; Niers et al., 2005; O'Mahony et al., 2006; Pochard et al., 2002; Rasche et al., 2007; Shida et al., 2006b; Timmerman et al., 2007).

Validation and standardisation of the conditions of cell culture is an important factor affecting the results. The growth conditions (media, temperature, atmosphere) as well as the optimal bacteria:host cell ratio in the cell culture are critical to the results. The optimal ratio was determined by dose-response experiments in the beginning of

the study, and the differences in the kinetics of the chosen cytokines were taken into account by choosing several timepoints in the experiments.

6.1.3 Subjects

The subjects were considered to represent the general, healthy adult population without any major chronic diseases. Healthy adults participating in a marathon were used as a sensitized model to study infections as hard-training individuals are known to be more prone to respiratory infections and the risk of infections is particularly high after competitive events (for review, see Gleeson, 2007). Because strenuous exercise is also known to cause many gastrointestinal symptoms such as vomiting, nausea, diarrhoea and heartburn (Peters et al., 2001; Simons and Kennedy, 2004), adults participating in a marathon comprised a good model for evaluating the effect of probiotics on the reduction of gastrointestinal symptoms.

6.1.4 Designs of intervention studies

The clinical intervention studies were carried out in randomized, double-blind and placebo-controlled settings. The sample size of the studies was rather small. The required sample size for the studies was not calculated as the a priori information was not available. All recruited subjects who fulfilled the inclusion criteria, were included in these studies and therefore the largest possible subject material was used.

A sufficiently long run-in period is an important factor in clinical interventions. In previous studies, probiotics were detected in faeces after a two-week run-in period (Alander et al., 1999; Myllyluoma et al., 2007; Suomalainen et al., 2008). A three-week run-in period with a restriction to use any probiotic products was therefore used in studies II and III. However, even this period of time may not have been sufficiently long as detectable, albeit very low levels of studied probiotics were found in the baseline samples in study II. These results indicate a need for sufficiently long run-in and follow-up periods in probiotic interventions. Consequently, the run-in period of studies IV and V was set to four weeks.

The immunomodulatory effects were studied both in the short three-week intervention and in the longer three-month intervention. The optimal length of intervention for the investigation of different immune measures is difficult to determine. In the short three-week intervention, also the acute effects were taken into account by several measurement timepoints (day 0, 1, 7, 21 and in addition day 42 as a follow-up sample). In addition to the markers of immunomodulation, the incidence of respiratory infections and gastrointestinal symptoms were studied in the longer, three-month intervention. The length of the previous studies investigating the effect

of probiotics and respiratory infections have varied a lot, ranging from three weeks (Turchet et al., 2003) to approximately three months (de Vrese et al., 2005b; de Vrese et al., 2006; Tubelius et al., 2005; Weizman et al., 2005; Winkler et al., 2005) while the longest interventions have lasted from six to seven months (Hatakka et al., 2007; Hatakka et al., 2001).

The probiotics were given in milk-based fruit-drinks and the taste and appearance of the probiotic and placebo products were identical. The viability of probiotic bacteria were measured from each study product batch immediately after manufacture and on the best before date (three weeks from manufacture). The adherence to the probiotic interventions was good reflecting a high motivation of the subjects. Compliance was measured by the self-reported consumption of study products by means of a study diary and also by measuring the ingested probiotic strains from faeces.

6.1.5 Immunological and biochemical measurements

In studies investigating the effect of nutritional intervention on measurements of immune function, it is critical to ensure that the subjects do not fall ill during the intervention (for review, see Albers et al., 2005). In the short-term three-week intervention, this was taken into account by means of daily study symptom diaries which revealed the subjects having no respiratory or other infections during the intervention period. There is no single marker to study the effect of nutrition intervention on the immune function (for review, see Albers et al., 2005; Calder and Kew, 2002; Cummings et al., 2004). Therefore, to gain a comprehensive understanding of the immune function a combination of measures of the basal status of the immune system, gut-associated markers and *ex vivo* measurements reflecting the functional capacity of cells of innate and adaptive immunity were selected as parameters in this thesis. To gain a broad picture on the effect of studied probiotics on cytokine production, a wide selection of cytokines with representatives of pro- (TNF-α, IL-1β, IL-6) and anti-inflammatory cytokines (IL-10) as well as Th1 (IL-2, IFN-γ, IL-12) and Th2-type cytokines (IL-4, IL-5) and chemokines (CXCL8, CXCL10, CCL2), was used. In addition, well-established immunological methods, including novel lipidomics profiling analysis using UPLC/MS technique, were applied. Lipidomics is a rapidly evolving branch of metabolomics (Schnackenberg and Beger, 2006; Wiest and Watkins, 2007) which enables the functional characterization of endogenous lipid metabolites. However, the results should be interpreted with caution as it is not yet known how the changes in lipid metabolites are associated with the individual metabolic health or major metabolic and inflammatory diseases.

6.1.6 Questionnaires

Infections can be measured either by self-reported study diaries or by diagnoses made by physician. There is no exact definition for a respiratory infection. Furthermore, the definitions vary from one study to another ranging from one symptomatic day (de Vrese et al., 2005b; de Vrese et al., 2006) to symptoms on at least two consecutive days (Tiollier et al., 2007), and these studies fail to define what is considered as a separate infection. A relatively widely used definition for the separation of two infection episodes is a symptom-free period of at least three days in between (Uhari and Möttönen, 1999) which was adopted also in the present study. The symptom diary used in this study was not systematically validated, but it has been used in previous studies by our group. Respiratory infections were recorded in a subjective daily symptom diary with ready-made questions that has already been used in previous studies by our group in children (Hatakka et al., 2007; Hatakka et al., 2001) and the elderly (Hatakka, 2007). Based on our previous experience in clinical trials in measuring gastrointestinal symptoms in children, (Hatakka et al., 2007; Hatakka et al., 2001), adults (Kajander et al., 2005; Myllyluoma et al., 2005) and the elderly (Hatakka, 2007), we used the same methodology also in the present study.

> *To summarize, the selection of probiotic strains for clinical studies was based on systematical immunological screening in an extensively used PBMC model. The clinical interventions were performed in a randomized, double-blind, placebo-controlled manner, which is considered to be the golden standard for intervention trials. A broad range of well-established immunological measurements along with novel lipidomics technique were utilized to get a comprehensive picture on possible immunomodulatory effects of probiotics.*

6.2 Main findings

6.2.1 Production of cytokines in peripheral blood mononuclear cells *in vitro*

Probiotic strains from six different genera were clearly different in their ability to induce cytokine responses in PBMC *in vitro*. All strains were able to induce TNF-α production, which is consistent with previous studies in human PBMC (Foligne et al., 2007; Gackowska et al., 2006; Miettinen et al., 1998; Miettinen et al., 1996; Niers et al., 2005; Shida et al., 2006a; Timmerman et al., 2007). Interestingly, *Lactobacillus* and *Bifidobacterium* strains which have previously been shown to stimulate IL-12 and IFN-γ production in human PBMC (Haller et al., 2000; Hessle et al., 2000; Hessle et al., 1999; Miettinen et al., 1998; Niers et al., 2005), were found be relatively poor inducers of these cytokines by the present study. Instead, it was found that potentially probiotic *S. thermophilus* and *Leuconostoc* strains were extremely good inducers of the said Th1-type cytokines. Cytokine profiles may provide mechanistic explanations for the observed clinical effects. A strong Th1-type cytokine response promoting cell-mediated immunity and especially IFN-γ due to its anti-viral activity is important in the defence against viral infections. In allergic diseases, the immune response is skewed towards Th2-type responses and balancing the response towards Th1-type prominent responses would thus be helpful. Different *Lactobacillus* strains have previously been shown to prevent and ameliorate the symptoms of respiratory infections (de Vrese et al., 2005b; de Vrese et al., 2006; Hatakka et al., 2001; Turchet et al., 2003; Winkler et al., 2005). LGG has also been used successfully in the treatment of rotavirus diarrhoea (Isolauri et al., 1994; Majamaa et al., 1995; Pant et al., 2007) as well as in the prevention or treatment of atopic diseases (Isolauri et al., 2000; Kalliomäki et al., 2001; Viljanen et al., 2005c). It is of interest that LGG has been effective in different immune-mediated diseases even though in the present study LGG induced only moderate cytokine production compared to the other strains. This may be due to the fact that cytokine induction is only one of the proposed mechanisms of action of probiotics. The effects of potentially probiotic *S. thermophilus* and *Leuconostoc* strains warrant further attention in the prevention of infections and allergy in clinical studies. In addition, other probiotic characteristics should be studied before these new strains can be called probiotics.

Bifidobacterium and *Propionibacterium* strains induced the highest IL-10 and the lowest IL-12 production indicating high anti-inflammatory capacity. This observation is consistent with previous findings where bifidobacteria were shown to induce higher IL-10 production as compared to lactobacilli (Helwig et al., 2006; Lammers et al., 2003; Timmerman et al., 2007). There is preliminary evidence that probiotics could be used in the treatment of inflammatory diseases, such as ulcerative colitis, pouchitis and rheumatoid arthritis (for review, see Jones and Foxx-Orenstein, 2007; Saxelin et al.,

2005) and the amelioration of these inflammatory diseases could be due to the ability of probiotics to induce IL-10. Recent studies indicate a possible role of low-grade mucosal inflammation also in the pathogenesis of irritable bowel syndrome (Chadwick et al., 2002; O'Mahony et al., 2005). Preliminary evidence indicating that a combination of probiotics, including anti-inflammatory *Bifidobacterium* and *Propionibacterium* strains in addition to two different *L. rhamnosus* strains, relieves the symptoms of irritable bowel syndrome is available (Kajander et al., 2005; Kajander et al., 2008). *Bifidobacterium* and *Propionibacterium*, able to induce anti-inflammatory IL-10 production in this study, could thus be used to treat different types of inflammatory diseases.

The use of probiotic bacterial combinations in clinical trials has shown great promise highlighting the importance of understanding the immunological properties of a single strain versus different bacterial combinations (Kajander et al., 2005; Kukkonen et al., 2007; Myllyluoma et al., 2005). However, there is virtually no comparative data on the immunomodulatory properties of probiotic multispecies within the same experimental system (Castellazzi et al., 2007; Drouault-Holowacz et al., 2006). Published data is generally limited to the comparisons of probiotic monostrains of the *Lactobacillus* and *Bifidobacterium* genera (Foligne et al., 2007; Helwig et al., 2006; Miettinen et al., 1998; Timmerman et al., 2007). In the present study, combinations of different Gram-positive probiotic bacteria did not induce any additive or synergistic cytokine production in PBMCs and the responses were an average of the responses of the individual strains. This could be due to the fact that all Gram-positive bacteria are likely to use the same or similar intracellular signal transduction mechanisms to induce cytokine gene expression. Gram-positive bacteria or their structural components activate host cells via TLR2, whereas Gram-negative bacteria and their major structural component lipopolysaccharide activate host cells via TLR4 (Akira, 2001). It is, however, likely that other receptor systems apart from TLRs take part in host cell responses to different microbes (Robinson et al., 2006). Different bacteria seem to compete with each other during bacteria-host cell interactions.

> *In summary, probiotics differ in their ability to induce cytokine responses. The Bifidobacterium and Propionibacterium strains possessed the best anti-inflammatory potential while the Streptococcus and Leuconostoc strains were good inducers of Th1-type cytokines in PBMC model. Screening of the immunomodulatory properties of probiotics can help in selecting strains for clinical trials for different purposes. Effects of probiotic multispecies should be studied as their responses differ from the probiotic monostrains.*

6.2.2 Immunomodulatory effects of probiotics in healthy adults

Monocytes, IL-6 and CRP

This is the first study to show that LGG may result in reduced serum highly sensitive CRP levels even in healthy adults. Previously, the effect of probiotics on CRP has only been studied in different disease conditions. A combination of *L. casei*, *B. breve* and prebiotic galactooligosaccharides (Sugawara et al., 2006) and *B. longum* (Furrie et al., 2005) have reduced serum CRP levels in immunocompromised patients and also improved the overall clinical appearance of chronic inflammation (Furrie et al., 2005). In contrast to the studies above and to results of the present study, LGG increased serum sensitive CRP levels compared to placebo in infants with IgE-associated atopic eczema (Viljanen et al., 2005b). However, LGG had no effect on serum CRP levels in patients with rheumatoid arthritis (Hatakka et al., 2003). It is of interest that a combination of four probiotic bacteria (LGG, Lc705, Bb99, PJS) had no effect on sensitive CRP (Viljanen et al., 2005b) in the same clinical setting with allergic children. In immunocompromised patients undergoing surgical procedures, *L. plantarum* 299V (McNaught et al., 2005; McNaught et al., 2002) or a combination of *L. acidophilus* La5, Bb12, *S. thermophilus* and *L. bulgaricus* (Anderson et al., 2004; Reddy et al., 2007) did not change serum CRP concentrations, either. The effect of probiotics on CRP appears controversial and the comparison of effects is rendered difficult by the differences in the measurement technique (highly sensitive vs. normal CRP measurement), the different patient materials (healthy vs. various diseases) and the different probiotic strains that have been used. It seems that age, the individual immunological status and the probiotic strain used in the study have a great impact on the immunomodulatory effects. The present finding on CRP lowering effect warrants further research, also during inflammatory processes and in individuals suffering from various types of inflammatory or autoimmune diseases.

In addition to a lowered sensitive CRP level, the number of circulating monocytes and the serum IL-6 concentration was lower in the LGG group. IL-6 is produced by monocytes and it plays a key role in acute-phase responses (Bruunsgaard, 2005; Gabay, 2006). IL-6 is an important mediator of inflammation, being able to induce the production of CRP, as well as a sensitive marker of inflammation produced by hepatocytes and other acute-phase proteins and inflammatory factors (Kishimoto, 2006; Volanakis, 2001). The finding of the present study that LGG reduced serum IL-6 levels is supported by another study in healthy adults showing a decrease in IL-6 production in peripheral blood mononuclear cells after LGG consumption (Schultz et al., 2003). Furthermore, another *Lactobacillus* strain, *L. plantarum* 299v has also reduced serum IL-6 levels in critically ill patients (McNaught et al., 2005) and in heavy smokers (Naruszewicz et al., 2002).

Production of cytokines by peripheral blood mononuclear cells ex vivo

LGG reduced Gram-positive pathogen-induced TNF-α responses in the *ex vivo* cultured PBMC. TNF-α is secreted by the monocytes, and it acts as an inflammatory mediator activating many types of cells. The present finding is supported by another clinical study carried out in healthy adults, showing that LGG treatment leads to decreased TNF-α production in PBMC (Schultz et al., 2003). In addition, when the cytokine expression pattern in the small bowel mucosa of healthy adults was studied, it was found that LGG induced the expression of genes involved in immune response and inflammation (TGF-beta and TNF family members, cytokines, nitric oxide synthase 1, defensin alpha 1) (Di Caro et al., 2005). LGG appears to modulate PBMC responses differently in healthy and allergic individuals as LGG increased the Th1-type cytokine IFN-γ production in PBMC in allergic infants (Pohjavuori et al., 2004).

Additionally, Bb12 was found by the present study to decrease IL-2 production in influenza virus-stimulated PBMC, indicating an anti-inflammatory effect. This finding is new, as a combination of Bb12 and *L. paracasei* ssp. *paracasei* CRL-431 had no effect in *ex vivo*-stimulated blood cytokine production in healthy adults (Christensen et al., 2006). Activating NK cells and inducing activation and proliferation of T lymphocytes, IL-2 is a very important cytokine in viral infections and inflammatory responses. Therefore, IL-2 production might be an important factor for a probiotic fighting against respiratory tract infections. Based on the present results, the *Bifidobacterium* strain might not be the most optimal strain against respiratory infections. Indeed, it is mainly probiotic strains from the *Lactobacillus* genera — *L. rhamnosus* GG (Hatakka et al., 2001), *L. casei* DN-114001 (Turchet et al., 2003), a combination of *L. gasseri* PA 16/8, *B. longum* SP 07/3 and *B. bifidum* MF 20/5 (de Vrese et al., 2005b; Winkler et al., 2005), and *L. reuteri* (Tubelius et al., 2005) — that have reduced the incidence or symptoms of respiratory infections.

> *To summarize, probiotics are able to induce strain-specific immunomodulatory effects that are reflected even to the systemic level and that may possess an anti-inflammatory potential in healthy individuals. LGG appears to reduce inflammatory mediators, such as sensitive CRP and IL-6. LGG and Bb12 seem to have a role in modulating the functional responses of PBMC.*

6.2.3 Global serum lipidomics profiling

This study is the first to apply lipidomics technique to analyze global lipidomics profiles in healthy individuals after a probiotic intervention. Lipid-derived compounds are important mediators of inflammation. LGG intervention seemed to cause changes in global lipidomic profiles reflected as decreased proinflammatory lysophosphatidylcholines and sphingomyelins and elevated triacylglycerols and glycerophosphocholines. Sphingomyelins are known to be involved in the immune cell function as well as in the regulation of inflammation (Melendez, 2008; Olivera and Rivera, 2005; Won and Singh, 2006). Previously, lysophosphatidylcholines have induced an increase in several inflammatory cytokines (IL-1β, IL-6, TNF-α) in human PBMC (Shi et al., 2007). The reductions observed in the present study in both proinflammatory lysophosphatidylcholines and sphingomyelins may thus be related to the observed anti-inflammatory actions of LGG in general. High concentrations of both lysophosphatidylcholines (Haapamäki et al., 1999; Minami et al., 1994) and lipids of the sphingomyelin/ceramide pathway (Homaidan et al., 2002; Sakata et al., 2007) have been associated with inflammatory bowel disease as well as impaired mucosal barrier function and increased gut permeability (Karlqvist et al., 1986; Otamiri et al., 1986; Sawai et al., 2002; Tagesson et al., 1985). Interestingly, previous studies have shown LGG to maintain the remission in patients with ulcerative colitis (Zocco et al., 2006) as well as to normalize gut permeability (Isolauri et al., 1993b) and enhance the mucosal integrity and epithelial cell survival (Lam et al., 2007; Yan et al., 2007).

> *In summary, novel global lipidomics profiling revealed a tendency towards decreased proinflammatory lysophosphatidylcholines and sphingomyelins after LGG intervention which might be related to the observed anti-inflammatory effects of LGG in general.*

6.2.4 Clinical outcome

Prevention of respiratory infections
The effect of LGG on respiratory infections has not been studied in adults before. In the present study, LGG did not reduce the incidence or duration of respiratory infections in healthy adults training for a marathon, although the number of healthy days during the intervention was slightly, but not significantly, higher in the LGG group. On the contrary to the present study, LGG reduced respiratory infections and the absence from day-care due to illness in healthy day-care children (Hatakka et al., 2001) and a combination of LGG, Lc705, Bb99 and PJS reduced the occurrence of recurrent respiratory infections in otitis-prone children (Hatakka, 2007). It may be that the effect of LGG on respiratory

infections is different in adults and children. Again, contrary to the present study, *L. fermentum* VRI 003 reduced the number of days with respiratory symptoms in elite distance runners (Cox et al., 2008). In contrast, *L. casei* DN114001 had no effect on the incidence or symptoms of respiratory infections in healthy men in French Commando training (Tiollier et al., 2007). It is possible that the level of training in the present study and in the study by Tiollier et al. (2007) was not high enough to depress the immune system as compared with elite distance runners investigated in the study by Cox et al. (2008). In addition, previous studies in children (Cobo Sanz et al., 2006; Hatakka et al., 2007; Hatakka et al., 2001; Weizman et al., 2005), adults (de Vrese et al., 2005b; de Vrese et al., 2006; Winkler et al., 2005) and the elderly (Hatakka, 2007; Turchet et al., 2003) have been carried out in wintertime whereas the present study was performed during the summer months due to the marathon race. It may be that the study being performed at summer and the number of subjects being relatively small compared with previous studies (141 vs. 200-500) counteracted the minor beneficial effects of LGG on the measured variables in healthy adults.

Prevention of gastrointestinal symptoms

The effect of probiotics on community-acquired diarrhoea or gastrointestinal symptoms has not received much attention although LGG as well as other probiotics have been effective in prevention and treatment of acute infectious diarrhoea, travellers' diarrhoea and antibiotic-associated diarrhoea (for review, see Sazawal et al., 2006). In the present study, LGG shortened the duration of gastrointestinal symptom episodes (including diarrhoea, vomiting and/or stomach ache) in more than half of the healthy adults training for a marathon. This finding is in accordance to previous studies, although they were conducted in the elderly (Turchet et al., 2003) and in children (Chouraqui et al., 2004; Pedone et al., 1999; Weizman et al., 2005). A reduction in the duration of gastrointestinal symptoms may be especially significant for athletes, such as long distance runners, because they are known to suffer from gastrointestinal disturbances (for review, see Riddoch and Trinick, 1988; Simons and Kennedy, 2004). Furthermore, long-distance running is known to affect the integrity of the gastric and intestinal mucosa and to increase gut permeability (Oktedalen et al., 1992; Smetanka et al., 1999). There are also indications that LGG could have beneficial effects on mucosal permeability in animal (Isolauri et al., 1993a; Isolauri et al., 1993b; Pessi et al., 1998) and in clinical studies (Gotteland et al., 2001). The effect of LGG on the gut barrier function should be evaluated in further studies.

Interestingly, subjects in the LGG group reported to exercise significantly more often per week than the placebo group during the three-month period before the marathon even though the randomization to groups was done according to the level of exercise. The higher rate of exercise in the LGG group may be related to the higher,

although insignificant, number of healthy days without gastrointestinal symptoms or symptoms of respiratory infections. The higher rate of exercise in the LGG group may also be reflected in the higher cortisol level as a marker of exercise-induced stress at the end of the intervention, although the other measured hormones, prolactin and testosterone, were at a comparable level in both groups.

> *In summary, LGG had no effect on the incidence or duration of respiratory infections, but it was able to shorten the duration of gastrointestinal symptoms in healthy exercising adults.*

6.2.5 Relationship between *in vitro*, *ex vivo* and *in vivo* results

In the primary cell culture study model using PBMC, the most potential anti-inflammatory probiotics were *Bifidobacterium* and *Propionibacterium* strains based on their ability to induce high IL-10 (anti-inflammatory cytokine) and low IL-12 (Th1-type cytokine) production. In addition, LGG was selected for further studies as a well-documented probiotic even though it was not particularly active in inducing cytokine responses. The results of the *in vivo* setting did not reflect the *in vitro* results. In the clinical intervention study, the best anti-inflammatory strain was LGG, which induced very moderate IL-10 production *in vitro* compared with Bb12 and PJS strains. In the clinical setting, LGG had the most promising anti-inflammatory effects as the sensitive CRP level and TNF-α produced by PBMC *ex vivo* were at the lowest level during the LGG intervention. Also Bb12 and PJS exerted some anti-inflammatory activity *in vivo*, as IL-2 levels produced by PBMC *ex vivo* were lower in the Bb12 group than in other groups during the intervention and the CRP levels were at comparable levels with the LGG group and the PJS group. At present, attempts to compare the *in vitro* results of one probiotic to its results in an *in vivo* setting are limited to comparisons between *in vitro* and experimental animal studies. In these studies, the anti-inflammatory effects seen as a high IL-10/IL-12 ratio *in vitro* have been reflected as protective effects in TNBS colitis model *in vivo* (Drouault-Holowacz et al., 2006; Foligne et al., 2007) and the modification of the cytokine response towards Th1-type response *in vitro* has been reflected as prevention of allergic response *in vivo* (Hisbergues et al., 2007). Furthermore, this study showed no correlation between serum cytokine levels and cytokines produced by PBMC *ex vivo*. Moreover, most of the changes were observed in the *ex vivo* produced cytokines. This raises the question on the relevance of serum cytokine measurements in probiotic interventions, especially in healthy adults.

Taken together, screening of the immunomodulatory properties of probiotics in vitro did not reflect the effects in vivo. The PBMC in vitro should not be used as the only screening method for immunomodulatory properties of probiotics. However, the ex vivo production of cytokines after probiotic intervention could offer a relatively easy and quick method for screening of immunomodulatory effects.

6.3 Clinical relevance and future aspects

All subjects investigated in the present study were healthy adults. The study covered a range of measurements of immunomodulation and inflammatory mediators as well as respiratory infections and gastrointestinal symptoms as clinical outcomes. Several small decreases in the inflammatory mediators (sensitive CRP, serum IL-6, TNF-α and IL-2 produced by PBMC *ex vivo*, sphingomyelin, lysophosphatidylcholine) were observed, indicating anti-inflammatory potential of probiotics. The clinical relevance of inflammatory mediators in healthy individuals and the relevance of observed small changes within a normal range, if established, can be questioned. It is clearly evident that a small change in one measurement does not necessarily denote better defence against inflammatory agents or infection. However, if small changes can be observed in several measurements and especially if the markers are clearly linked with each other, this gives indications of biologically meaningful events. The real impact of probiotics as anti-inflammatory substances thus warrants further research during inflammatory processes and in individuals suffering from various types of inflammatory diseases. For future probiotic interventions, screening of subjects for imbalance in inflammatory mediators would be advisable, even in apparently healthy adults. Additionally, the shortening of the duration of gastrointestinal symptoms with more than half after probiotic intervention can be considered as clinically meaningful. As the actions of probiotics are most probably mediated via the gut mucosal immune system, it would be extremely important to clarify the actual events in the gut mucosa. Lipidomics and other approaches of systems biology, such as gene expression analysis, proteomics and metabolomics, may provide powerful means for identifying new biomarkers behind the clinical effects of different nutritional interventions. They may also better characterize the clusters of small changes in markers associated with one other as they are able to detect changes in numerous markers from various sample materials simultaneously.

7. Summary and conclusions

The present study investigated the immunomodulatory effects of probiotics in a primary cell culture model using human PBMC as well as in healthy adults in randomized, double-blind, placebo-controlled clinical intervention studies. The main findings in the present study were as follows:

1. Probiotic strains from six different genera showed clear differences in their ability to induce cytokine responses in PBMC *in vitro*. Strains belonging to the *Streptococcus* and *Leuconostoc* genera were the most potent inducers of Th1-type cytokines, whereas strains from the *Bifidobacterium* and *Propionibacterium* genera induced anti-inflammatory IL-10 production. No combinations of probiotics resulted in enhanced cytokine production suggesting that different bacteria compete with each other during host cell-probiotic interactions.

2. Probiotics, especially LGG, appeared to have anti-inflammatory effects in healthy adults reflected as a small decrease in inflammatory mediators, such as sensitive CRP, inflammatory cytokines as well as inflammatory lipid-derived mediators, namely lysophosphatidylcholines and sphingomyelins, in serum. Both LGG and Bb12 seem to have a role in modulating the functional responses of PBMC.

3. The *in vitro* screening of cytokine responses in a primary cell culture using human PBMC should not be used as the only indicator of immunomodulatory properties of probiotics, as the *in vitro* model did not reflect the effects *in vivo*. Instead, the *ex vivo* production of cytokines in PBMC after probiotic intervention could offer a relatively easy and quick model for screening of immunomodulatory effects of probiotics. Calculations of the ratios between pro- and anti-inflammatory or Th1/Th2 cytokines could give useful information on the specific properties of probiotics.

4. LGG had no effect on the incidence or duration of respiratory infections in healthy adults. However, LGG was able to reduce the duration of gastrointestinal symptoms.

In conclusion, probiotics are strain-specifically able to modulate the release and actions of inflammatory mediators in healthy adults. Immunomodulatory effects of probiotic multispecies should be studied as the effects differ from single strains. The *ex vivo* production of cytokines in PBMC after probiotic intervention could be used as a screening method for immunomodulatory properties of probiotics. New cutting-edge systems biology technologies may provide new tools for further investigation of the mechanisms of specific host-probiotic interactions in the gut resulting in systemic and clinical effects.

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References

Acheson DW, Luccioli S. Microbial-gut interactions in health and disease. Mucosal immune responses. Best Pract Res Clin Gastroenterol 2004; 18: 387-404.

Akira S. Toll-like receptors and innate immunity. Adv Immunol 2001; 78: 1-56.

Alander M, Satokari R, Korpela R, Saxelin M, Vilpponen-Salmela T, Mattila-Sandholm T, et al. Persistence of colonization of human colonic mucosa by a probiotic strain, *Lactobacillus rhamnosus* GG, after oral consumption. Appl Environ Microbiol 1999; 65: 351-4.

Albers R, Antoine JM, Bourdet-Sicard R, Calder PC, Gleeson M, Lesourd B, et al. Markers to measure immunomodulation in human nutrition intervention studies. Br J Nutr 2005; 94: 452-81.

Alexopoulou L, Kontoyiannis D. Contribution of microbial-associated molecules in innate mucosal responses. Cell Mol Life Sci 2005; 62: 1349-58.

Anderson AD, McNaught CE, Jain PK, MacFie J. Randomised clinical trial of synbiotic therapy in elective surgical patients. Gut 2004; 53: 241-5.

Arunachalam K, Gill HS, Chandra RK. Enhancement of natural immune function by dietary consumption of *Bifidobacterium lactis* (HN019). Eur J Clin Nutr 2000; 54: 263-7.

Arvå E, Andersson B. Induction of phagocyte-stimulating and Th1-promoting cytokines by *in vitro* stimulation of human peripheral blood mononuclear cells with *Streptococcus pneumoniae*. Scand J Immunol 1999; 49: 417-23.

Banasaz M, Norin E, Holma R, Midtvedt T. Increased enterocyte production in gnotobiotic rats mono-associated with *Lactobacillus rhamnosus* GG. Appl Environ Microbiol 2002; 68: 3031-4.

Barker M, Rayens W. Partial least squares for discrimination. J Chemometr 2003; 17: 166-73.

Barksby HE, Lea SR, Preshaw PM, Taylor JJ. The expanding family of interleukin-1 cytokines and their role in destructive inflammatory disorders. Clin Exp Immunol 2007; 149: 217-25.

Baumgart DC, Dignass AU. Intestinal barrier function. Curr Opin Clin Nutr Metab Care 2002; 5: 685-94.

Bertazza L, Mocellin S. Tumor necrosis factor (TNF) biology and cell death. Front Biosci 2008; 13: 2736-43.

Beutler B. Innate immunity: an overview. Mol Immunol 2004; 40: 845-59.

Bismuth J, Lin P, Yao Q, Chen C. Ceramide: a common pathway for atherosclerosis? Atherosclerosis 2008; 196: 497-504.

Blaut M, Clavel T. Metabolic diversity of the intestinal microbiota: implications for health and disease. J Nutr 2007; 137: S751-5.

Bono MR, Elgueta R, Sauma D, Pino K, Osorio F, Michea P, et al. The essential role of chemokines in the selective regulation of lymphocyte homing. Cytokine Growth Factor Rev 2007; 18: 33-43.

Boyle RJ, Tang ML. The role of probiotics in the management of allergic disease. Clin Exp Allergy 2006; 36: 568-76.

Braat H, van den Brande J, van Tol E, Hommes D, Peppelenbosch M, van Deventer S. *Lactobacillus rhamnosus* induces peripheral hyporesponsiveness in stimulated CD4+ T cells via modulation of dendritic cell function. Am J Clin Nutr 2004; 80: 1618-25.

Brigden M. The erythrocyte sedimentation rate. Still a helpful test when used judiciously. Postgrad Med 1998; 103: 257-62, 272-4.

Bruunsgaard H. Physical activity and modulation of systemic low-level inflammation. J Leukoc Biol 2005; 78: 819-35.

Caballero-Franco C, Keller K, De Simone C, Chadee K. The VSL#3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells. Am J Physiol Gastrointest Liver Physiol 2007; 292: G315-22.

Calder PC, Kew S. The immune system: a target for functional foods? Br J Nutr 2002; 88 Suppl 2: S165-77.

Calder PC, Krauss-Etschmann S, de Jong EC, Dupont C, Frick JS, Frokiaer H, et al. Early nutrition and immunity - progress and perspectives. Br J Nutr 2006; 96: 774-90.

Castanheira LG, Castro JM, Martins-Filho Ode A, Nicoli JR, Vieira LQ, Afonso LC. *Lactobacillus delbrueckii* as a potential skin adjuvant for induction of type 1 immune responses. Front Biosci 2007; 12: 1300-7.

Castellazzi AM, Valsecchi C, Montagna L, Malfa P, Ciprandi G, Avanzini MA, et al. *In vitro* activation of mononuclear cells by two probiotics: *Lactobacillus paracasei* I 1688, *Lactobacillus salivarius* I 1794, and their mixture (PSMIX). Immunol Invest 2007; 36: 413-21.

Chadwick VS, Chen W, Shu D, Paulus B, Bethwaite P, Tie A, et al. Activation of the mucosal immune system in irritable bowel syndrome. Gastroenterology 2002; 122: 1778-83.

Chaplin DD. 1. Overview of the human immune response. J Allergy Clin Immunol 2006; 117: S430-5.

Chen H. Cellular inflammatory responses: novel insights for obesity and insulin resistance. Pharmacol Res 2006; 53: 469-77.

Chiang BL, Sheih YH, Wang LH, Liao CK, Gill HS. Enhancing immunity by dietary consumption of a probiotic lactic acid bacterium (*Bifidobacterium lactis* HN019): optimization and definition of cellular immune responses. Eur J Clin Nutr 2000; 54: 849-55.

Chouraqui JP, Van Egroo LD, Fichot MC. Acidified milk formula supplemented with *Bifidobacterium lactis*: impact on infant diarrhea in residential care settings. J Pediatr Gastroenterol Nutr 2004; 38: 288-92.

Christensen HR, Larsen CN, Kaestel P, Rosholm LB, Sternberg C, Michaelsen KF, et al. Immunomodulating potential of supplementation with probiotics: a dose-response study in healthy young adults. FEMS Immunol Med Microbiol 2006; 47: 380-90.

Clancy RL, Gleeson M, Cox A, Callister R, Dorrington M, D'Este C, et al. Reversal in fatigued athletes of a defect in interferon gamma secretion after administration of *Lactobacillus acidophilus*. Br J Sports Med 2006; 40: 351-4.

Cloez-Tayarani I, Changeux JP. Nicotine and serotonin in immune regulation and inflammatory processes: a perspective. J Leukoc Biol 2007; 81: 599-606.

Cobo Sanz JM, Mateos JA, Munoz Conejo A. Effect of *Lactobacillus casei* on the incidence of infectious conditions in children. Nutr Hosp 2006; 21: 547-51.

Collado MC, Jalonen L, Meriluoto J, Salminen S. Protection mechanism of probiotic combination against human pathogens: *in vitro* adhesion to human intestinal mucus. Asia Pac J Clin Nutr 2006; 15: 570-5.

Collado MC, Meriluoto J, Salminen S. Role of commercial probiotic strains against human pathogen adhesion to intestinal mucus. Lett Appl Microbiol 2007; 45: 454-60.

Couper KN, Blount DG, Riley EM. IL-10: The master regulator of immunity to infection. J Immunol 2008; 180: 5771-7.

Cox AJ, Pyne DB, Saunders PU, Fricker PA. Oral administration of the probiotic *Lactobacillus fermentum* VRI-003 and mucosal immunity in endurance athletes. Br J Sports Med 2008 Feb 13 [Epub ahead of print].

Cummings JH, Antoine JM, Azpiroz F, Bourdet-Sicard R, Brandtzaeg P, Calder PC, et al. PASSCLAIM--gut health and immunity. Eur J Nutr 2004; 43 Suppl 2: II118-II173.

de Vrese M, Rautenberg P, Laue C, Koopmans M, Herremans T, Schrezenmeir J. Probiotic bacteria stimulate virus-specific neutralizing antibodies following a booster polio vaccination. Eur J Nutr 2005a; 44: 406-13.

de Vrese M, Winkler P, Rautenberg P, Harder T, Noah C, Laue C, et al. Effect of *Lactobacillus gasseri* PA 16/8, *Bifidobacterium longum* SP 07/3, *B. bifidum* MF 20/5 on common cold episodes: a double blind, randomized, controlled trial. Clin Nutr 2005b; 24: 481-91.

de Vrese M, Winkler P, Rautenberg P, Harder T, Noah C, Laue C, et al. Probiotic bacteria reduced duration and severity but not the incidence of common cold episodes in a double blind, randomized, controlled trial. Vaccine 2006; 24: 6670-4.

Di Caro S, Tao H, Grillo A, Elia C, Gasbarrini G, Sepulveda AR, et al. Effects of *Lactobacillus* GG on genes expression pattern in small bowel mucosa. Dig Liver Dis 2005; 37: 320-9.

Donnet-Hughes A, Rochat F, Serrant P, Aeschlimann JM, Schiffrin EJ. Modulation of nonspecific mechanisms of defense by lactic acid bacteria: effective dose. J Dairy Sci 1999; 82: 863-9.

Drouault-Holowacz S, Foligne B, Dennin V, Goudercourt D, Terpend K, Burckel A, et al. Anti-inflammatory potential of the probiotic dietary supplement Lactibiane Tolerance: *in vitro* and *in vivo* considerations. Clin Nutr 2006; 25: 994-1003.

Dubois B, Goubier A, Joubert G, Kaiserlian D. Oral tolerance and regulation of mucosal immunity. Cell Mol Life Sci 2005; 62: 1322-32.

Egert M, de Graaf AA, Smidt H, de Vos WM, Venema K. Beyond diversity: functional microbiomics of the human colon. Trends Microbiol 2006; 14: 86-91.

Emmer PM, van der Vlag J, Adema GJ, Hilbrands LB. Dendritic cells activated by lipopolysaccharide after dexamethasone treatment induce donor-specific allograft hyporesponsiveness. Transplantation 2006; 81: 1451-9.

Ewaschuk JB, Dieleman LA. Probiotics and prebiotics in chronic inflammatory bowel diseases. World J Gastroenterol 2006; 12: 5941-50.

FAO/WHO. Guidelines for the evaluation of probiotics in food. Report of a joint FAO/WHO working group on drafting guidelines for the evaluation of probiotics in food. Vol 2002. London Ontario, Canada: World Health Oraganization, 2002.

Favier CF, Vaughan EE, De Vos WM, Akkermans AD. Molecular monitoring of succession of bacterial communities in human neonates. Appl Environ Microbiol 2002; 68: 219-26.

Feleszko W, Jaworska J, Rha RD, Steinhausen S, Avagyan A, Jaudszus A, et al. Probiotic-induced suppression of allergic sensitization and airway inflammation is associated with an increase of T regulatory-dependent mechanisms in a murine model of asthma. Clin Exp Allergy 2007; 37: 498-505.

Feng WH, Tompkins MB, Xu JS, Zhang HX, McCaw MB. Analysis of constitutive cytokine expression by pigs infected in-utero with porcine reproductive and respiratory syndrome virus. Vet Immunol Immunopathol 2003; 94: 35-45.

Foligne B, Nutten S, Grangette C, Dennin V, Goudercourt D, Poiret S, et al. Correlation between *in vitro* and *in vivo* immunomodulatory properties of lactic acid bacteria. World J Gastroenterol 2007; 13: 236-43.

Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 1972; 18: 499-502.

Fukushima Y, Kawata Y, Hara H, Terada A, Mitsuoka T. Effect of a probiotic formula on intestinal immunoglobulin A production in healthy children. Int J Food Microbiol 1998; 42: 39-44.

Furrie E, Macfarlane S, Kennedy A, Cummings JH, Walsh SV, O'Neil D A, et al. Synbiotic therapy (*Bifidobacterium longum*/Synergy 1) initiates resolution of inflammation in patients with active ulcerative colitis: a randomised controlled pilot trial. Gut 2005; 54: 242-9.

Gabay C. Interleukin-6 and chronic inflammation. Arthritis Res Ther 2006; 8 Suppl 2: S3.

Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. N Engl J Med 1999; 340: 448-54.

Gackowska L, Michalkiewicz J, Krotkiewski M, Helmin-Basa A, Kubiszewska I, Dzierzanowska D. Combined effect of different lactic acid bacteria strains on the mode of cytokines pattern expression in human peripheral blood mononuclear cells. J Physiol Pharmacol 2006; 57 Suppl 9: 13-21.

Galpin L, Manary MJ, Fleming K, Ou CN, Ashorn P, Shulman RJ. Effect of *Lactobacillus* GG on intestinal integrity in Malawian children at risk of tropical enteropathy. Am J Clin Nutr 2005; 82: 1040-5.

Gill HS, Rutherfurd KJ, Cross ML. Dietary probiotic supplementation enhances natural killer cell activity in the elderly: an investigation of age-related immunological changes. J Clin Immunol 2001a; 21: 264-71.

Gill HS, Rutherfurd KJ, Cross ML, Gopal PK. Enhancement of immunity in the elderly by dietary supplementation with the probiotic *Bifidobacterium lactis* HN019. Am J Clin Nutr 2001b; 74: 833-9.

Gleeson M. Mucosal immune responses and risk of respiratory illness in elite athletes. Exerc Immunol Rev 2000; 6: 5-42.

Gleeson M. Immune function in sport and exercise. J Appl Physiol 2007; 103: 693-9.

Gotteland M, Cruchet S, Verbeke S. Effect of *Lactobacillus* ingestion on the gastrointestinal mucosal barrier alterations induced by indometacin in humans. Aliment Pharmacol Ther 2001; 15: 11-7.

Grönlund MM, Lehtonen OP, Eerola E, Kero P. Fecal microflora in healthy infants born by different methods of delivery: permanent changes in intestinal flora after cesarean delivery. J Pediatr Gastroenterol Nutr 1999; 28: 19-25.

Guandalini S. Probiotics for children: use in diarrhea. J Clin Gastroenterol 2006; 40: 244-8.

Guarner F. Enteric flora in health and disease. Digestion 2006; 73 Suppl 1: 5-12.

Gurney KB, Elliott J, Nassanian H, Song C, Soilleux E, McGowan I, et al. Binding and transfer of human immunodeficiency virus by DC-SIGN+ cells in human rectal mucosa. J Virol 2005; 79: 5762-73.

Haapamäki MM, Grönroos JM, Nurmi H, Irjala K, Alanen KA, Nevalainen TJ. Phospholipase A2 in serum and colonic mucosa in ulcerative colitis. Scand J Clin Lab Invest 1999; 59: 279-87.

Haller D, Blum S, Bode C, Hammes WP, Schiffrin EJ. Activation of human peripheral blood mononuclear cells by nonpathogenic bacteria *in vitro*: evidence of NK cells as primary targets. Infect Immun 2000; 68: 752-9.

Hammerman C, Bin-Nun A, Kaplan M. Safety of probiotics: comparison of two popular strains. BMJ 2006; 333: 1006-8.

Hatakka K. Probiotics in the prevention of clinical manifestations of common infectious diseases in children and in the elderly. Medical Faculty. Helsinki: University of Helsinki, 2007.

Hatakka K, Blomgren K, Pohjavuori S, Kaijalainen T, Poussa T, Leinonen M, et al. Treatment of acute otitis media with probiotics in otitis-prone children-a double-blind, placebo-controlled randomised study. Clin Nutr 2007; 26: 314-21.

Hatakka K, Martio J, Korpela M, Herranen M, Poussa T, Laasanen T, et al. Effects of probiotic therapy on the activity and activation of mild rheumatoid arthritis--a pilot study. Scand J Rheumatol 2003; 32: 211-5.

Hatakka K, Savilahti E, Pönkä A, Meurman JH, Poussa T, Näse L, et al. Effect of long term consumption of probiotic milk on infections in children attending day care centres: double blind, randomised trial. BMJ 2001; 322: 1327.

He F, Tuomola E, Arvola H, Salminen S. Modulation of humoral immune response through probiotic intake. FEMS Immunol Med Microbiol 2000; 29: 47-52.

Helwig U, Lammers KM, Rizzello F, Brigidi P, Rohleder V, Caramelli E, et al. Lactobacilli, bifidobacteria and *E. coli* nissle induce pro- and anti-inflammatory cytokines in peripheral blood mononuclear cells. World J Gastroenterol 2006; 12: 5978-86.

Hessle C, Andersson B, Wold AE. Gram-positive bacteria are potent inducers of monocytic interleukin-12 (IL-12) while gram-negative bacteria preferentially stimulate IL-10 production. Infect Immun 2000; 68: 3581-6.

Hessle C, Hanson LA, Wold AE. Lactobacilli from human gastrointestinal mucosa are strong stimulators of IL-12 production. Clin Exp Immunol 1999; 116: 276-82.

Hisbergues M, Magi M, Rigaux P, Steuve J, Garcia L, Goudercourt D, et al. *In vivo* and *in vitro* immunomodulation of Der p 1 allergen-specific response by *Lactobacillus plantarum* bacteria. Clin Exp Allergy 2007; 37: 1286-95.

Holm SE, Falsen E. An antigen free medium for cultivation of beta-hemolytic streptococci. Acta Pathol Microbiol Scand 1967; 69: 264-7.

Homaidan FR, El-Sabban ME, Chakroun I, El-Sibai M, Dbaibo GS. IL-1 stimulates ceramide accumulation without inducing apoptosis in intestinal epithelial cells. Mediators Inflamm 2002; 11: 39-45.

Huurre A, Kalliomäki M, Rautava S, Rinne M, Salminen S, Isolauri E. Mode of delivery - effects on gut microbiota and humoral immunity. Neonatology 2007; 93: 236-40.

Isolauri E, Arvola T, Sütas Y, Moilanen E, Salminen S. Probiotics in the management of atopic eczema. Clin Exp Allergy 2000; 30: 1604-10.

Isolauri E, Joensuu J, Suomalainen H, Luomala M, Vesikari T. Improved immunogenicity of oral D x RRV reassortant rotavirus vaccine by *Lactobacillus casei* GG. Vaccine 1995; 13: 310-2.

Isolauri E, Kaila M, Arvola T, Majamaa H, Rantala I, Virtanen E, et al. Diet during rotavirus enteritis affects jejunal permeability to macromolecules in suckling rats. Pediatr Res 1993a; 33: 548-53.

Isolauri E, Kaila M, Mykkänen H, Ling WH, Salminen S. Oral bacteriotherapy for viral gastroenteritis. Dig Dis Sci 1994; 39: 2595-600.

Isolauri E, Majamaa H, Arvola T, Rantala I, Virtanen E, Arvilommi H. *Lactobacillus casei* strain GG reverses increased intestinal permeability induced by cow milk in suckling rats. Gastroenterology 1993b; 105: 1643-50.

Itoh M, Uchimura K, Yamamoto K, Makino M, Imamura S, Kobayashi T, et al. Distinctive response of thyroid-infiltrating mononuclear cells to B cell activation through CD40 and interleukin-4 in Graves' patients. Cytokine 2002; 19: 107-14.

Izcue A, Powrie F. Special regulatory T-cell review: Regulatory T cells and the intestinal tract--patrolling the frontier. Immunology 2008; 123: 6-10.

Jernberg C, Löfmark S, Edlund C, Jansson JK. Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. ISME J 2007; 1: 56-66.

Johnson-Henry KC, Donato KA, Shen-Tu G, Gordanpour M, Sherman PM. *Lactobacillus rhamnosus* strain GG prevents enterohemorrhagic *Escherichia coli* O157:H7-induced changes in epithelial barrier function. Infect Immun 2008; 76: 1340-8.

Jones JL, Foxx-Orenstein AE. The role of probiotics in inflammatory bowel disease. Dig Dis Sci 2007; 52: 607-11.

Jutel M, Blaser K, Akdis CA. The role of histamine in regulation of immune responses. Chem Immunol Allergy 2006; 91: 174-87.

Kaila M, Isolauri E, Soppi E, Virtanen E, Laine S, Arvilommi H. Enhancement of the circulating antibody secreting cell response in human diarrhea by a human *Lactobacillus* strain. Pediatr Res 1992; 32: 141-4.

Kajander K, Hatakka K, Poussa T, Färkkilä M, Korpela R. A probiotic mixture alleviates symptoms in irritable bowel syndrome patients: a controlled 6-month intervention. Aliment Pharmacol Ther 2005; 22: 387-94.

Kajander K, Myllyluoma E, Rajilic-Stojanovic M, Kyrönpalo S, Rasmussen M, Järvenpää S, et al. Clinical trial: multispecies probiotic supplementation alleviates the symptoms of irritable bowel syndrome and stabilizes intestinal microbiota. Aliment Pharmacol Ther 2008; 27: 48-57.

Kalliomäki M, Salminen S, Arvilommi H, Kero P, Koskinen P, Isolauri E. Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. Lancet 2001; 357: 1076-9.

Karlqvist PA, Franzen L, Sjödahl R, Tagesson C. Lysophosphatidylcholine and taurodeoxycholate increase stomach permeability to different-sized molecules. Scand J Gastroenterol 1986; 21: 1039-45.

Katajamaa M, Miettinen J, Oresic M. MZmine: toolbox for processing and visualization of mass spectrometry based molecular profile data. Bioinformatics 2006; 22: 634-6.

Khanapure SP, Garvey DS, Janero DR, Letts LG. Eicosanoids in inflammation: biosynthesis, pharmacology, and therapeutic frontiers. Curr Top Med Chem 2007; 7: 311-40.

Khovidhunkit W, Kim MS, Memon RA, Shigenaga JK, Moser AH, Feingold KR, et al. Effects of infection and inflammation on lipid and lipoprotein metabolism: mechanisms and consequences to the host. J Lipid Res 2004; 45: 1169-96.

Kidd P. Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. Altern Med Rev 2003; 8: 223-46.

Kishimoto T. Interleukin-6: discovery of a pleiotropic cytokine. Arthritis Res Ther 2006; 8 Suppl 2: S2.

Klein A, Friedrich U, Vogelsang H, Jahreis G. *Lactobacillus acidophilus* 74-2 and *Bifidobacterium animalis* subsp *lactis* DGCC 420 modulate unspecific cellular immune response in healthy adults. Eur J Clin Nutr 2007 Apr 18 [Epub ahead of print].

Kobayashi Y. The role of chemokines in neutrophil biology. Front Biosci 2008; 13: 2400-7.

Koopman LP, Savelkoul H, van Benten IJ, Gerritsen J, Brunekreef B, H JN. Increased serum IL-10/IL-12 ratio in wheezing infants. Pediatr Allergy Immunol 2003; 14: 112-9.

Korhonen R, Korpela R, Saxelin M, Mäki M, Kankaanranta H, Moilanen E. Induction of nitric oxide synthesis by probiotic *Lactobacillus rhamnosus* GG in J774 macrophages and human T84 intestinal epithelial cells. Inflammation 2001; 25: 223-32.

Kougias P, Chai H, Lin PH, Lumsden AB, Yao Q, Chen C. Lysophosphatidylcholine and secretory phospholipase A2 in vascular disease: mediators of endothelial dysfunction and atherosclerosis. Med Sci Monit 2006; 12: RA5-16.

Kuisma J, Mentula S, Järvinen H, Kahri A, Saxelin M, Färkkilä M. Effect of *Lactobacillus rhamnosus* GG on ileal pouch inflammation and microbial flora. Aliment Pharmacol Ther 2003; 17: 509-15.

Kukkonen K, Savilahti E, Haahtela T, Juntunen-Backman K, Korpela R, Poussa T, et al. Probiotics and prebiotic galacto-oligosaccharides in the prevention of allergic diseases: a randomized, double-blind, placebo-controlled trial. J Allergy Clin Immunol 2007; 119: 192-8.

Lam EK, Tai EK, Koo MW, Wong HP, Wu WK, Yu L, et al. Enhancement of gastric mucosal integrity by *Lactobacillus rhamnosus* GG. Life Sci 2007; 80: 2128-36.

Lammers KM, Brigidi P, Vitali B, Gionchetti P, Rizzello F, Caramelli E, et al. Immunomodulatory effects of probiotic bacteria DNA: IL-1 and IL-10 response in human peripheral blood mononuclear cells. FEMS Immunol Med Microbiol 2003; 38: 165-72.

Leblanc J, Fliss I, Matar C. Induction of a humoral immune response following an *Escherichia coli* O157:H7 infection with an immunomodulatory peptidic fraction derived from *Lactobacillus helveticus*-fermented milk. Clin Diagn Lab Immunol 2004; 11: 1171-81.

Legg JP, Hussain IR, Warner JA, Johnston SL, Warner JO. Type 1 and type 2 cytokine imbalance in acute respiratory syncytial virus bronchiolitis. Am J Respir Crit Care Med 2003; 168: 633-9.

Lenoir-Wijnkoop I, Sanders ME, Cabana MD, Caglar E, Corthier G, Rayes N, et al. Probiotic and prebiotic influence beyond the intestinal tract. Nutr Rev 2007; 65: 469-89.

Lievin-Le Moal V, Servin AL. The front line of enteric host defense against unwelcome intrusion of harmful microorganisms: mucins, antimicrobial peptides, and microbiota. Clin Microbiol Rev 2006; 19: 315-37.

Limdi JK, O'Neill C, McLaughlin J. Do probiotics have a therapeutic role in gastroenterology? World J Gastroenterol 2006; 12: 5447-57.

Link-Amster H, Rochat F, Saudan KY, Mignot O, Aeschlimann JM. Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intake. FEMS Immunol Med Microbiol 1994; 10: 55-63.

Lorea Baroja M, Kirjavainen PV, Hekmat S, Reid G. Anti-inflammatory effects of probiotic yogurt in inflammatory bowel disease patients. Clin Exp Immunol 2007; 149: 470-9.

Mack DR, Ahrne S, Hyde L, Wei S, Hollingsworth MA. Extracellular MUC3 mucin secretion follows adherence of *Lactobacillus* strains to intestinal epithelial cells *in vitro*. Gut 2003; 52: 827-33.

Mack DR, Michail S, Wei S, McDougall L, Hollingsworth MA. Probiotics inhibit enteropathogenic *E. coli* adherence *in vitro* by inducing intestinal mucin gene expression. Am J Physiol 1999; 276: G941-50.

Madsen K, Cornish A, Soper P, McKaigney C, Jijon H, Yachimec C, et al. Probiotic bacteria enhance murine and human intestinal epithelial barrier function. Gastroenterology 2001; 121: 580-91.

Majamaa H, Isolauri E, Saxelin M, Vesikari T. Lactic acid bacteria in the treatment of acute rotavirus gastroenteritis. J Pediatr Gastroenterol Nutr 1995; 20: 333-8.

Malin M, Suomalainen H, Saxelin M, Isolauri E. Promotion of IgA immune response in patients with Crohn's disease by oral bacteriotherapy with *Lactobacillus* GG. Ann Nutr Metab 1996; 40: 137-45.

Marcos A, Warnberg J, Nova E, Gomez S, Alvarez A, Alvarez R, et al. The effect of milk fermented by yogurt cultures plus *Lactobacillus casei* DN-114001 on the immune response of subjects under academic examination stress. Eur J Nutr 2004; 43: 381-9.

Marschan E, Kuitunen M, Kukkonen K, Poussa T, Sarnesto A, Haahtela T, et al. Probiotics in infancy induce protective immune profiles that are characteristic for chronic low-grade inflammation. Clin Exp Allergy 2008; 38: 611-8.

McCracken VJ, Lorenz RG. The gastrointestinal ecosystem: a precarious alliance among epithelium, immunity and microbiota. Cell Microbiol 2001; 3: 1-11.

McFarland LV. Meta-analysis of probiotics for the prevention of traveler's diarrhea. Travel Med Infect Dis 2007; 5: 97-105.

McNaught CE, Woodcock NP, Anderson AD, MacFie J. A prospective randomised trial of probiotics in critically ill patients. Clin Nutr 2005; 24: 211-9.

McNaught CE, Woodcock NP, MacFie J, Mitchell CJ. A prospective randomised study of the probiotic *Lactobacillus plantarum* 299V on indices of gut barrier function in elective surgical patients. Gut 2002; 51: 827-31.

Medina M, Izquierdo E, Ennahar S, Sanz Y. Differential immunomodulatory properties of *Bifidobacterium longum* strains: relevance to probiotic selection and clinical applications. Clin Exp Immunol 2007; 150: 531-8.

Melendez AJ. Sphingosine kinase signalling in immune cells: Potential as novel therapeutic targets. Biochim Biophys Acta 2008; 1784: 66-75.

Meyer AL, Micksche M, Herbacek I, Elmadfa I. Daily intake of probiotic as well as conventional yogurt has a stimulating effect on cellular immunity in young healthy women. Ann Nutr Metab 2006; 50: 282-9.

Miettinen M, Lehtonen A, Julkunen I, Matikainen S. Lactobacilli and Streptococci activate NF-kappa B and STAT signaling pathways in human macrophages. J Immunol 2000; 164: 3733-40.

Miettinen M, Matikainen S, Vuopio-Varkila J, Pirhonen J, Varkila K, Kurimoto M, et al. Lactobacilli and streptococci induce interleukin-12 (IL-12), IL-18, and gamma interferon production in human peripheral blood mononuclear cells. Infect Immun 1998; 66: 6058-62.

Miettinen M, Vuopio-Varkila J, Varkila K. Production of human tumor necrosis factor alpha, interleukin-6, and interleukin-10 is induced by lactic acid bacteria. Infect Immun 1996; 64: 5403-5.

Minami T, Tojo H, Shinomura Y, Matsuzawa Y, Okamoto M. Increased group II phospholipase A2 in colonic mucosa of patients with Crohn's disease and ulcerative colitis. Gut 1994; 35: 1593-8.

Muzio M, Polentarutti N, Bosisio D, Prahladan MK, Mantovani A. Toll-like receptors: a growing family of immune receptors that are differentially expressed and regulated by different leukocytes. J Leukoc Biol 2000; 67: 450-6.

Myllyluoma E, Kajander K, Mikkola H, Kyrönpalo S, Rasmussen M, Kankuri E, et al. Probiotic intervention decreases serum gastrin-17 in *Helicobacter pylori* infection. Dig Liver Dis 2007; 39: 516-23.

Myllyluoma E, Veijola L, Ahlroos T, Tynkkynen S, Kankuri E, Vapaatalo H, et al. Probiotic supplementation improves tolerance to *Helicobacter pylori* eradication therapy--a placebo-controlled, double-blind randomized pilot study. Aliment Pharmacol Ther 2005; 21: 1263-72.

Naruszewicz M, Johansson ML, Zapolska-Downar D, Bukowska H. Effect of *Lactobacillus plantarum* 299v on cardiovascular disease risk factors in smokers. Am J Clin Nutr 2002; 76: 1249-55.

Niers LE, Hoekstra MO, Timmerman HM, van Uden NO, de Graaf PM, Smits HH, et al. Selection of probiotic bacteria for prevention of allergic diseases: immunomodulation of neonatal dendritic cells. Clin Exp Immunol 2007; 149: 344-52.

Niers LE, Timmerman HM, Rijkers GT, van Bleek GM, van Uden NO, Knol EF, et al. Identification of strong interleukin-10 inducing lactic acid bacteria which down-regulate T helper type 2 cytokines. Clin Exp Allergy 2005; 35: 1481-9.

Nordmark EL, Yang Z, Huttunen E, Widmalm G. Structural studies of the exopolysaccharide produced by *Propionibacterium freudenreichii* ssp. *shermanii* JS. Biomacromolecules 2005; 6: 521-3.

O'Hara AM, O'Regan P, Fanning A, O'Mahony C, Macsharry J, Lyons A, et al. Functional modulation of human intestinal epithelial cell responses by *Bifidobacterium infantis* and *Lactobacillus salivarius*. Immunology 2006; 118: 202-15.

O'Keefe SJ. Nutrition and colonic health: the critical role of the microbiota. Curr Opin Gastroenterol 2008; 24: 51-8.

O'Mahony L, McCarthy J, Kelly P, Hurley G, Luo F, Chen K, et al. *Lactobacillus* and *bifidobacterium* in irritable bowel syndrome: symptom responses and relationship to cytokine profiles. Gastroenterology 2005; 128: 541-51.

O'Mahony L, O'Callaghan L, McCarthy J, Shilling D, Scully P, Sibartie S, et al. Differential cytokine response from dendritic cells to commensal and pathogenic bacteria in different lymphoid compartments in humans. Am J Physiol Gastrointest Liver Physiol 2006; 290: G839-45.

Oktedalen O, Lunde OC, Opstad PK, Aabakken L, Kvernebo K. Changes in the gastrointestinal mucosa after long-distance running. Scand J Gastroenterol 1992; 27: 270-4.

Olivares M, Diaz-Ropero MP, Gomez N, Lara-Villoslada F, Sierra S, Maldonado JA, et al. The consumption of two new probiotic strains, *Lactobacillus gasseri* CECT 5714 and *Lactobacillus coryniformis* CECT 5711, boosts the immune system of healthy humans. Int Microbiol 2006a; 9: 47-52.

Olivares M, Diaz-Ropero MP, Sierra S, Lara-Villoslada F, Fonolla J, Navas M, et al. Oral intake of *Lactobacillus fermentum* CECT5716 enhances the effects of influenza vaccination. Nutrition 2007; 23: 254-60.

Olivares M, Paz Diaz-Ropero M, Gomez N, Sierra S, Lara-Villoslada F, Martin R, et al. Dietary deprivation of fermented foods causes a fall in innate immune response. Lactic acid bacteria can counteract the immunological effect of this deprivation. J Dairy Res 2006b; 73: 492-8.

Olivera A, Rivera J. Sphingolipids and the balancing of immune cell function: lessons from the mast cell. J Immunol 2005; 174: 1153-8.

Ono SJ, Nakamura T, Miyazaki D, Ohbayashi M, Dawson M, Toda M. Chemokines: roles in leukocyte development, trafficking, and effector function. J Allergy Clin Immunol 2003; 111: 1185-99.

Otamiri T, Sjodahl R, Tagesson C. Lysophosphatidylcholine potentiates the increase in mucosal permeability after small-intestinal ischaemia. Scand J Gastroenterol 1986; 21: 1131-6.

Ouwehand AC. Antiallergic effects of probiotics. J Nutr 2007; 137: S794-7.

Palmer C, Bik EM, Digiulio DB, Relman DA, Brown PO. Development of the human infant intestinal microbiota. PLoS Biol 2007; 5: e177.

Pant N, Marcotte H, Brussow H, Svensson L, Hammarstrom L. Effective prophylaxis against rotavirus diarrhea using a combination of *Lactobacillus rhamnosus* GG and antibodies. BMC Microbiol 2007; 7: 86.

Park JH, Um JI, Lee BJ, Goh JS, Park SY, Kim WS, et al. Encapsulated *Bifidobacterium bifidum* potentiates intestinal IgA production. Cell Immunol 2002; 219: 22-7.

Parkin J, Cohen B. An overview of the immune system. Lancet 2001; 357: 1777-89.

Parra D, De Morentin BM, Cobo JM, Mateos A, Martinez JA. Monocyte function in healthy middle-aged people receiving fermented milk containing *Lactobacillus casei*. J Nutr Health Aging 2004a; 8: 208-11.

Parra MD, Martinez de Morentin BE, Cobo JM, Mateos A, Martinez JA. Daily ingestion of fermented milk containing *Lactobacillus casei* DN114001 improves innate-defense capacity in healthy middle-aged people. J Physiol Biochem 2004b; 60: 85-91.

Pawelec G. Immunity and ageing in man. Exp Gerontol 2006; 41: 1239-42.

Pedone CA, Arnaud CC, Postaire ER, Bouley CF, Reinert P. Multicentric study of the effect of milk fermented by *Lactobacillus casei* on the incidence of diarrhoea. Int J Clin Pract 2000; 54: 568-71.

Pedone CA, Bernabeu AO, Postaire ER, Bouley CF, Reinert P. The effect of supplementation with milk fermented by *Lactobacillus casei* (strain DN-114 001) on acute diarrhoea in children attending day care centres. Int J Clin Pract 1999; 53: 179-84.

Pelto L, Isolauri E, Lilius EM, Nuutila J, Salminen S. Probiotic bacteria down-regulate the milk-induced inflammatory response in milk-hypersensitive subjects but have an immunostimulatory effect in healthy subjects. Clin Exp Allergy 1998; 28: 1474-9.

Pereg D, Kimhi O, Tirosh A, Orr N, Kayouf R, Lishner M. The effect of fermented yogurt on the prevention of diarrhea in a healthy adult population. Am J Infect Control 2005; 33: 122-5.

Pessi T, Sütas Y, Marttinen A, Isolauri E. Probiotics reinforce mucosal degradation of antigens in rats: implications for therapeutic use of probiotics. J Nutr 1998; 128: 2313-8.

Peters HP, De Vries WR, Vanberge-Henegouwen GP, Akkermans LM. Potential benefits and hazards of physical activity and exercise on the gastrointestinal tract. Gut 2001; 48: 435-9.

Pirhonen J, Sareneva T, Kurimoto M, Julkunen I, Matikainen S. Virus infection activates IL-1 beta and IL-18 production in human macrophages by a caspase-1-dependent pathway. J Immunol 1999; 162: 7322-9.

Pochard P, Gosset P, Grangette C, Andre C, Tonnel AB, Pestel J, et al. Lactic acid bacteria inhibit TH2 cytokine production by mononuclear cells from allergic patients. J Allergy Clin Immunol 2002; 110: 617-23.

Pohjavuori E, Viljanen M, Korpela R, Kuitunen M, Tiittanen M, Vaarala O, et al. *Lactobacillus* GG effect in increasing IFN-gamma production in infants with cow's milk allergy. J Allergy Clin Immunol 2004; 114: 131-6.

Pujol P, Huguet J, Drobnic F, Banquells M, Ruiz O, Galilea P, et al. The effect of fermented milk containing *Lactobacillus casei* on the immune response to exercise. Sports Med Train Rehab 2000; 9: 209-23.

Rasche C, Wolfram C, Wahls M, Worm M. Differential immunomodulating effects of inactivated probiotic bacteria on the allergic immune response. Acta Derm Venereol 2007; 87: 305-11.

Rautava S, Arvilommi H, Isolauri E. Specific probiotics in enhancing maturation of IgA responses in formula-fed infants. Pediatr Res 2006; 60: 221-4.

Rautava S, Walker WA. Commensal bacteria and epithelial cross talk in the developing intestine. Curr Gastroenterol Rep 2007; 9: 385-92.

Reddy BS, Macfie J, Gatt M, Larsen CN, Jensen SS, Leser TD. Randomized clinical trial of effect of synbiotics, neomycin and mechanical bowel preparation on intestinal barrier function in patients undergoing colectomy. Br J Surg 2007; 94: 546-54.

Riddoch C, Trinick T. Gastrointestinal disturbances in marathon runners. Br J Sports Med 1988; 22: 71-4.

Robinson MJ, Sancho D, Slack EC, LeibundGut-Landmann S, Reis e Sousa C. Myeloid C-type lectins in innate immunity. Nat Immunol 2006; 7: 1258-65.

Roessler A, Friedrich U, Vogelsang H, Bauer A, Kaatz M, Hipler UC, et al. The immune system in healthy adults and patients with atopic dermatitis seems to be affected differently by a probiotic intervention. Clin Exp Allergy 2008; 38: 93-102.

Sakata A, Yasuda K, Ochiai T, Shimeno H, Hikishima S, Yokomatsu T, et al. Inhibition of lipopolysaccharide-induced release of interleukin-8 from intestinal epithelial cells by SMA, a novel inhibitor of sphingomyelinase and its therapeutic effect on dextran sulphate sodium-induced colitis in mice. Cell Immunol 2007; 245: 24-31.

Salminen MK, Tynkkynen S, Rautelin H, Saxelin M, Vaara M, Ruutu P, et al. *Lactobacillus* bacteremia during a rapid increase in probiotic use of *Lactobacillus rhamnosus* GG in Finland. Clin Infect Dis 2002; 35: 1155-60.

Salminen S, Benno Y, de Vos W. Intestinal colonisation, microbiota and future probiotics? Asia Pac J Clin Nutr 2006; 15: 558-62.

Sansonetti PJ. War and peace at mucosal surfaces. Nat Rev Immunol 2004; 4: 953-64.

Sawai T, Lampman R, Hua Y, Segura B, Drongowski RA, Coran AG, et al. Lysophosphatidylcholine alters enterocyte monolayer permeability via a protein kinase C/Ca2+ mechanism. Pediatr Surg Int 2002; 18: 591-4.

Saxelin M, Tynkkynen S, Mattila-Sandholm T, de Vos WM. Probiotic and other functional microbes: from markets to mechanisms. Curr Opin Biotechnol 2005; 16: 204-11.

Sazawal S, Hiremath G, Dhingra U, Malik P, Deb S, Black RE. Efficacy of probiotics in prevention of acute diarrhoea: a meta-analysis of masked, randomised, placebo-controlled trials. Lancet Infect Dis 2006; 6: 374-82.

Schiffrin EJ, Blum S. Interactions between the microbiota and the intestinal mucosa. Eur J Clin Nutr 2002; 56 Suppl 3: S60-4.

Schiffrin EJ, Brassart D, Servin AL, Rochat F, Donnet-Hughes A. Immune modulation of blood leukocytes in humans by lactic acid bacteria: criteria for strain selection. Am J Clin Nutr 1997; 66: S515-20.

Schnackenberg LK, Beger RD. Monitoring the health to disease continuum with global metabolic profiling and systems biology. Pharmacogenomics 2006; 7: 1077-86.

Schoenborn JR, Wilson CB. Regulation of interferon-gamma during innate and adaptive immune responses. Adv Immunol 2007; 96: 41-101.

Schultz M, Linde HJ, Lehn N, Zimmermann K, Grossmann J, Falk W, et al. Immunomodulatory consequences of oral administration of *Lactobacillus rhamnosus* strain GG in healthy volunteers. J Dairy Res 2003; 70: 165-73.

Servin AL. Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS Microbiol Rev 2004; 28: 405-40.

Sheih YH, Chiang BL, Wang LH, Liao CK, Gill HS. Systemic immunity-enhancing effects in healthy subjects following dietary consumption of the lactic acid bacterium *Lactobacillus rhamnosus* HN001. J Am Coll Nutr 2001; 20: 149-56.

Shi Y, Zhang P, Zhang L, Osman H, Mohler ER, Macphee C, et al. Role of lipoprotein-associated phospholipase A2 in leukocyte activation and inflammatory responses. Atherosclerosis 2007; 191: 54-62.

Shida K, Kiyoshima-Shibata J, Nagaoka M, Watanabe K, Nanno M. Induction of interleukin-12 by *Lactobacillus* strains having a rigid cell wall resistant to intracellular digestion. J Dairy Sci 2006a; 89: 3306-17.

Shida K, Suzuki T, Kiyoshima-Shibata J, Shimada S, Nanno M. Essential roles of monocytes in stimulating human peripheral blood mononuclear cells with *Lactobacillus casei* to produce cytokines and augment natural killer cell activity. Clin Vaccine Immunol 2006b; 13: 997-1003.

Siigur U, Tamm E, Torm S, Lutsar I, Salminen S, Midtvedt T. Effect of bacterial infection and administration of a probiotic on faecal short-chain fatty acids. Microb Ecol Health Dis 1996; 9: 271-77.

Simons SM, Kennedy RG. Gastrointestinal problems in runners. Curr Sports Med Rep 2004; 3: 112-6.

Smetanka RD, Lambert GP, Murray R, Eddy D, Horn M, Gisolfi CV. Intestinal permeability in runners in the 1996 Chicago marathon. Int J Sport Nutr 1999; 9: 426-33.

Smits HH, Engering A, van der Kleij D, de Jong EC, Schipper K, van Capel TM, et al. Selective probiotic bacteria induce IL-10-producing regulatory T cells *in vitro* by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin. J Allergy Clin Immunol 2005; 115: 1260-7.

Snydman DR. The safety of probiotics. Clin Infect Dis 2008; 46 Suppl 2: S104-11; discussion S144-51.

Sobko T, Huang L, Midtvedt T, Norin E, Gustafsson LE, Norman M, et al. Generation of NO by probiotic bacteria in the gastrointestinal tract. Free Radic Biol Med 2006; 41: 985-91.

Spanhaak S, Havenaar R, Schaafsma G. The effect of consumption of milk fermented by *Lactobacillus casei* strain Shirota on the intestinal microflora and immune parameters in humans. Eur J Clin Nutr 1998; 52: 899-907.

Staaf M, Yang Z, Huttunen E, Widmalm G. Structural elucidation of the viscous exopolysaccharide produced by *Lactobacillus helveticus* Lb161. Carbohydr Res 2000; 326: 113-9.

Sugawara G, Nagino M, Nishio H, Ebata T, Takagi K, Asahara T, et al. Perioperative synbiotic treatment to prevent postoperative infectious complications in biliary cancer surgery: a randomized controlled trial. Ann Surg 2006; 244: 706-14.

Sun JL, Le GW, Hou LX, Wang NF, Chang GF, Shi YH. Nonopsonic phagocytosis of Lactobacilli by mice Peyer's patches' macrophages. Asia Pac J Clin Nutr 2007; 16 Suppl 1: 204-7.

Suomalainen T, Sigvart-Mattila P, Mättö J, Tynkkynen S. *In vitro* and *in vivo* gastrointestinal survival, antibiotic susceptibility and genetic identification of *Propionibacterium freudenreichii* ssp. *shermanii* JS. Int Dairy J 2008; 18: 271-8.

Szajewska H, Kotowska M, Mrukowicz JZ, Armanska M, Mikolajczyk W. Efficacy of *Lactobacillus* GG in prevention of nosocomial diarrhea in infants. J Pediatr 2001; 138: 361-5.

Tagesson C, Franzen L, Dahl G, Westrom B. Lysophosphatidylcholine increases rat ileal permeability to macromolecules. Gut 1985; 26: 369-77.

Takeda K, Okumura K. Effects of a fermented milk drink containing *Lactobacillus casei* strain Shirota on the human NK-cell activity. J Nutr 2007; 137: 791S-3S.

Taylor A, Verhagen J, Blaser K, Akdis M, Akdis CA. Mechanisms of immune suppression by interleukin-10 and transforming growth factor-beta: the role of T regulatory cells. Immunology 2006; 117: 433-42.

Thibault H, Aubert-Jacquin C, Goulet O. Effects of long-term consumption of a fermented infant formula (with *Bifidobacterium breve* c50 and *Streptococcus thermophilus* 065) on acute diarrhea in healthy infants. J Pediatr Gastroenterol Nutr 2004; 39: 147-52.

Timmerman HM, Niers LE, Ridwan BU, Koning CJ, Mulder L, Akkermans LM, et al. Design of a multispecies probiotic mixture to prevent infectious complications in critically ill patients. Clin Nutr 2007; 26: 450-9.

Tiollier E, Chennaoui M, Gomez-Merino D, Drogou C, Filaire E, Guezennec CY. Effect of a probiotics supplementation on respiratory infections and immune and hormonal parameters during intense military training. Mil Med 2007; 172: 1006-11.

Tlaskalova-Hogenova H, Stepankova R, Hudcovic T, Tuckova L, Cukrowska B, Lodinova-Zadnikova R, et al. Commensal bacteria (normal microflora), mucosal immunity and chronic inflammatory and autoimmune diseases. Immunol Lett 2004; 93: 97-108.

Tosi MF. Innate immune responses to infection. J Allergy Clin Immunol 2005; 116: 241-9.

Townsend SM, Hurrell E, Gonzalez-Gomez I, Lowe J, Frye JG, Forsythe S, et al. *Enterobacter sakazakii* invades brain capillary endothelial cells, persists in human macrophages influencing cytokine secretion and induces severe brain pathology in the neonatal rat. Microbiology 2007; 153: 3538-47.

Trinchieri R. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol 2003; 3: 133-46.

Tripathi P, Tripathi P, Kashyap L, Singh V. The role of nitric oxide in inflammatory reactions. FEMS Immunol Med Microbiol 2007; 51: 443-52.

Tubelius P, Stan V, Zachrisson A. Increasing work-place healthiness with the probiotic *Lactobacillus reuteri*: a randomised, double-blind placebo-controlled study. Environ Health 2005; 4: 25.

Turchet P, Laurenzano M, Auboiron S, Antoine JM. Effect of fermented milk containing the probiotic *Lactobacillus casei* DN-114001 on winter infections in free-living elderly subjects: a randomised, controlled pilot study. J Nutr Health Aging 2003; 7: 75-7.

Uematsu S, Akira S. Toll-Like receptors (TLRs) and their ligands. Handb Exp Pharmacol 2008: 1-20.

Uhari M, Möttönen M. An open randomized controlled trial of infection prevention in child day-care centers. Pediatr Infect Dis J 1999; 18: 672-7.

Ukena SN, Singh A, Dringenberg U, Engelhardt R, Seidler U, Hansen W, et al. Probiotic *Escherichia coli* Nissle 1917 inhibits leaky gut by enhancing mucosal integrity. PLoS ONE 2007; 2: e1308.

Underhill DM. Collaboration between the innate immune receptors dectin-1, TLRs, and Nods. Immunol Rev 2007; 219: 75-87.

Wakabayashi H, Nariai C, Takemura F, Nakao W, Fujiwara D. Dietary supplementation with lactic acid bacteria attenuates the development of atopic-dermatitis-like skin lesions in NC/Nga mice in a strain-dependent manner. Int Arch Allergy Immunol 2008; 145: 141-51.

Valeur N, Engel P, Carbajal N, Connolly E, Ladefoged K. Colonization and immunomodulation by *Lactobacillus reuteri* ATCC 55730 in the human gastrointestinal tract. Appl Environ Microbiol 2004; 70: 1176-81.

Veckman V, Miettinen M, Matikainen S, Lande R, Giacomini E, Coccia EM, et al. Lactobacilli and streptococci induce inflammatory chemokine production in human macrophages that stimulates Th1 cell chemotaxis. J Leukoc Biol 2003; 74: 395-402.

Veckman V, Miettinen M, Pirhonen J, Siren J, Matikainen S, Julkunen I. *Streptococcus pyogenes* and *Lactobacillus rhamnosus* differentially induce maturation and production of Th1-type cytokines and chemokines in human monocyte-derived dendritic cells. J Leukoc Biol 2004; 75: 764-71.

Weizman Z, Asli G, Alsheikh A. Effect of a probiotic infant formula on infections in child care centers: comparison of two probiotic agents. Pediatrics 2005; 115: 5-9.

Wiest MM, Watkins SM. Biomarker discovery using high-dimensional lipid analysis. Curr Opin Lipidol 2007; 18: 181-6.

Vilcek J. The cytokines: an overview. In: Thomson A, editor. The Cytokine Handbook: Academic Press, 2003: 3-18.

Viljanen M, Kuitunen M, Haahtela T, Juntunen-Backman K, Korpela R, Savilahti E. Probiotic effects on faecal inflammatory markers and on faecal IgA in food allergic atopic eczema/dermatitis syndrome infants. Pediatr Allergy Immunol 2005a; 16: 65-71.

Viljanen M, Pohjavuori E, Haahtela T, Korpela R, Kuitunen M, Sarnesto A, et al. Induction of inflammation as a possible mechanism of probiotic effect in atopic eczema-dermatitis syndrome. J Allergy Clin Immunol 2005b; 115: 1254-9.

Viljanen M, Savilahti E, Haahtela T, Juntunen-Backman K, Korpela R, Poussa T, et al. Probiotics in the treatment of atopic eczema/dermatitis syndrome in infants: a double-blind placebo-controlled trial. Allergy 2005c; 60: 494-500.

Vinderola G, Matar C, Perdigon G. Role of intestinal epithelial cells in immune effects mediated by gram-positive probiotic bacteria: involvement of toll-like receptors. Clin Diagn Lab Immunol 2005; 12: 1075-84.

Vinderola G, Matar C, Perdigon G. Milk fermentation products of L. *helveticus* R389 activate calcineurin as a signal to promote gut mucosal immunity. BMC Immunol 2007; 8: 19.

Winkler P, de Vrese M, Laue C, Schrezenmeir J. Effect of a dietary supplement containing probiotic bacteria plus vitamins and minerals on common cold infections and cellular immune parameters. Int J Clin Pharmacol Ther 2005; 43: 318-26.

Winkler P, Ghadimi D, Schrezenmeir J, Kraehenbuhl JP. Molecular and cellular basis of microflora-host interactions. J Nutr 2007; 137: 756S-72S.

Volanakis JE. Human C-reactive protein: expression, structure, and function. Mol Immunol 2001; 38: 189-97.

Wold S, Esbensen, K., Geladi, P. Principal component analysis. Chemometr Intell Lab Syst 2, 1987: 37-52.

Won JS, Singh I. Sphingolipid signaling and redox regulation. Free Radic Biol Med 2006; 40: 1875-88.

Wu K, Bi Y, Sun K, Wang C. IL-10-producing type 1 regulatory T cells and allergy. Cell Mol Immunol 2007; 4: 269-75.

Xie H, He SH. Roles of histamine and its receptors in allergic and inflammatory bowel diseases. World J Gastroenterol 2005; 11: 2851-7.

Yan F, Cao H, Cover TL, Whitehead R, Washington MK, Polk DB. Soluble proteins produced by probiotic bacteria regulate intestinal epithelial cell survival and growth. Gastroenterology 2007; 132: 562-75.

Yang Z, Huttunen E, Staaf M, Wildmalm G, Tenhu H. Separation, purification and characterisation of extracellular polysaccharides produced by slime-forming *Lactococcus lactis* ssp. *cremoris* strains. Int Dairy J 1999; 9: 631-8.

Yang Z, Staaf M, Huttunen E, Widmalm G. Structure of a viscous exopolysaccharide produced by *Lactobacillus helveticus* K16. Carbohydr Res 2000; 329: 465-9.

Yaqoob P, Newsholme EA, Calder PC. Comparison of cytokine production in cultures of whole human blood and purified mononuclear cells. Cytokine 1999; 11: 600-5.

Yetukuri L, Katajamaa M, Medina-Gomez G, Seppänen-Laakso T, Vidal-Puig A, Oresic M. Bioinformatics strategies for lipidomics analysis: characterization of obesity related hepatic steatosis. BMC Syst Biol 2007; 1: 12.

Zanini K, Marzotto M, Castellazzi M, Borsari M, Dellaglio F, Torriani S. The effects of fermented milks with simple and complex probiotic mixtures on the intestinal microbiota and immune response of healthy adults and children. Int Dairy J 2007; 17: 1332-43.

Zocco MA, dal Verme LZ, Cremonini F, Piscaglia AC, Nista EC, Candelli M, et al. Efficacy of *Lactobacillus* GG in maintaining remission of ulcerative colitis. Aliment Pharmacol Ther 2006; 23: 1567-74.

Zoetendal EG, Akkermans AD, De Vos WM. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl Environ Microbiol 1998; 64: 3854-9.

Zou H, Hastie, T. Regularization and variable selection via the elastic net. J R Statist Soc 2005; B67: 301-20.

Zyrek AA, Cichon C, Helms S, Enders C, Sonnenborn U, Schmidt MA. Molecular mechanisms underlying the probiotic effects of *Escherichia coli* Nissle 1917 involve ZO-2 and PKCzeta redistribution resulting in tight junction and epithelial barrier repair. Cell Microbiol 2007; 9: 804-16.

Original publications