Interactions of lactobacilli with the host immune system

M. Meijerink

Thesis committee

Thesis supervisors
Prof. dr. Jerry M. Wells
Professor of Host Microbe Interactomics
Wageningen University, The Netherlands

Prof. dr. ir. Huub F. J. Savelkoul
Professor of Cell Biology and Immunology
Wageningen University, The Netherlands

Thesis co-supervisor
Dr. Jolanda van Bilsen
Project Manager Food Allergy and Experimental Immunology
TNO Earth, Environmental and Life Sciences, Zeist, The Netherlands

Other members
Prof. dr. Willem M. de Vos, Wageningen University
Dr. Janneke N. Samson, Erasmus University Medical Centre, Rotterdam
Dr. Annick Mercenier, Nestlé Research Center, Lausanne, Switzerland
Dr. Karen Robinson, The University of Nottingham, United Kingdom

This research was conducted under the auspices of the graduate school VLAG (Voeding, Levensmiddelentechnologie, Agrotechnologie en Gezondheid)

Interactions of lactobacilli with the host immune system

M. Meijerink

Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. dr. M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 11 November 2011
at 4 p.m. in the Aula.

M. Meijerink
Interactions of lactobacilli with the host immune system, 224 pages.
Thesis, Wageningen University, Wageningen, NL (2011)
With references, with summaries in Dutch and English
ISBN 978-94-6173-044-2

Voor mijn lieve pa en ma

The research described in this thesis was financially supported by Top Institute Food and Nutrition
Financial support from Wageningen University, and Top Institute Food and Nutrition for printing this thesis is gratefully acknowledged.

Table of contents

Chapter 1

General introduction

Marjolein Meijerink

Part of the introduction is from the review: Epithelial crosstalk at the microbiota-mucosal interface. Wells JM, Rossi O, Meijerink M, van Baarlen P. Proc Natl Acad Sci U S A. 2011 Mar 15;108 Suppl 1:4607-14. Epub 2010 Sep 8

The gastro-intestinal tract

The largest and most complex immune organ in the body is the gastro-intestinal (GI) tract. Its vast surface area can be divided into three major components: the intestinal microbiota, the mucosal barrier and the gut-associated lymphoid tissue (GALT). The microbiota varies considerably in composition and in numbers along the human GI tract. Bacterial numbers are the lowest ($<10^3$ colony-forming units (CFU) per gram of content) in the stomach, because of the low pH and relatively fast transit time but increase to around 10^5 to 10^6 CFU per gram intestinal content in the terminal ileum. In the colon bacterial numbers reach up to 10^{10} to 10^{12} CFU per gram content in the colon. The upper part of the small intestine supports lower numbers of commensal bacteria due to pancreatic enzymes, bile and motility patterns that hamper colonization, while in the colon the number of bacteria can be high due to a slower transit time, favorable pH, substrate availability and oxygen levels [1]. More than 500 species of predominantly anaerobic bacteria are estimated to comprise the human intestinal microbiota and in total these outnumber host cells by approximate a factor of ten [2, 3]. The intestinal microbes compete with each other for nutrients and adhesion sites and are thus important in colonization resistance to pathogens as well as via competition and the production of bacteriocins or other inhibitory molecules [4-6].

The mucosal barrier consists of physical and chemical components, including epithelial cells (ECs) and the mucus layer. The mucus layer is important in limiting bacterial translocation (alive and dead) and passage of harmful substances from the gut lumen into the body. Host chemical defensins include gastric and pancreatic juices as well as deconjugated bile salt and antimicrobial enzymes, peptides and polypeptides (e.g. lysozyme, defensins and Reg proteins) that are secreted by Paneth cells and/or enterocytes.

The GALT comprises all the elements relating to the immune system in the GI tract, including organized lymphoid aggregates such as the Peyer's patches (PP), isolated lymphoid follicles (ILFs) containing lymphocytes, dendritic cells (DCs) and macrophages.

Secretory IgA produced by plasma B cells in the lamina propria (LP) and transported across the epithelium into the lumen serves as an important factor to exclude antigens, neutralize pathogens or neutralize microbial toxins and viruses [7]. The follicular associated epithelium (FAE) contains specialized microfold cells (M cells) that transport special or particulate antigens to the underlying immune cells to induce mucosal immune responses. T and B cell responses induced at one location in the GALT can are disseminated to other locations in the mucosa via the expression of specific homing integrins on mucosal lymphocytes and the circulation via the lymphatics and bloodstream. This concept known as the common immune system serves to dissipate protective immune responses over our vulnerable mucosal surfaces although recent studies indicate selectivity in the homing of lymphocytes depending on the sites of induction [8].

The gut is part of the main route of contact with the external environment and encounters thousands of different foreign molecules every day. Therefore the mucosal immune system is constantly challenged with external stimuli, such as pathogens (bacteria, vi-

ruses, fungi, protozoa) or toxic substances, as well as harmless food or commensal/ probiotic antigens. The GALT is capable to avoid potentially harmful adverse responses to food and commensal microbiota while still retaining a capacity to mount inflammatory responses towards invading and harmful pathogens. The typical response to harmless antigens that appear in the gut is the induction of local and systemic immunological tolerance, known as oral tolerance. Oral tolerance is an effective way to induce peripheral tolerance to antigens. Most dietary antigens are degraded, but some can reach the small intestine in a partially degraded or even intact form. After absorption these molecules are transported into the blood stream and distributed systemically and are able to induce tolerance. The underlying mechanism is still not completely resolved and suggested to rest on repeated low dose induction of regulatory T-cell populations that secrete immunosuppressive cytokines, anergy induction in antigen-specific T-cells by presenting the antigens on cells lacking co-stimulatory capacity, and/ or lack of the presence of danger signals that stimulate DCs. Dysfunctional regulation of tolerance to harmless antigens may be involved in the development of food allergy. The epithelium has developed mechanisms to control bacterial growth, limit direct contact with the bacteria, and prevent bacterial dissemination into underlying tissue, to protect the body from uncontrolled inflammatory responses. In susceptible individuals a dysfunctional barrier can lead to loss of immune tolerance to the microbiota and an inappropriate inflammatory response, as is thought to occur in the inflammatory bowel diseases (IBD) ulcerative colitis and Crohn's disease [9].

Mucosal immunity

Luminal antigens are recognized and taken up by bacterial sampling by DCs or by adhesion to specialized M cells in the PP. In mice and humans, the intestinal DCs are located throughout intestinal LP and in the radial muscle layer [10]. They also accumulate in the lymphoid tissues of the mucosa, namely PP, ILFs, and mesenteric lymph node (MLNs) [11]. DCs are the most important professional antigen presenting cells and express up to 100 times more MHCII and are more effective at differentiating naïve T cells than other antigen presenting cells (APCs) [12, 13]. In the PP and ILF DCs are considered to be primarily responsible for T cell-dependent IgA responses [14]. In the LP, two major subsets of DCs can be discriminated that perform different immune functions. These two populations can be discriminated by the differential expression of CD103 (also known as integrin $\alpha E\beta 7$) and the fractalkine receptor CX3CR1 giving rise to the CD103+ CX3CR1- and CD103- CX3CR1+ subsets. The LP, CD103+ expressing DCs have recently been shown to play a key role in regulating oral tolerance through the induction of regulatory Foxp3+ T cells expressing gut-homing receptors in the MLNs (Fig. 1) [15, 16].
A subset of LP CD103− cells which share phenotypic traits with both macrophages and DCs express the fractalkine receptor (CX3CR1) and bind to the membrane form of fractalkine on IEC [17, 18]. The ability of DCs to produce protrusions and sample antigens through the epithelium is reported to depend on CX3CR1 expression [19], although this

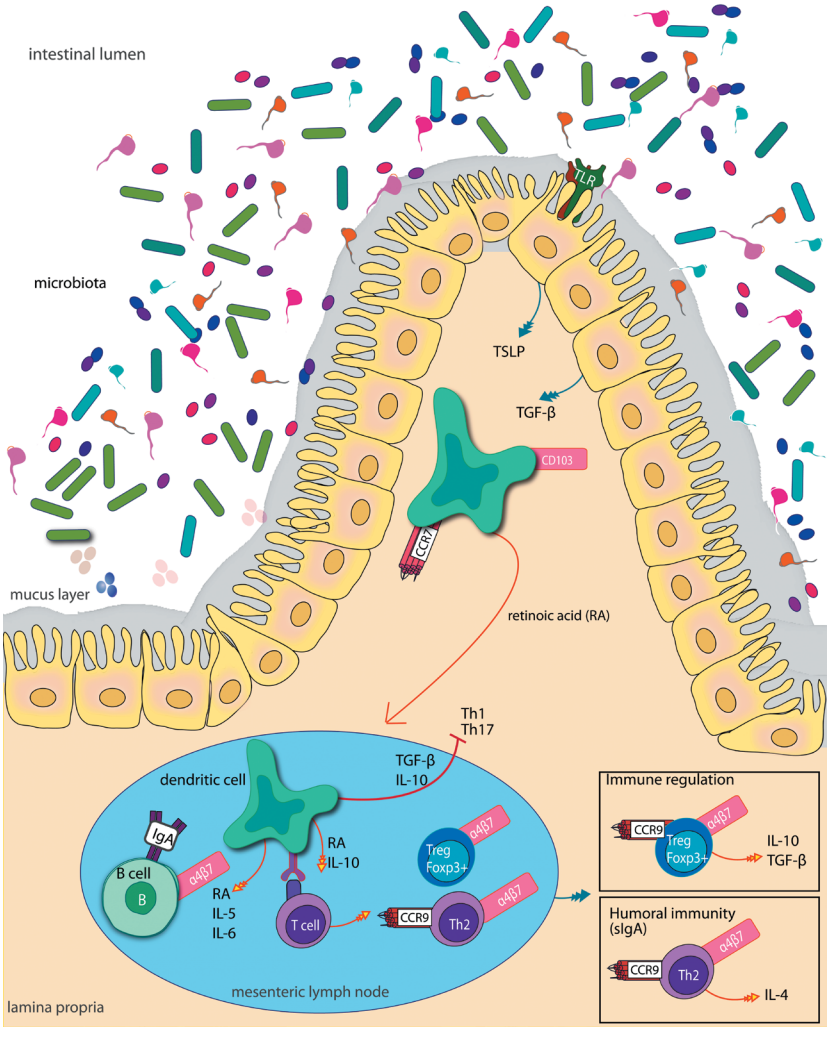

Fig. 1. Mucosal CD103+ dendritic cells (DCs) are conditioned in the peripheral tissues by epithelial production of thymic stromal lymphopoietin (TSLP) and transforming growth factor beta (TGF-β), which endows DCs with the ability to prime non-inflammatory responses and induce regulatory T cells. When they receive an inflammatory or danger signal, they begin to mature, and the expression of CCR7 increases, allowing the DCs to enter lymph vessels and migrate to the draining lymph nodes. In the T cell areas retinoic acid (RA), plays an important role in the ability of DCs to up-regulate homing receptors on lymphocytes. RA is also an important cofactor for the differentiation of Foxp3+ regulatory T cells (Treg) and has been shown to inhibit the generation of Th17 cells.

is somewhat controversial, and it is possible that other subsets of DCs can sample antigens in this way [20]. Recently, the CD103− CX3CR1+ subset of LP DCs were shown to be derived from a different cell linage than the CD103+ population [21] and seem to

support
inflammatory immune responses [22, 23]. Taken together these findings suggest that the mucosal CD103+ DCs serve classical DC functions and promote T cell responses in the draining lymph nodes, whereas the CX3CR1+ DCs are retained in the LP to kill colonizing and invading microorganisms. Several studies have implicated a role for ECs in endowing DCs with their ability to prime non-inflammatory responses and induce Tregs [15, 24, 25]. DCs that express CCR6, a chemokine receptor that binds the chemokine CCL20, are found only in the domes of the PP and acquire antigen solely by M-cell mediated transcytosis. The mucosa, in particular the intestinal villi, are inherently fragile structures that can easily be injured by the products (tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ)) and actions of cells activated during inflammation. Potential invaders, including commensal bacteria in the lumen, which do not normally harm the host must be prevented from penetrating the epithelium, e.g. by the constant production of commensal-specific sIgA. The nature of the mucosal DC subsets that reside in the dome region of the PP may inherently skew by virtue of their high production of IL-10, the differentiation of the Th0 cells that they encounter towards regulatory Th3 and Tr1 cells producing transforming growth factor beta (TGF-β) and IL-10 or Th2 effector cells that produce IL-4, IL-5 and IL-10. This way effective protection is provided without overt inflammation.
Conditioning of monocyte-derived DCs with IEC supernatants *in vitro* abolishes the ability of DCs to produce IL-12 and prime naïve T cells toward T helper (Th)1 polarization in response to microbial stimuli [25]. In contrast, EC-conditioned DCs produce high amounts of IL-10 and promote Treg and Th2 cell responses. *In vitro* the conditioning of DCs was shown to depend on epithelial production of thymic stromal lymphopoietin (TSLP) (Fig. 1) [25]. Another important immunoregulatory cytokine produced abundantly by IEC and stromal cells in the intestine is TGF-β [24]. This cytokine inhibits NF-κB dependent gene expression and the production of pro-inflammatory cytokines by macrophages and DCs [26, 27]. Additionally, TGF-β acts in concert with TSLP to induce a tolerogenic phenotype in monocyte-derived DCs *in vitro* [28]. DCs purified from the LP have also been shown to promote a high level of Tregs conversion relative to lymphoid organ derived DCs via a TGF-β and retinoic acid-dependent mechanism. Consistent with these findings is the fact that TSLP deletion in mice leads to constitutive overexpression of IL-12p40 by intestinal DCs and inability to generate protective regulatory and Th2 responses against the nematode parasite *Trichuris muris* [29]. TSLP mRNA is constitutively expressed by ECs and can be up-regulated by NF-κB-dependent pathways [30]. Thus, one may expect that recognition of microbiota by epithelial pattern recognition receptors (PRRs) would also regulate TSLP production. Support for this idea comes from *in vitro* IEC-DC co-culture studies where it was shown that composition of the microbiota exposed to the apical side of the IEC influenced production of TSLP and TGF-β and, hence, the function of the underlying DCs [28]. In an *in vivo* expression profiling study where healthy adult humans consumed preparations of viable lactic acid bacteria, a central role was uncovered for the NF-κB signaling cascade in the regulation of

tolerance in the small intestine [31]. In this study, it was found that NF-κB signaling up-regulated the expression of downstream effectors such as chemokines but also factors that regulate cell survival of B and T cells and DCs as well as regulators that suppress inappropriate immune responses. In addition to the epithelial cytokines influencing B cell and DCs functions mentioned above, the intestinal epithelium expresses a range of metabolic enzymes that can impact on immune cell function. Non-bone marrow-derived stromal cells located predominantly in the villi of proximal small intestine have been shown to constitutively produce cyclooxygenase (COX)-2 and abundantly produce the COX-2-dependent arachidonic acid (AA) metabolite, prostaglandin E2 (PGE2) (79). Although the production of COX-2 and COX-2-dependent metabolites does not appear to be regulated by pro-inflammatory stimuli or the microbiota, its production in the epithelium could contribute to the default immunoregulatory tone of the LP [32].

The detection of pathogens by the host is achieved through the families of PRRs that recognize conserved molecular structures known as pathogen-associated molecular patterns (PAMPs) and induce production of innate effector molecules. Because these structures are also found on non-pathogenic microorganisms, the term microbe-associated molecular patterns (MAMPs) is increasingly used, particularly in the context of host–commensal interactions. These signaling receptors can be divided into three families: TLR, retinoic acid inducible gene I (RIG-I)-like receptors (RLRs), and nucleotide oligomerization domain (NOD)-like receptors (NLRs). The TLR family is the best characterized, and 13 receptors have been reported in mice and humans. For TLR, it has been shown that ligand sensing and specificity is achieved through the arrangement and sequence variation in the conserved leucine-rich repeat (LRR) domains. TLR-2 can form heterodimers with TLR-1 or TLR-6 to detect different but related ligands (Table 1). TLR are localized in the cell membrane and/or endosomal membrane components to recognize extracellular and endocytosed MAMPs (Table 1). The ubiquitously expressed RIG-I-like receptor (RLR) family of RNA helicases are cytoplasmic proteins that recognize viral RNAs and induce innate antiviral responses, including the activation of pro-inflammatory cytokines and type I interferon (IFN) [33]. The third family, containing >20 cytoplasmic NLR in humans and mice, is divided into four major groups based on the nature of the N-terminal activation domains involved in signal transduction. The NLR characterized to date recognize a wide range of bacterial ligands and toxins as well as certain damage-associated molecular patterns (DAMPs) of the host cell [34]. NLR proteins can signal through different multicomponent signal complexes to activate alternative signaling pathways, including caspase activation, cell death, and NF-κB leading to cytokine, chemokine, and defensin expression. In the intestine, only the functions of NOD1 and NOD2 have been well characterized, and these NLR respond to the synthetic peptidoglycan components meso-diaminopimelic acid (DAP) and muramyl dipeptide (MDP), respectively (Table 1).

Recently, several lectin and C-type lectin receptors (CLRs) have been characterized that are involved in the recognition and capture of antigens by antigen presenting cells (APCs). One such CLR known as DC-specific intracellular adhesion molecule-3 grabbing

Table 1: The PRRs, subcellular localization, and recognized ligands

Receptor	Subcellular localization	Ligand	Origin of ligand
TLR2	Cell surface	Lipoteichoic acid	G (+) bacteria
		Lipoprotein/ lipopeptides	Various pathogens
		Hemoagglutinin protein	Viruses (Measles Virus)
		Glycosyl-phosphatidylinositols	Parasites (*Toxoplasma gondii*)
TLR2/1	Cell surface	Triacyl lipopeptides	G(-) bacteria and mycobacteria
TLR2/6	Cell surface	Diacyl lipopeptides	G (+) bacteria and mycobacteria
		Zymosan	Fungi
TLR3	Cellular compartment	dsRNA	Viruses
TLR4	Cell surface	Lipopolysaccharide	G (-) bacteria
		Envelope proteins	Viruses (Respiratory Syncytial Virus)
		Glycosyl-phosphatidylinositols	Parasites (*Toxoplasma gondii*)
TLR5	Cell surface	Flagellin	Bacteria
TLR7/8	Cellular compartment	ssRNA	Viruses
TLR9	Cellular compartment/cell surface	CpG-containing DNA	Bacteria and viruses
TLR11	Cell surface	Uropathogenic bacteria component	Bacteria (uropathogenic *Escherichia coli*)
		Profilin	Parasites
NOD1	Cell cytoplasm	Meso-diaminopimelic acid	PGN of G (−) and some G (+) bacteria
NOD2	Cell cytoplasm	Muramyl dipeptide	PGN of G (+) and G (−) bacteria
RIG-I	Cell cytoplasm	5′-triphosphate-bearing RNAs	Viruses

G (+), Gram-positive; G (−), Gram-negative; dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; PGN, peptidoglycan; Meso-DAP, γ-D-glutamyl-meso-diaminopimelic acid; MDP, muramyl dipeptide NOD1, nucleotide oligomerization domain-like receptor 1; NOD2 nucleotide oligomerization domain-like receptor 2.

non-integrin (DC-SIGN) recognizes high-mannose-containing structures.

Probiotics and their modes of action

Over the last years there has been an increasing awareness of the role played by commensal bacteria in modulating mucosal immune responses which has greatly increased interest in the therapeutic potential of probiotics for a range of immune disorders.

Probiotics are defined as living organisms that, when administered in sufficient numbers, are beneficial to the host [35] Current evidence indicates that probiotic strains mediate their effects by a variety of different mechanisms that are dependent on the dose as well as the route and frequency of delivery [36] (Fig. 2).

Probiotics may act directly on the host or indirectly via modulation of the composition and/ or activity of the commensal microbiota, and/ or their stimulation of the immune system. By changing the conditions in the gut and improving colonization resistance, probiotics make the gut environment more favorable to the growth of genera generally considered as beneficial (e.g. lactobacilli and bifidobacteria) and less favorable for pathogens and other harmful microbes. Some probiotics act in the lumen of the gut by producing antibacterial molecules such as bacteriocins; others enhance the mucosal barrier by increasing the production of innate immune molecules, including goblet cell–derived mucins and trefoil factors and defensins produced by intestinal Paneth cells. Probiotics may also mediate beneficial effects by promoting adaptive immune responses (secretory IgA, Tregs, IL-10).
In animal models or human studies probiotics have been shown to prevent or treat disorders such as IBD, irritable bowel syndrome, infectious diarrhea, and infection by *Salmonella* or *Helicobacter pylori*. Probiotics might also be useful in treating or preventing allergic diseases, which could be a good alternative to the pharmacological approach in patients who experience serious side effects or drug resistance. Currently, allergen-specific immunotherapy is the only causal treatment of type I allergies (18) and involves frequent injections of gradually increasing amounts of an allergen to diminish type I sensitivity reactions to the allergen. There is an inherent risk of allergic reactions during treatment and the patients compliance is often poor due to the frequency and duration of the treatment.
In addition, probiotics have shown promising results in enhancing immune responses to vaccination in young children and in adults. For example, probiotics have been shown to increase the antigen specific antibody titers of orally administered vaccines such as rotavirus [37], *Salmonella* [38], polio [39] and cholera [40].

Commonly used probiotic species of bacteria

Many probiotic strains are lactic acid bacteria (LAB) which are bacteria which produce lactic acid as their major fermentation product, and includes species of *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Lactococcus*, *Bifidobacterium* and *Leuconostoc*. They are widely distributed in the intestinal tract. The largest genus in this order is *Lactobacillus* and are used in fermentation products such as pickle, sauerkraut, beet, wine, juices, cheese, yoghurt and sausage [41]. The dietary LAB are generally regarded as safe (GRAS status) owing to their long history of use or association with humans and food [42] and are therefore often selected as candidate probiotics. *Lactobacillus plantarum* WCFS1 was the first *Lactobacillus* species for which the genome was completely sequenced

and published [43]. The genome is the largest known among LAB, namely 3.3 Mb, and is therefore a good candidate to act as a model organism for other lactobacilli. *L. plantarum* is found in a variety of niches, including the human GI-tract and can promote health and some strains (e.g. 299v and R1012) have been marketed as probiotics.

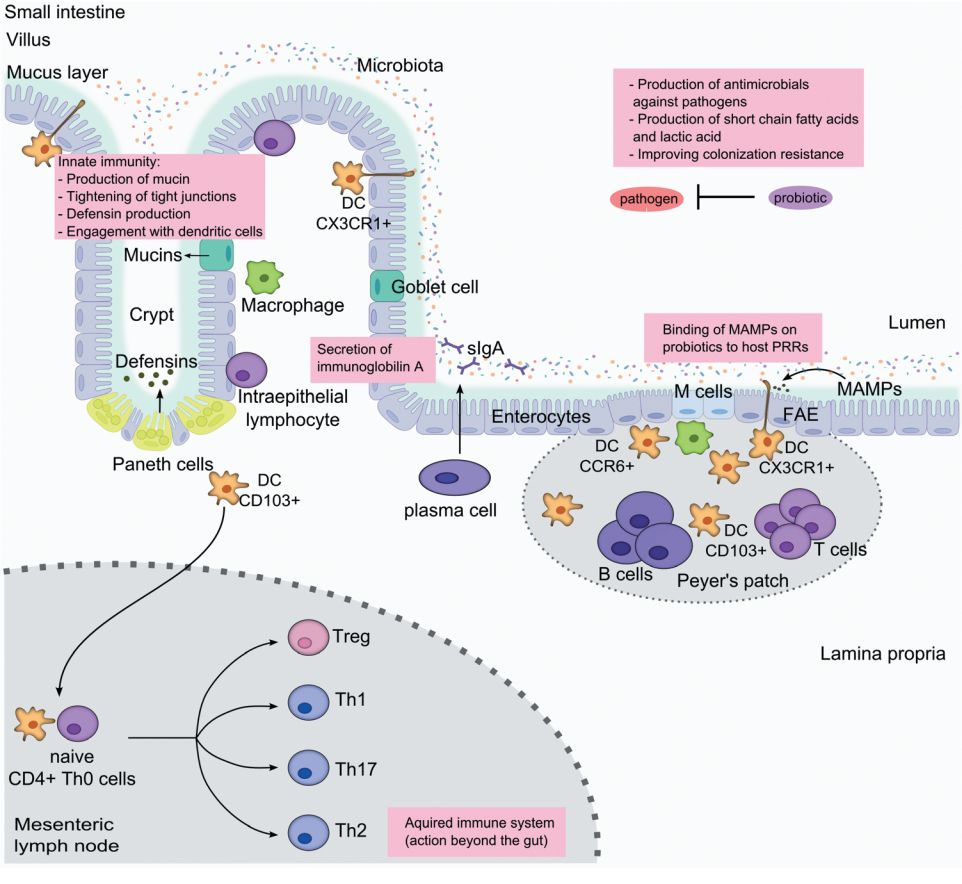

Fig. 2 Proposed mechanisms of probiotic action: within lumen, mucosal surface, and by stimulation of innate and adaptive immunity. The predominant mechanism varies among different probiotic strains (Adapted from Rossi et al. [7]).

Probiotics and peanut allergy

In this thesis a model of sensitization to peanut extract was used to assess the potential of selected *Lactobacillus* strains to prevent allergy. Currently, the prevalence of peanut allergic sensitisation varies between 1-2% (US and Canada) to 10% in the UK, of whom 2% have a clinical peanut allergy [44]. Peanut allergy accounts for the majority of severe food-related allergic reactions, and is often associated with anaphylaxis, which can have

a fatal outcome. Most childhood food allergies generally resolve spontaneously with age, but peanut allergy resolves in only 20% of cases, resulting in a life-long allergy with a high impact on quality of life [45, 46]. Over the past several decades the developed countries have seen a steady increase in the proportion of children that develop food allergies, but there are reports indicating that this trend has stabilized [47-50].

Sensitization and allergic reaction

Peanut allergy occurs in two phases: sensitization and allergic reaction. The development of IgE-mediated allergy is always initiated with a sensitization phase to a certain allergen. Allergens are taken up by antigen presenting cells, such as DCs, after entering the body via the epithelial barrier of the skin, airway, or gut [51]. The allergens are processed and the allergen-derived peptides are bound to major histocompatibility complex class II (MHC-II) molecules on the surface of the APCs. The MHC-II-bound peptides are presented to the T cell receptor on naïve CD4+ antigen specific T cells which will lead to the differentiation into Th1, Th2, Th17 cells or Tregs. In persons predisposed to allergic sensitization, this process leads to an exaggerated Th2 response to the allergen with an accompanying cytokine profile that includes IL-4, IL-5, IL-10, and IL-13. These cytokines drive B cell heavy-chain class-switching to IgE production and secretion of peanut-specific IgE. Secreted peanut-specific IgE antibodies are distributed systemically and bind to the high affinity receptor FcεRI of mast cells in the tissue or basophils in the blood.

In sensitized individuals re-exposure to peanut elicits an allergic reaction. This is mediated by binding of the food antigen to cell bound IgE thereby cross -linking two or more FcεR1 receptors on the surface of mast cells or basophils to induce activation and release of effectors. The release of inflammatory mediators and cytokines from mast cells, and the increase in vascular permeability promotes the subsequent recruitment of other effector cells, such as eosinphils, neutrophils, basophils and Th2 lymphocytes [52]. In order to induce mast cell and/ or basophil activation, the number and duration of cross-links per mast cell or basophils should be at least 100 for at least 100 seconds, provoking degranulation and release of mediators such as histamine, leukotrienes, cytokines, platelet-activating factors, and prostaglandin [53]. (Fig. 3)

The release of inflammatory mediators can lead to a variety of cutaneous (urticaria, angiodema, eczema), gastrointestinal (nausea, vomiting, abdominal pain, diarrhoea), respiratory (cough, wheeze), and systemic (hypotension) symptoms within minutes to hours after ingestion of the food. The anaphylactic reactions are life-threatening and are the leading cause of anaphylactic reactions treated in emergency departments in Westernized countries. In certain persons, the recruitment of effector cells can lead to a cell-mediated response, after 6-24h, which is often seen in allergic nasal respiratory and skin disease (atopic eczema). If an antigen persists and stimulates allergen-specific Th2 cells, this can convert into a chronic inflammatory response, like chronic asthma, because of the enhanced IgE production.

The precise combination of factors leading to allergic sensitization in some individu-

als are complex and not well understood in most cases. There are probably many factors involved, such as genetic predisposition, type of allergen, allergen concentration in the environment, route of exposure and whether allergen exposure is accompanied by agents that can enhance or down regulate the initiation phase [54].
Among the several factors involved in the development of IgE-mediated allergic diseases, the most important seems to be genetic predisposition to allergies (atopy) [55]. The presence of an atopic phenotype in one or two parents will substantially increase the likelihood of an atopic phenotype in the child. However, atopy itself does not determine if a child will become allergic and to which particular protein an allergic response will develop, implicating environmental factors in the outcome of an allergic disease.
These environmental factors may include the age at which an antigen is introduced, the composition of the gut microbiota, the infection status of the gastrointestinal tract at the time of antigen introduction, the dietary composition, and formula versus breastfeeding [56]. Food allergies affect only susceptible individuals and are most commonly IgE-mediated.

Potential therapies for peanut allergy
Currently, allergen avoidance is the only way to prevent allergic responses [57] but in the case of peanut allergy, accidental ingestion is common due to the trace amount of peanut in many food products and cross-contamination during food processing [58]. In case of an unintended ingestion, epinephrine injection is used against anaphylaxis [44]. At this moment no curative therapy is available for patients that already have an established peanut allergy. Recently the potential therapies for peanut allergy were reviewed by Stahl et al. [59]. The use of oral immunotherapy, anti-IgE therapy, soy-based immunotherapy, Chinese medicine, cellular mediators, engineered allergen immunotherapy, plasmid DNA immunotherapy and immunostimulatory sequence and oligodeoxynucleotide-based immunotherapy and probiotics were extensively discussed. Most explored therapies aim, at least in part, to reverse Th2 predominance towards a Th1 profile. In patients that outgrew their peanut allergy, cytokine levels more closely resembled the Th1 profile (IFN-γ and TNF-α) of individuals without history of peanut allergy. In contrast persons with active peanut allergy have a Th2 profile (IL-4, IL-5, and IL-13). Liposomal delivery of IL-12 during and after oral sensitization with peanut allergen enhanced allergen-specific Th1 responses as shown by increased IFN-γ responses in re-stimulated splenocytes although the Th2 cytokine responses were unaffected [60]. This strategy proved to be effective in both prevention and treatment of allergy.
Given the potential for probiotics to modulate host immune responses there is ongoing interest in their application in the treatment and prevention of allergy including food allergy. A review of the literature on probiotics and allergy is beyond the scope of this introduction but this has been recently reviewed in relation to human studies [61]. Most success has been obtained in primary prevention of atopic eczema. However after almost a decade of research on this topic it is clear that more research is needed to understand probiotic mechanisms in humans [62]. There have been some clear successes

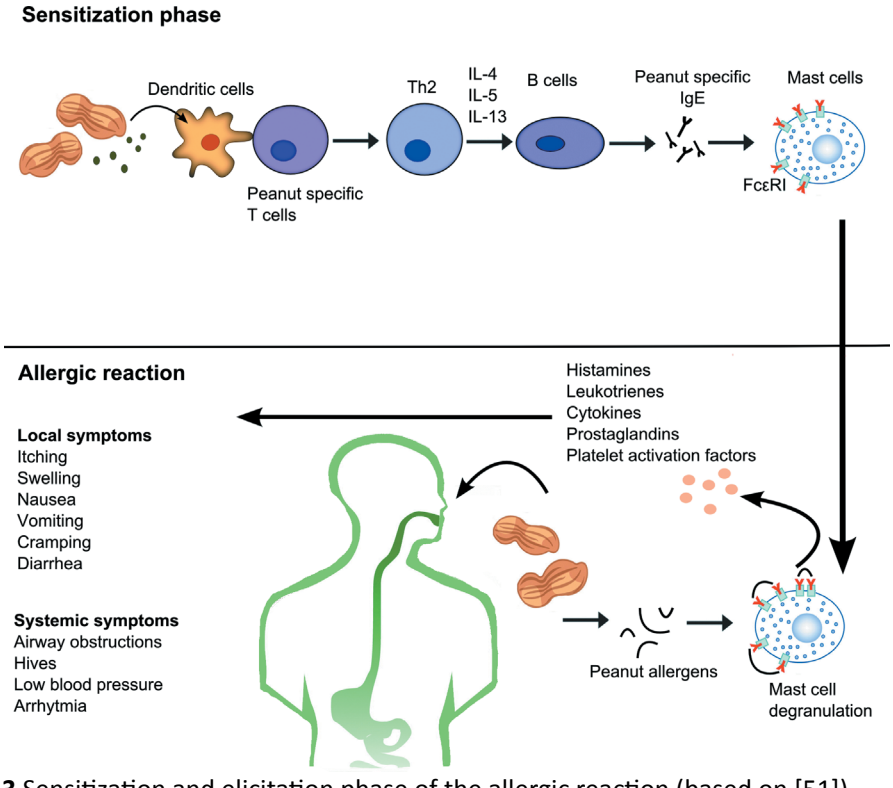

Fig. 3 Sensitization and elicitation phase of the allergic reaction (based on [51]).

but also negative results and the field would benefit from more comparative studies using different strains that have been extensively characterized *in vitro* using immune assays and animal models.

Peanut sensitization model

Due to ethical considerations for testing directly *in vivo* in humans, animal models of food allergy have been used to study the mechanisms involved in the development of hyper-sensitization reactions to food proteins as well as the immunologic mechanisms of the adverse reactions to allergen re-exposure [63, 64]. The use of mucosal adjuvants and manipulation of the epithelial barrier has been frequently been used to break oral tolerance in animal models [65]. Cholera toxin (CT) is the most common adjuvant used for oral sensitization in rodent models of food allergy [64, 65]. CT is composed of an A subunit with ADP ribosyltransferase activity and a pentameric B subunit. The B subunit mediates the binding to the cell membrane through a high-affinity receptor, the ganglioside GM-I [66]. After internalization of the CT by the cells, the A subunit ADP-ribosylates the α subunit of Gs protein and causes intracellular increase of cAMP. The mechanisms of the adjuvanticity are believed to be a complex multistep phenomenon resulting from the interaction of CT with different cell types. The binding of CT to mucosal ECs [67] and

the increase of the mucosal barrier permeability [68] might be critical, as this allow the CT and the co-delivered antigen to cross the mucosal barrier and to come in contact with the cells of the immune system. The induction of allergic sensitization by CT is accompanied by the up-regulation of MHC-II and co-stimulatory molecules on monocyte and bone-marrow derived DCs, resulting in DCs that preferably prime naïve CD4+ T cells to a Th2 phenotype [69]. Furthermore, the matured DCs express functional chemokine receptors CCR7 and CXR4, which are involved in the migration of DCs from the intestine to the MLNs, and the migration from the sub-epithelial dome to T cell areas of the PP [70-72].
In this thesis a peanut sensitization model was used to determine the immunomodulatory effects of different strains of lactobacilli. C3H/HeOuJ mice were sensitized with peanut extract (PE) in combination with CT for three consecutive days, following weekly sensitizations for a further three weeks with an oral challenge of PE alone on the fifth week. This model has been used succesfully for different studies showing that an oral challenge in C3H/HeOuJ mice resulted in measureable mast cell degranulation but not in clinical signs of anaphylaxis (unpublished data, TNO). This hypersensitivity model has been proven very suitable to study the mechanisms of oral sensitization, including challenge responses (such as mast cell activity).

Probiotics as adjuvants for vaccination
In this thesis intranasal vaccination with the Influvac 2010/2011 influenza vaccine was used as a model to investigate the potential adjuvant effects of different selected lactobacilli. Influenza is an interesting model because it could reveal beneficial effects of a probiotic in healthy subjects. Furthermore influenza is a relevant vaccine target given its global importance as a respiratory pathogen and the high degree of morbidity and mortality associated with influenza infections each year. Both mucosal and systemic immunity contributes to resistance to influenza infection and disease. Antibodies secreted locally in the upper respiratory tract are a major factor in resistance to natural infection. Secretory IgA (SIgA) is involved in protection of the upper respiratory tract and serum IgG in the protection of the lower respiratory tract by the production of neutralizing antibodies directed against important viral proteins. The humoral immune system, both mucosal and systemic, plays a major role in immunity to influenza infection, and the cell-mediated immune response is particularly effective in the clearing of virus-infected cells. The cytotoxic T-cell response is mainly directed against nucleoprotein (NP) proteins [73, 74], which are more conserved than the surface hemagglutinin and neuraminidase glycoproteins [74-76]. This response is important for the clearance of virus and recovery from illness, but does not confer protection against infection. The influenza vaccine has to be reformulated almost every year because the virus is continuously undergoing antigenic drift and shift to escape the host's acquired immunity. The efficacy of influenza vaccine varies each year depending on the match between vaccine subtypes and circulating viral strains, patient's age and pre-existing immunity. In young healthy adults, the influenza vaccine provides a protective clinical efficacy in 70-90%

of cases, which is reduced to only 17-53% in elderly individuals. In addition to the inactivated vaccine, a live attenuated influenza vaccine (LAIV) has been licensed by the Food and Drug Administration (FDA) since 2003. The LAIV contains live but attenuated (weakened) influenza viruses, and is sprayed into the nostrils rather than injected into the muscle. Since 2007 the FDA approved use of the LAIV in healthy children and adults from 2-49 years of age allowing it to be used to control pandemic outbreaks. To improve the efficacy of vaccines several approaches have been investigated such as increasing the dose of antibody in the vaccine (Centers for Disease Control and Prevention, 2010), using alternative routes of administration such as intradermal injection [77] and using immune adjuvants. In general, new strategies are needed for mucosal adjuvants to increase the efficacy of oral vaccines such as polio, rotavirus and influenza. Probiotics have shown to enhance specific immune responses to vaccination in young children and in adults. They increased the immunogenicity of orally administered vaccines such as rotavirus [37], *Salmonella* [38], polio [39] and cholera [40]. Furthermore a recent study on LGG as an immune adjuvant for LAIV in healthy adults showed significantly improved protective serum responses to one of the three viruses present in the vaccine [78]. This has important implications for the use of probiotics to stimulate immunity to respiratory pathogens and enhance responses to intranasally delivered vaccines.

IFN-γ and IL-12, which are produced by Th1 cells, play crucial parts in the stimulation of natural-killer-cell activity and maturation of cytotoxic lymphocytes, and thus in protection against infectious diseases and vaccination. Systemic administration of IL-12 has pleiotropic effects on T and B cells and is a key regulator of Th1 differentiation. As our understanding of the immunomodulatory properties of different LAB increases, it might be possible to select potential probiotic that influence the balance between Th1- and Th2-cytokine production and by that enhance the vaccine specific immune response.

Table 2. Frequently measured immune parameters in this thesis

Immune parameter	Function
IFN-Y	Pro-inflammatory cytokine produced by Th1 cells and NK cells. Activates macrophages and suppresses Th2 cells
IL-2	Pro-inflammatory cytokine produced by Th1 cells. Promotes T cell proliferation
IL-4	Allergy related cytokine produced by Th2 cells and mast cells. Increases eosinophils growth and differentiation
IL-5	Allergy related cytokine produced by Th2 cells and mast cells. Increases eosinophils growth and differentiation
IL-6	Pro-inflammatory cytokine, acute phase protein, produced by T cells and macrophages. Promotes T and B cell growth; impact on IgA production
IL-10	Anti-inflammatory cytokine produced by Th2 cells and Tregs cells. Suppresses macrophages.
IL-12	Produced by macrophages and dendritic cells. Activates NK cells, induces Th1 responses. Marker for innate immunity
NK cell activity	Fast and non-specific elimination of virus-infected and tumour cells. Marker for innate immunity
TNF-α	Pro-inflammatory cytokine produced by Th1 cells, macrophages and NK cells. Endothelial activation
IgG1	Th2-type Ig subclass in mouse
IgG2a	Th1-associated Ig subclass in mouse and could promote the rapid clearance of the allergen from the organism or directly inhibit mast cell and basophil degranulation by triggering inhibitory signal transduction by means of FcγRII receptors
IgE	Involved in allergic reactions - As a consequence of its binding to basophils an mast cells, IgE is involved in allergic reactions. Binding of the allergen to the IgE on the cells results in the release of various pharmacological mediators that result in allergic symptoms
Foxp3	Protein involved in immune system responses and a regulator in the development and function of Tregs.

Research aim and thesis outline

The research aim of this thesis is to better understand the molecular mechanisms of host responses to probiotics. As mentioned above probiotics can be used to stimulate or regulate immune responses in epithelial and immune cells of the intestinal mucosa and generate beneficial effects on the immune system. Carefully selected probiotics should be able to steer the activity of the immune response in a predetermined manner by increasing or decreasing the activity of different aspects of the immune system (e.g. T helper subsets). Beneficial effects of strains of probiotics have been established in the treatment and prevention of various intestinal disorders, including allergic diseases and diarrhea. However the precise molecular mechanisms and the strain dependent factors involved are poorly understood. Here *in vitro* molecular studies and *in vivo* mechanistic studies were combined in different mouse models to generate new insights into the beneficial mechanisms of selected lactobacilli and identify novel bacterial genes influencing the immune response. A further aim was to investigate the predictive value of *in vitro* immune assays for the effects of probiotics *in vivo*.

Given the importance of DCs in mucosal tolerance and immunity and their use in immune assays with probiotics, the current knowledge and understanding of the immunomodulatory effects of different probiotic species and strains on DCs and the adaptive immune system is reviewed in **chapter 2**. The relevance and the implications of the *in vitro* studies for clinical trials or mechanistic research in animal models are also discussed.

Chapter 3 and **chapter 4** present new insights gained from research on the strain-dependent factors involved in probiotic immune modulation. These chapters describe the extensive variation in immune responses among a collection of 42 *L. plantarum* strains and the identification of genetic loci in the model organism *L. plantarum* WCFS1 that modulate the host cell immune response using gene-trait matching. In **chapter 3** we used more specialized antigen presenting cells, namely DCs and in **chapter 4** we used peripheral mononuclear cells (PBMCs). The amounts of cytokines secreted by the immune cells after stimulation (see Table 2 for immune parameters measured in this thesis) with 42 individual *L. plantarum* strains were measured and correlated with the strain-specific genomic composition using comparative genome hybridization and the Random Forest algorithm. This *in silico* "gene-trait matching" approach led to the identification of several candidate genes in the *L. plantarum* genome that might modulate the immune cytokine response to *L. plantarum*. Selective gene deletions mutants were constructed for the candidate genes in *L. plantarum* WCFS1 and compared to the wild-type strain in immune assays with PBMCs and DCs.

In vitro assays for pre-screening of candidate probiotics would benefit from standardized methods and cryopreservation techniques for immature DCs or precursor monocytes. Literature on the effects of cryopreservation and thawing of monocytes or monocyte-derived iDCs suggested that this strategy might be useful although bacteria had not been used as a stimulus. Thus in **chapter 5** we investigated the effects of cryopreser-

vation and thawing of precursor monocytes and immature DCs on the maturation and immune response of DCs to potential probiotic strains and bacterial TLR agonists.
As there are only a few studies comparing potential probiotics strains *in vitro* and *in vivo*, strains of *Lactobacillus* and *Bifidobacterium* were isolated from different commercially available products and were screened for their immunomodulatory properties in a co-culture assay with human PBMCs (**chapter 6**). Three of these strains were selected on the basis of their induced cytokine profiles and predicted immune modulatory mechanisms and tested for their potential to attenuate allergic sensitization *in vivo*.
In **chapter 7** we selected strains based on the results of **chapter 3** and **chapter 4** that might modulate systemic immune responses in a mouse influenza vaccination model. First we established an intranasal influenza vaccination model in young female Balb/c mice. As the administration of different *L. plantarum* strains could potentially lead to either down-regulation or up-regulation of the immune responses, conditions had to be selected in such a way that both types of modulation could be detected. Therefore, the vaccine dose was titrated in an initial experiment to induce a sub-maximal immune response. Modulation of cellular immune responses to vaccination by the *L. plantarum* strains was studied by delayed type hypersensitivity (DTH) responses, as a parameter for cellular Th1 dependent immunity, and by *ex vivo* ConA and vaccine-induced MLN response as a parameter for T-cell activation. Modulation of the humoral immune response to vaccination was measured by vaccine-specific serum antibody. In addition, mRNA levels of different cytokines, chemokines and receptors were measured in naïve MLNs (see Table 2 for immune parameters measured in this thesis). In this way we were able to compare different *L. plantarum* strains in a standardized and well characterized study.
Chapter 8 completes this thesis with a general discussion on the results and conclusions of the different chapters. It discusses the future perspectives for probiotic research with respect to strain characterization *in vitro* and *in vivo*, microbiota, peanut allergy, and influenza vaccination.

References

1. Ouwehand, A.C. and E. Vaughan, Gastrointestinal microbiology. 2006.
2. Eckburg, P.B., et al., Diversity of the human intestinal microbial flora. Science, 2005. 308(5728): p. 1635-8.
3. Hold, G.L., et al., Assessment of microbial diversity in human colonic samples by 16S rDNA sequence analysis. FEMS Microbiol Ecol, 2002. 39(1): p. 33-9.
4. Cursino, L., et al., Exoproducts of the Escherichia coli strain H22 inhibiting some enteric pathogens both *in vitro* and *in vivo*. J Appl Microbiol, 2006. 100(4): p. 821-9.
5. Filho-Lima, J.V., E.C. Vieira, and J.R. Nicoli, Antagonistic effect of *Lactobacillus acidophilus*, Saccharomyces boulardii and Escherichia coli combinations against experimental infections with Shigella flexneri and Salmonella enteritidis subsp. typhimurium in gnotobiotic mice. J Appl Microbiol, 2000. 88(3): p. 365-70.
6. Millette, M., et al., Capacity of human nisin- and pediocin-producing lactic acid bacteria to reduce intestinal colonization by vancomycin-resistant enterococci. Appl Environ Microbiol, 2008. 74(7): p. 1997-2003.
7. Rossi, O., P. Van Baarlen, and J. Wells, Mammalian Intestinal Host-Microbe Relationships. Curr Top Microbiol Immunol, 2011.
8. Koboziev, I., F. Karlsson, and M.B. Grisham, Gut-associated lymphoid tissue, T cell trafficking, and chronic intestinal inflammation. Ann N Y Acad Sci, 2010. 1207 Suppl 1: p. E86-93.
9. Shanahan, F., Probiotics in perspective. Gastroenterology, 2010. 139(6): p. 1808-12.
10. Coombes, J.L. and F. Powrie, Dendritic cells in intestinal immune regulation. Nat Rev Immunol, 2008. 8(6): p. 435-46.
11. Johansson, C. and B.L. Kelsall, Phenotype and function of intestinal dendritic cells. Semin Immunol, 2005. 17(4): p. 284-94.
12. Inaba, K., et al., High levels of a major histocompatibility complex II-self peptide complex on dendritic cells from the T cell areas of lymph nodes. J Exp Med, 1997. 186(5): p. 665-72.
13. Levin, D., et al., Role of dendritic cells in the priming of CD4+ T lymphocytes to peptide antigen *in vivo*. J Immunol, 1993. 151(12): p. 6742-50.
14. Macpherson, A.J. and T. Uhr, Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. Science, 2004. 303(5664): p. 1662-5.
15. Iliev, I.D., et al., Human intestinal epithelial cells promote the differentiation of tolerogenic dendritic cells. Gut, 2009. 58(11): p. 1481-9.
16. Jaensson, E., et al., Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. J Exp Med, 2008. 205(9): p. 2139-49.
17. Coombes, J.L., et al., A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med, 2007. 204(8): p. 1757-64.
18. Mora, J.R., et al., Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. Nature, 2003. 424(6944): p. 88-93.
19. Niess, J.H., et al., CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. Science, 2005. 307(5707): p. 254-8.
20. Chieppa, M., et al., Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. J Exp Med, 2006. 203(13): p. 2841-52.
21. Bogunovic, M., et al., Origin of the lamina propria dendritic cell network. Immunity, 2009. 31(3): p. 513-25.
22. Niess, J.H. and G. Adler, Enteric flora expands gut lamina propria CX3CR1+ dendritic cells supporting inflammatory immune responses under normal and inflammatory conditions.

J Immunol, 2010. 184(4): p. 2026-37.
23. Varol, C., et al., Intestinal lamina propria dendritic cell subsets have different origin and functions. Immunity, 2009. 31(3): p. 502-12.
24. Jarry, A., et al., Mucosal IL-10 and TGF-beta play crucial roles in preventing LPS-driven, IFN-gamma-mediated epithelial damage in human colon explants. J Clin Invest, 2008. 118(3): p. 1132-42.
25. Rimoldi, M., et al., Monocyte-derived dendritic cells activated by bacteria or by bacteria-stimulated epithelial cells are functionally different. Blood, 2005.106(8): p.2818-26.
26. Kalinski, P., et al., Prostaglandin E(2) is a selective inducer of interleukin-12 p40 (IL-12p40) production and an inhibitor of bioactive IL-12p70 heterodimer.Blood, 2001.97(11):p.3466-9.
27. Smythies, L.E., et al., Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. J Clin Invest, 2005. 115(1): p. 66-75.
28. Zeuthen, L.H., L.N. Fink, and H. Frokiaer, Epithelial cells prime the immune response to an array of gut-derived commensals towards a tolerogenic phenotype through distinct actions of thymic stromal lymphopoietin and transforming growth factor-beta. Immunology, 2008. 123(2): p. 197-208.
29. Zaph, C., et al., Epithelial-cell-intrinsic IKK-beta expression regulates intestinal immune homeostasis. Nature, 2007. 446(7135): p. 552-6.
30. Allakhverdi, Z., et al., Thymic stromal lymphopoietin is released by human epithelial cells in response to microbes, trauma, or inflammation and potently activates mast cells. J Exp Med, 2007. 204(2): p. 253-8.
31. van Baarlen, P., et al., Differential NF-kappaB pathways induction by *Lactobacillus plantarum* in the duodenum of healthy humans correlating with immune tolerance. Proc Natl Acad Sci U S A, 2009. 106(7): p. 2371-6.
32. Harris, S.G., et al., Prostaglandins as modulators of immunity. Trends Immunol,2002. 23(3): p. 144-50.
33. Rehwinkel, J. and C. Reis e Sousa, RIGorous detection: exposing virus through RNA sensing. Science, 2010. 327(5963): p. 284-6.
34. Williams, A., R.A. Flavell, and S.C. Eisenbarth, The role of NOD-like Receptors in shaping adaptive immunity. Curr Opin Immunol, 2010. 22(1): p. 34-40.
35. FAO and WHO, Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria. 2001.
36. Sherman, P.M., J.C. Ossa, and K. Johnson-Henry, Unraveling mechanisms of action of probiotics. Nutr Clin Pract, 2009. 24(1): p. 10-4.
37. Isolauri, E., et al., Improved immunogenicity of oral D x RRV reassortant rotavirus vaccine by *Lactobacillus casei* GG. Vaccine, 1995. 13(3): p. 310-2.
38. Fang, H., et al., Modulation of humoral immune response through probiotic intake. FEMS Immunol Med Microbiol, 2000. 29(1): p. 47-52.
39. de Vrese, M., et al., Probiotic bacteria stimulate virus-specific neutralizing antibodies following a booster polio vaccination. Eur J Nutr, 2005. 44(7): p. 406-13.
40. Paineau, D., et al., Effects of seven potential probiotic strains on specific immune responses in healthy adults: a double-blind, randomized, controlled trial. FEMS Immunol Med Microbiol, 2008. 53(1): p. 107-13.
41. Prescott, L.M., J.P. Harley, and D.A. Kelin, Microbiology, Bacteria: The Low G+ C Gram positives. (5th Ed.). Boston: McGraw Hill, 2002: p. 529–530.
42. Bernardeau, M., et al., Safety assessment of dairy microorganisms: the *Lactobacillus* genus. Int J Food Microbiol, 2008. 126(3): p. 278-85.
43. Kleerebezem, M., et al., Complete genome sequence of *Lactobacillus plantarum* WCFS1.

Proc Natl Acad Sci U S A, 2003. 100(4): p. 1990-5.
44. Sicherer, S.H., et al., US prevalence of self-reported peanut, tree nut, and sesame allergy: 11-year follow-up. J Allergy Clin Immunol, 2010. 125(6): p. 1322-6.
45. Le, T.M., et al., Reported food allergy to peanut, tree nuts and fruit: comparison of clinical manifestations, prescription of medication and impact on daily life. Allergy, 2008. 63(7): p. 910-6.
46. Sampson, H.A., L. Mendelson, and J.P. Rosen, Fatal and near-fatal anaphylactic reactions to food in children and adolescents. N Engl J Med, 1992. 327(6): p. 380-4.
47. Ronmark, E., et al., Major increase in allergic sensitization in schoolchildren from 1996 to 2006 in northern Sweden. J Allergy Clin Immunol, 2009. 124(2): p. 357-63, 63 e1-15.
48. Simpson, C.R., et al., Incidence and prevalence of multiple allergic disorders recorded in a national primary care database. J R Soc Med, 2008. 101(11): p. 558-63.
49. Tai, A., R. Volkmer, and A. Burton, Prevalence of asthma symptoms and atopic disorders in preschool children and the trend over a decade. J Asthma, 2009. 46(4): p. 343-6.
50. Simpson, C.R., et al., Trends in the epidemiology and prescribing of medication for eczema in England. J R Soc Med, 2009. 102(3): p. 108-17.
51. Burks, A.W., Peanut allergy. Lancet, 2008. 371(9623): p. 1538-46.
52. Prussin, C. and D.D. Metcalfe, 5. IgE, mast cells, basophils, and eosinophils. J Allergy Clin Immunol, 2006. 117(2 Suppl Mini-Primer): p. S450-6.
53. Knol, E.F., Requirements for effective IgE cross-linking on mast cells and basophils. Mol Nutr Food Res, 2006. 50(7): p. 620-4.
54. Rindsjo, E. and A. Scheynius, Mechanisms of IgE-mediated allergy. Exp Cell Res, 2010. 316(8): p. 1384-9.
55. Ruiz, R.G., D.M. Kemeny, and J.F. Price, Higher risk of infantile atopic dermatitis from maternal atopy than from paternal atopy. Clin Exp Allergy, 1992. 22(8): p. 762-6.
56. Kimber, I. and R.J. Dearman, Factors affecting the development of food allergy. Proc Nutr Soc, 2002. 61(4): p. 435-9.
57. Lee, L.A. and A.W. Burks, New insights into diagnosis and treatment of peanut food allergy. Front Biosci, 2009. 14: p. 3361-71.
58. Clark, A.T. and P.W. Ewan, Good prognosis, clinical features, and circumstances of peanut and tree nut reactions in children treated by a specialist allergy center. J Allergy Clin Immunol, 2008. 122(2): p. 286-9.
59. Stahl, M.C. and T.S. Rans, Potential therapies for peanut allergy. Ann Allergy Asthma Immunol, 2011. 106(3): p. 179-87; quiz 188.
60. Lee, S.Y., et al., Oral administration of IL-12 suppresses anaphylactic reactions in a murine model of peanut hypersensitivity. Clin Immunol, 2001. 101(2): p. 220-8.
61. Kalliomaki, M., et al., Guidance for Substantiating the Evidence for Beneficial Effects of Probiotics: Prevention and Management of Allergic Diseases by Probiotics. J Nutr, 2010.
62. Meijerink, M. and J. Wells, Probiotic modulation of dendritic cells and T cell responses in the intestine. Beneficial microbes, 2010. 1(4): p. 317-326.
63. Knippels, L.M., et al., Oral sensitization to food proteins: a Brown Norway rat model. Clin Exp Allergy, 1998. 28(3): p. 368-75.
64. Li, X.M., et al., A murine model of peanut anaphylaxis: T- and B-cell responses to a major peanut allergen mimic human responses. J Allergy Clin Immunol, 2000. 106(1 Pt 1): p. 150-8.
65. Dearman, R.J. and I. Kimber, Animal models of protein allergenicity: potential benefits, pitfalls and challenges. Clin Exp Allergy, 2009. 39(4): p. 458-68.
66. Spangler, B.D., Structure and function of cholera toxin and the related Escherichia coli heat-labile enterotoxin. Microbiol Rev, 1992. 56(4): p. 622-47.
67. Frey, A., et al., Role of the glycocalyx in regulating access of microparticles to apical plasma

membranes of intestinal epithelial cells: implications for microbial attachment and oral vaccine targeting. J Exp Med, 1996. 184(3): p. 1045-59.
68. Lycke, N., et al., The adjuvant action of cholera toxin is associated with an increased intestinal permeability for luminal antigens. Scand J Immunol, 1991. 33(6): p. 691-8.
69. Gagliardi, M.C., et al., Cholera toxin induces maturation of human dendritic cells and licences them for Th2 priming. Eur J Immunol, 2000. 30(8): p. 2394-403.
70. Blazquez, A.B. and M.C. Berin, Gastrointestinal dendritic cells promote Th2 skewing via OX40L. J Immunol, 2008. 180(7): p. 4441-50.
71. Anosova, N.G., et al., Cholera toxin, E. coli heat-labile toxin, and non-toxic derivatives induce dendritic cell migration into the follicle-associated epithelium of Peyer's patches. Mucosal Immunol, 2008. 1(1): p. 59-67.
72. Shreedhar, V.K., B.L. Kelsall, and M.R. Neutra, Cholera toxin induces migration of dendritic cells from the subepithelial dome region to T- and B-cell areas of Peyer's patches. Infect Immun, 2003. 71(1): p. 504-9.
73. Townsend, A.R. and J.J. Skehel, The influenza A virus nucleoprotein gene controls the induction of both subtype specific and cross-reactive cytotoxic T cells. J Exp Med, 1984. 160(2): p. 552-63.
74. Yewdell, J.W., et al., Influenza A virus nucleoprotein is a major target antigen for cross-reactive anti-influenza A virus cytotoxic T lymphocytes. Proc Natl Acad Sci U S A, 1985. 82(6): p. 1785-9.
75. McMichael, A., Cytotoxic T lymphocytes specific for influenza virus. Curr Top Microbiol Immunol, 1994. 189: p. 75-91.
76. McMichael, A.J., et al., Recognition of influenza A virus nucleoprotein by human cytotoxic T lymphocytes. J Gen Virol, 1986. 67 (Pt 4): p. 719-26.
77. Holland, D., et al., Intradermal influenza vaccine administered using a new microinjection system produces superior immunogenicity in elderly adults: a randomized controlled trial. J Infect Dis, 2008. 198(5): p. 650-8.
78. Davidson, L.E., et al., *Lactobacillus* GG as an immune adjuvant for live-attenuated influenza vaccine in healthy adults: a randomized double-blind placebo-controlled trial. Eur J Clin Nutr, 2011. 65(4): p. 501-7.

Chapter 2

Probiotic modulation of dendritic cells and T cell responses in the intestine

M. Meijerink and J.M. Wells

Beneficial Microbes (2010) 1, 317-26

Abstract

Over the past decade it has become clear that probiotic and commensal interactions with mucosal dendritic cells in the lamina propria or epithelial cells lining the mucosa can modulate specific functions of the mucosal immune system. Innate pattern-recognition receptors such as Toll-like receptors (TLRs), NOD-like receptors (NLRs) and C-type lectin receptors (CLRs) play a crucial role in the host recognition of probiotics and other microorganism. Signalling via these receptors directly influences the chemokine and cytokine response of dendritic cells as well as the crosstalk between the epithelium and the immune cells in the lamina propria. This can influence the population of effector and regulatory T cell subsets in the mucosa. Immune assays with probiotics have shown that the *in vitro* immune response is both species and strain-specific. Such assays may be useful for the selection of probiotic strains that have beneficial effects on the regulation of intestinal inflammation but more comparative studies are needed to confirm recent findings. A better understanding of the molecular mechanisms of probiotics, the effect of dose, and frequency of administration on microbial sampling by mucosal antigen presenting cells (APCs) will also help to clarify the value of immune assays as selection criteria for probiotics.

Mucosal dendritic cell and antigen sampling

The surface area of the intestinal epithelium is estimated to be around 400 m^2 and the major portal for the entry of pathogenic viruses and bacteria. The intestinal tract is also home to a myriad of commensal bacteria which are largely mutualistic and symbiotic in nature. Several host adaptations appear to have evolved to avoid potentially damaging inflammatory immune reactions to harmless food and commensal antigens while retaining the capacity to detect and protect against pathogens. It would be incorrect however, to consider the epithelium as impermeable to microbes even in the absence of inflammation. Antigen sampling occurs at mucosal sites associated with isolated lymphoid follicles (ILFs) in the large intestine and Peyer's patches (PP) in the small intestine which contain large aggregates of lymphoid follicles (LFs). Specialized antigen sampling cells, called M cells are present in the epithelium overlaying the mucosal lymphoid tissue and actively transport antigens to the underlying immune cells. In addition epithelial cells can transport antigens to the lamina propria (LP) albeit much less efficiently than M cells [1].
Dendritic cells (DCs) are present throughout the intestine in PP, ILFs and LP and play a key role in regulating tolerance and immunity [2]. DCs are the most important professional antigen-presenting cells and express up to 100x more MHC-class II and are more effective at differentiating naïve T cells than other antigen presenting cells (APCs) [3, 4]. DCs in the PP and ILFs are considered to be primarily responsible for T–cell dependent IgA responses [5]. The LP of both the small and large intestine is populated by two major DC subsets which perform different immune functions. These two populations can be discriminated by the differential expression of CD103 (also known as integrin $\alpha E\beta 7$) and the fractalkine receptor CX3CR1 giving rise to the CD103+ CX3CR1- and CD103- CX3CR1+ subsets. The CD103+ subset of DCs are derived from a common DC progenitor that gives rise to DCs in the lymphoid organs but not monocytes [6]. CD103+ DCs traffic from the mucosal tissues to the draining lymph nodes (LN) where they present luminal antigens including those derived from microorganisms to T–lymphocytes [7, 8]. The CD103+ DCs are primarily involved the induction of CD4+ Foxp3+ T regulatory cells (Tregs) via a transforming growth factor beta (TGF-β) and retinoic acid dependent mechanism [9]. In contrast the CD103- CX3CR1+ cells are longer lived but poor T cell stimulators [10]. Furthermore this cell population expresses the macrophage markers CD11b, F4/80 but also variable levels of CD11c, and CD14 suggesting that this population consists of several different cell subsets [10]. The CX3CR1+ cell population were recently reported to comprise both CD68+ and CD68- cells indicating the presence of both macrophage and DC cell types. CX3CR1+ 'DC' cells have been shown to extend protrusions through the paracellular space between epithelial cells to sample luminal antigens. However, the dependency of this mechanism on expression of CX3CR1 is somewhat controversial and it is possible that other LP cells can sample antigens in this way [11]. CX3CR1+ CD68- DCs are associated with inflammatory responses and IL-17/ IFN-γ responses [12, 13] and thus are considered as effector T cell inducing cells.

Microbial recognition by dendritic cells

DCs play a key role in mucosal immunity and tolerance, and, not surprisingly, have been implicated as key players in the interaction between probiotics and the immune system. Several studies have implicated a role for epithelial cells in endowing DCs with their ability to prime non-inflammatory responses and induce Tregs [14-17]. Conditioning of monocyte-derived DCs (MoDCs) with intestinal epithelial cell (EC) supernatants *in vitro* abolishes the ability of DCs to produce interleukin (IL)-12 and prime naïve T cells for T helper (Th) 1 cell polarization in response to microbial stimuli [16]. Consequently the EC conditioned DCs produce high amounts of IL-10 and promote Tregs and Th2 cell responses. *In vitro* this was shown to be dependent on epithelial production of thymic stromal lymphopoietin (TSLP) which acts in concert with epithelial produced transforming growth factor beta (TGF-β) to induce a tolerogenic phenotype in MoDCs *in vitro* [14, 18]. TSLP mRNA is constitutively expressed by epithelial cells, but is upregulated by NF-κB dependent pathways [19]. Thus one may expect that recognition of microbiota by epithelial pattern recognition receptors would also modulate TSLP production. Support for this comes from *in vitro* EC-DC co-culture studies where it was shown that composition of the microbiota exposed to the apical side of the EC influenced production of TSLP and TGF-β and hence the function of the underlying DCs [18]. Microbial stimulation of DCs in the LP and PP is mediated via the binding of microbe-associated molecular patterns (MAMPs) to pattern recognition receptors (PRRs) expressed by mucosal dendritic cells and intestinal epithelial cells. One such family of PRRs comprises the Toll-like receptors (TLRs) which are expressed by a variety of cells of the innate immune system including immature DCs. Each TLR family member is endowed with the ability to recognize a distinct class of conserved MAMPs. With respect to microbial recognition, TLR 1, 2, 4, 5, 6, and 9 signal via binding to common bacterial structures whereas TLR 3, 7 and 8 are aimed at primarily at viral detection. In DCs TLRs 2, 4, 1, 5, and 6 participate in innate signalling on the cell surface and TLR 9, 7, 8 and 3 in endosomes.

A different set of receptors known as the nucleotide binding and oligomerization domain-like receptors (NLRs) of which there are more than 20 family members recognize intracellular ligands. NOD1 and NOD2 have been shown to detect the synthetic mimics of peptidoglycan (PGN) meso-DAP and muramyl dipeptide respectively, whereas the specificities of most of the other NLRs remains uncharacterized.

Activation of TLRs and NLRs can trigger the release of cytokines and the differentiation of immature to mature dendritic cells, making a link to the adaptive arm of the immune system. During maturation DCs up-regulate expression of MHC and co-stimulatory molecules required for antigen presentation and activation of T cells [20]. The cytokines produced by activated DCs are also influenced by the nature of the TLR and NLR stimulus which has important consequences for the induction of different T cell subsets (Fig. 1). The activation of Th1 responses by DCs has often been associated with the production of IL-12 and elicits IFN-γ production by T cells and by NK cells [21]. IL-10 is an anti-inflammatory cytokine that suppresses IL-12 production and consequently IFN-γ

production, thus favouring a Th2 or Treg response. Furthermore, IL-10 down-regulates antigen (Ag) presentation by blocking translocation of Ag-MHC class II complex [22] and acts on macrophages to prevent their activation and elaboration of pro-inflammatory molecules and chemokines, thus inhibiting T cell recruitment into the intestine.

TLR2 is important for the innate recognition of bacterial MAMPs and can form heterodimers with TLR-1 or TLR-6 to detect different but related ligands. TLR-2/1 recognizes tri-acyl lipoproteins found predominantly in Gram-negative bacteria and TLR-2/6 the diacyl groups on lipoteichoic acid and lipoproteins of Gram-positive bacteria. Signalling through these different receptors can have important functional consequences as shown in a recent study where TLR-2/6 agonists induced high amounts of IL-10 and low amounts of IL-12 whereas TLR-2/1 agonists have the opposite effect [23]. Several studies report that preparations of macromolecular PGN (PGNpolymer) can activate NF-κB through human Toll-like receptors 2 (TLR-2). However, a recent study showed that purified PGN isolated from *S. aureus* mutant lacking prolipoprotein diacylglyceryl transferase (Lgt), which couples the diacyl groups to lipoproteins in Gram-positive bacteria, fails to stimulate TLR-2 signalling [24]. This demonstrates that lipoproteins within the macromolecular structures of PGNpolymer, but not PGN itself, activate TLR-2. HPLC-purified monomeric PGN (PGNmonomer) lacked TLR-2 activity but acted as a potent co-stimulator of the innate immune system exclusively in the presence of TLR signals [24]. This synergistic effect was lost in NOD2(-/-) DCs demonstrating that NOD2 is the natural ligand for PGNmonomer [24].

Recently, several lectin and C-type lectin receptors (CLRs) have been characterized that are involved in the recognition and capture of antigens by APCs. One such CLR known as DC-specific intracellular adhesion molecule-3 grabbing non-integrin (DC-SIGN) recognizes high-mannose-containing structures. Some viral pathogens such as HIV-1, and non-viral pathogens, such as *Mycobacterium tuberculosis*, subvert DC functions to escape immune surveillance by targeting the C-type lectin DC-SIGN. For example, the interaction of the abundant mannose containing cell wall component of *M. tuberculosis* to DC-SIGN inhibits DC maturation and increases expression of IL-10, thereby preventing the induction of cell-mediated responses against the pathogen. DC-SIGN binding activity has also been reported for certain strains of *Lactobacillus* species and shown to have an immunomodulatory effect on DC cytokine responses and T cell priming *in vitro* [25, 26]. Other polysaccharides expressed on commensals may also influence mucosal immunity as demonstrated for the PSA polysaccharide of a non-toxin producing *Bacteroides fragilis* strain. Colonization of germ-free mice with the polysaccharide producing strain leads to the induction of Tregs and IL-10 production [27].

Given the complex array of PRRs involved in microbial recognition and the possibilities for crosstalk between the different signalling pathways there are conceivably many ways in which interaction of DCs with a microbe could lead to different outcomes. Here we aim to specifically review our knowledge and understanding of the immunomodulatory effects of different probiotic species and strains on dendritic cells and the adaptive immune system and discuss the relevance and implications of the *in vitro* studies for

clinical trials or mechanistic research in animal models.

Immunomodulation by probiotic strains

It is clear that some intestinal bacteria such as lactobacilli and bifidobacteria, frequently used as probiotics, can play a beneficial role on the health of the host. Probiotics have been shown to enhance barrier function, alter epithelial signaling, compete with pathogens for mucosal colonisation, and directly modify the activity of immune cells [28-30] (Fig. 1). The positive effects of probiotics on inflammatory diseases seem to be largely mediated by an increase in the population of Foxp3+ Tregs as well as the induction of other lymphocytes producing regulatory cytokines. For example, treatment of colitic mice with VSL#3 cocktail of probiotics increases the number of TGF-β and IL-10 producing T cells [31]. Administration of VSL#3 in patients with ileal pouch-anal anastomosis due to ulcerative colitis was associated with a significant increase of Tregs and an increased expression of Foxp3 mRNA in mucosal biopsies [32]. *Bifidobacterium infantis* induced Tregs were also shown to protect against pathogen induced inflammation [33]. Such effects are likely to be mediated by interactions with mucosal DCs [34] although interactions with other immune cells may also play a role. A recent study demonstrated that probiotics selected on the ability to induce high IL-10/IL-12 ratios and enhanced Foxp3 expression in MLNs, modulated CD103+ DCs to induce Tregs *in vivo* [35].
Probiotics and even fragments of dead or lysed bacteria can influence the immune system with their cell wall components, metabolites and their DNA (Fig. 1). Several *in vitro* studies have been conducted on the effect of probiotic bacteria on immune function using different types of immune cells, such as human MoDCs, human peripheral blood mononuclear cells (PBMCs) and mouse bone marrow derived DCs. Different *Lactobacillus* species and even strains of the same species have been shown to differ strikingly in their capacity to induce the cytokines IL-10, IL-12p70 and TNF-α in co-culture with DCs [36, 37]. Many studies on the co-culture of bacteria with immune cells measured cytokine production only after one particular time point of stimulation despite the fact that the kinetics of production are cytokine-specific [38]. To determine the optimal time point for cytokine measurement several studies have been published. For example, Lammers et al. found a time-dependent effect for *L. casei* and *Bifidobacterium breve* by exposing PBMCs for 24, 48 and 72 h to 20 mg/mL probiotic DNA. IL-1β, IL-6 and IL-10 secretion reached maximum levels after 24h incubation and thereafter remained at this level [39]. Chen et al. investigated the kinetics of IL-10 and TNF-α secretion by PBMCs co-cultured with cell wall fragments isolated from *Eubacterium aerofaciens* and *Streptococcus pyogenes*. IL-10 peaked at about 24h incubation, while TNF-α reached the highest level already after 6h [38]. Latvala et al. showed the kinetics of bacteria-induced cytokine mRNA expression and cytokine secretion by DCs co-cultured with *S. thermophilus* THS, *B. breve* Bb99, and *L. lactis* subsp. *cremoris* ARH7. The expression of CCL20 and TNF-α mRNAs, CCL20, TNF-α, and IL-6 production was detected 8h after stimulation with these three probiotic bacteria. While IL-10, IL-12 and IFN-γ were produced 24h

after stimulation with *S. thermophilus* and the probiotic strains [40]. In general most cytokines accumulate in the culture supernatant after stimulation of the immune cells, therefore 24h or 48h incubations are typically used and represent a good time point for immune screening. Quantitative PCR (qPCR) is a more sensitive method to measure cytokine expression, and can be used to measure induction after only a few hours but may not reflect the amount of cytokines that is actually secreted. Additionally feedback signalling of secreted cytokines on immune cells will be missed by this method, while ELISA will measure the accumulation after a certain time.

Probiotics are defined as viable microorganisms which when supplied in sufficient quantities confer a health benefit on the host, but *in vitro* immune assays often utilize dead bacteria and bacterial DNA. According to Rachmilewitz et al., γ-irradiated non-viable bacteria and live bacteria showed similar effects in PBMC co-culture assays [41]. Heat-killed bacteria have been shown to reduce IL-4 and IL-5 production by staphylococcal enterotoxin A stimulated PBMCs [42]. UV-killed bacteria are also often used in studies to investigate the immunomodulatory effects of bacteria [43, 44], but the results do not always correlate with those obtained with live bacteria. Purified bacterial DNA has been shown to induce the same immune responses as live bacteria [41, 45] by the binding of non-methylated CpG motifs in bacterial DNA to TLR-9. The study of Rachmilewitz showed that DNA isolated from VSL#3, a probiotic mixture, but not DNAse-treated probiotics and *E. coli* genomic DNA ameliorated the severity of colitis in DSS-induced and TNBS-induced colitis models as well as spontaneous colitis in IL-10 knockout mice. Ghadimi et al. showed that live probiotic bacteria as well as their genomic DNA inhibited IL-4 and IL-5 and increased the IFN-γ production by PBMCs in healthy donors. The bacterial DNA appears to contribute to about 50% of the immunomodulatory effects of these probiotics [45]. *Bifidobacterium* genomic DNA decreased IL-1β secretion and induced the secretion of IL-10 by PBMCs from healthy donors, suggesting that purified bacterial DNA can have immunomodulatory effects [39]. In contrast Medina et al. showed that some *B. longum* strains and their genomic DNA increased IL-12 and IFN-γ production by PBMCs [46]. In TLR9-/- mice the Treg frequencies are dramatically increased suggesting that commensal DNA constrains the generation of Tregs [47]. However this is not comparable to studies with individual probiotic DNA samples and may also involve the effects of TLR-9 signalling on other cell types such as the epithelium. Furthermore, it is unlikely that the purified DNA of bacteria will be presented in the same way *in vivo* and in the context of whole bacteria the induced cytokines could be quite different due to the synergistic or inhibitory effects of other MAMPs. Most immune co-culture assays with bacteria employ antibiotics to prevent overgrowth and acidification of the culture, and therefore the probiotic strains will be killed, or arrested in growth during the incubation period [36, 37, 45, 48]. Mileti et al. used an elegant method by which the live bacteria are in co-culture with DCs and after 1h incubation, the cells are washed and the medium is replaced by an antibiotic containing medium [49]. The bacteria to cell ratios (BCR) used in probiotic co-culture experiments differs a lot between *in vitro*

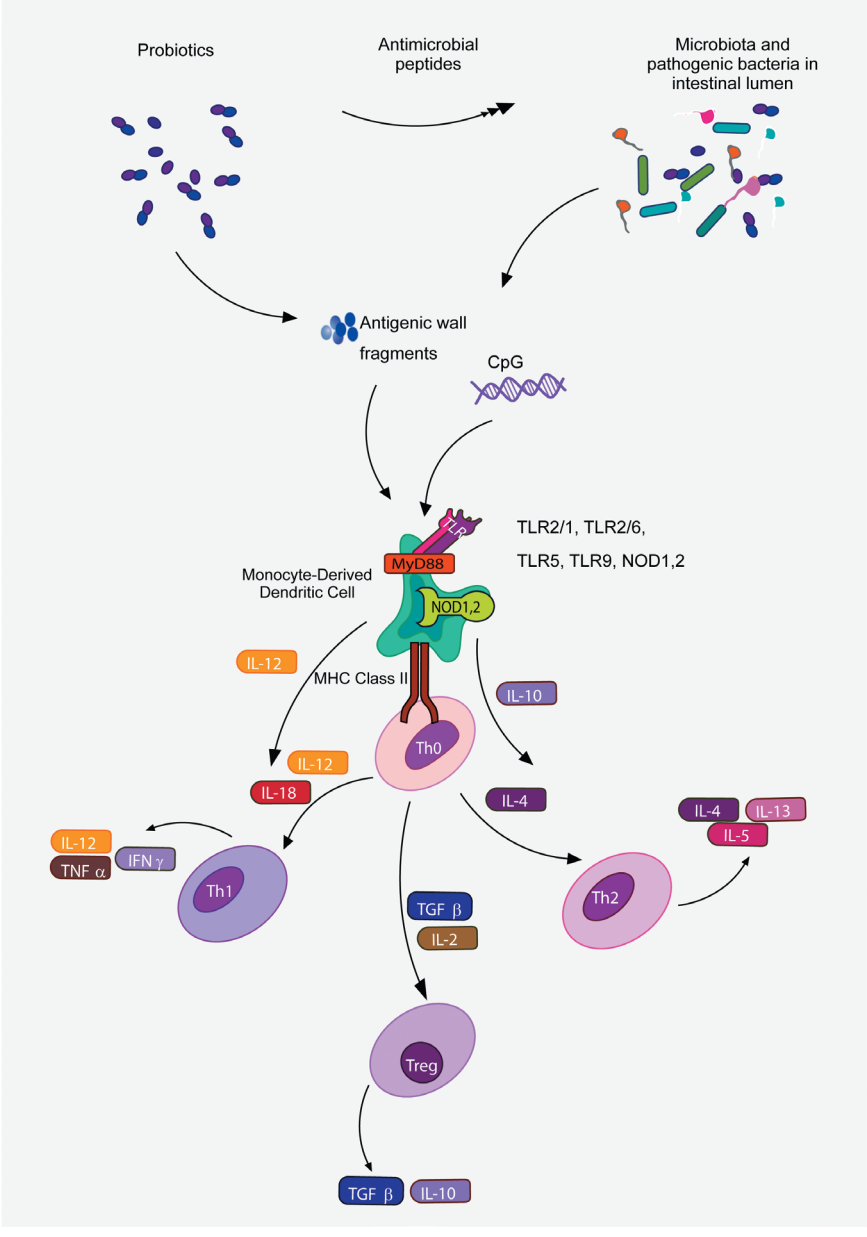

Figure 1. Microbe associated molecular patterns derived from bacteria (including probiotics) are recognised by pattern recognition receptors (PRRs), such as Toll like receptors and NOD like receptors. Initiation of dendritic cell maturation starts after ligation of PRRs. The integration of different signaling via PRRs determines the selective priming of DCs for production of Th1, Th2 or Tregs polarizing cytokines.

matured DCs, expressing high levels of CD86 [25], other groups used BCR of 1:1 or 10:1 studies. Whereas Konstantinov et al. matured MoDCs with BCR 100:1 and obtained fully to maturate DCs or stimulate their immune cells [26, 36, 37, 48, 50]. *In vivo* it is likely that DCs will only be in contact with low numbers of bacteria (or fragments) that are either actively sampled by M cells or intra- and transepithelial DCs [5]. Therefore BCR of 1:1 or 10:1 seem to be more relevant to the *in vivo* situation.

The phase of growth also can influence the immunomodulating capacity of the bacteria. Strikingly a recent study showed that exponential phase and stationary phase *L. plantarum* WCFS1 cells elicited distinct human duodenal transcript profiles. This appeared to result from differential modulation of canonical NF-κB-dependent signalling pathways associated with mucosal immune tolerance in epithelial tissue biopsies [51]. Hemert et al. showed *in vitro* that PBMC cytokine responses were also differently induced by exponential- and stationary-phase grown bacteria [48]. Commercial probiotic products are typically in stationary growth phase and thus are used as such in immune assays. However, it cannot be ruled out that some probiotics persist in the intestine and will therefore be sampled in different growth phases by the mucosal immune system.

Both species-specific and strain-specific effects of different lactobacilli and bifidobacteria on PBMCs and DCs have been described in several studies [36, 37, 40, 46, 48, 50, 52-56]. For example, Christensen et al. showed that different species of *Lactobacillus* exert very different and even opposing effects on DC activation [52]. Similarly, co-culture of DCs with different strains of *B. longum* led to variable cytokine responses and co-culture with PBMCs led to the secretion of either pro-inflammatory or regulatory cytokines [46]. Foligne et al. demonstrated large strain-specific variations in cytokine production by PBMCs co-cultured with 13 different bacterial strains and strain-specific differences in their protective capacity in a mouse TNBS-induced model of colitis. Taken together these findings suggest probiotics intended for modulation of immune responses should be selected on a strain by strain basis in a comparative study [36, 48, 50]. This does not take into account the possibility that different probiotic species and even strains may also differ in their fate *in vivo*. Probiotic sampling may occur at several places along the GI tract but the efficiency is likely to vary substantially within the entire small intestine due to the anatomical differences in the epithelium and lymphoid tissue. Competition for binding and adhesion of probiotic organisms will also be influenced by competing microorganisms which reach more than a million fold higher numbers in the colon than in the small intestine. Bacterial translocation or sampling of probiotic strains, could be very different in healthy individuals compared to individuals with colitis or intestinal damage thereby leading to different effects on the host immune system.

Linking results from *in vitro* and *in vivo* studies

Although studies on the *in vitro* immunomodulatory properties of probiotics usually precede their application in animals or humans, there is a dearth of systematic comparative studies linking *in vitro* data to *in vivo* data for different strains.

Animal models

The ability of different lactobacilli to induce a high ratio of IL-10/ IL-12 or IL-10/ TNF-α in immune cells correlates with their capacity to provide significant protection in TNBS induced colitis in mice and rats [37, 57, 58]. Foligne et al. showed that the ranking of strains obtained on the basis of an *in vitro* IL-10/IL-12 cytokine induction ratio closely correlates with the ranking of the *in vivo* ability of the strains to attenuate experimental colitis. However, this study did not include strains exhibiting a moderate or low IL-10/ IL-12 profile in the *in vivo* study, which might have validated the proposed screening strategy. In contrast Mileti et al., demonstrated that *L. paracasei* and LGG induced a similar cytokine ratio in DCs, but did not show comparable protective effects *in vivo* in the mouse DSS model of colitis. Mice receiving *L. paracasei* showed a delay in colitis development and a reduced severity of disease whereas LGG exacerbated colitis [49]. The protection of *L. paracasei* could work via another mechanism than the induction of an anti-inflammatory response, which might explain the contradictory results. Additionally the probiotics used could also have different colonization patterns. Two different strains of *Lactobacillus* with similar *in vitro* growth, survival and adherence properties showed distinct differences in colonization patterns, resulting in distinct host immune responses at both the mucosal as well as systemic level [59]. The study by Mastrangeli et al. investigated the immunomodulatory effects of VSL#3 on bone marrow derived dendritic cells and spleen cells from naïve and allergen-sensitized mice and tested the ability of VSL#3 to prevent *in vivo* development of allergen-specific sensitization in a mouse model. Co-culture of DCs with VSL#3 induced maturation of DCs and production of IL-10 and IL-12. IL-10 and IFN-γ secretion by spleen cells from both naïve and *Parietaria judaica*-sensitized mice was increased by co-culture with VSL#3. *In vivo* prophylactic treatment with VSL#3 induced IgG1 levels in serum, and in the lung IL-13 and IL-4 mRNA expression were remarkably decreased, while IL-10 expression was increased [60].

Kwon et al. demonstrated that probiotics induce regulatory DCs that expand the Treg population in the MLNs [35]. Tregs are important in immunological tolerance by the suppression of effector T cells at inflammatory sites. This study underlines the important role of DCs in the induction of Tregs and how *in vitro* assays can be used for the selection of probiotic strains and species that have therapeutic effects in models of inflammatory bowel disease, atopic dermatitis and rheumatoid arthritis. Nevertheless the human gastrointestinal tract is a complex and variable environment and other host factors such as TGF-β and TSLP produced by epithelial cells can influence the mucosal immune functions [30]. DCs conditioned with EC supernatants *in vitro* are blocked to produce IL-12 and to prime naïve T cells for Th1 polarization in response to bacteria. The conditioned DCs secrete more IL-10 and promote Treg and Th2 responses, which was shown to be dependent on TSLP production [16].

Although there is evidence that *in vitro* data can be used to predict *in vivo* action, specific probiotic strains need to be investigated *in vitro* and *in vivo* using more than one murine disease model to select suitable strains, as the activity of certain strains depends on the type of colitis model. For example Shibolet et al. showed variable responses to

VSL#3 and LGG in two models of experimental colitis in rats, where the two probiotis significantly ameliorated the severity of the colitis induced by sulfhydryl-blocker iodactamide but had no effect on the immune mediated DNBS-induced colitis. Sulfhydryl plays an important role in maintaining the mucosal integrity, blocking sulfhydryl compounds will therefore induce colitis, while DNBS induces severe transmural necrosis. Thus the differences in (severity of) colitis induction might explain the differences in protection [61]. Contrasting results have also been obtained for the same probiotic mixture VSL#3 in the DSS-induced colitis model. Whereas Rachmilewitz et al. showed that the protective effect of VSL#3 was due to interaction of bacterial CpG DNA with TLR-9, Gaudier et al. reported no protective efficacy. The two study designs look similar with respect to daily dose and the colitis-inducing agent, but the start of VSL#3 administration differed between the studies and thus could be an explanation for the conflicting results [41, 62]. Differences between studies with the same probiotic could also potentially be affected by the method of culturing the bacteria or the schedule of probiotic administration. Furthermore, the techniques used for cytokine quantification (RT-PCR, immunohistochemical analysis, ELISA or cytometric bead arrays) differ a lot between published studies, sometimes making data comparison difficult.

Human trials

A few studies have been performed to directly compare the *in vitro* properties of probiotics with the *in vivo* response in humans. Flinterman et al. studied both the *in vitro* immunological effects of a mixture of bacterial strains and the *ex vivo* immunological effects after oral administration of a probiotic mixture in children with food allergy [63]. This showed that the probiotics had a different potential to modulate the immune response *in vitro* and *ex vivo*. Co-culture of PBMCs isolated from the food allergic children resulted in an increased T-cell proliferation with enhanced production of IFN-γ, IL-10, TNF-α and to a lesser extent IL-6. After oral treatment for three months, proliferation of the T-cells was increased, whereas *in vitro* IgE production decreased in the probiotic treatment group compared to the base line. The cytokine production of IL-10, TNF-α, and IL-6 tended to decrease, but other Th1 and Th2 cytokines were not altered. The study by Kopp et al. investigated the protective effect of LGG on the development of atopic dermatitis in neonates with a family history of atopy [64]. They showed that LGG co-cultured with mononuclear cells enhanced IL-10 production and decreased IFN-γ release, but supplementation with LGG during pregnancy did not alter the proliferation capacity or cytokine pattern in their recipients. Taken together these studies reveal discrepancies between the data obtained *in vitro* with PBMCs and the clinical effects of probiotics.

In vitro VSL#3 induced IL-10 and down regulated IL-12p40 production in colonic DCs and this correlated well with the results of an *in vivo* study in ulcerative colitis (UC) patients [65]. The VSL#3 treated group showed improved intestinal function, an increase in regulatory cytokines and lowered pro-inflammatory cytokines and TLR expression compared to placebo, which might have contributed to the therapeutic benefit [65,

66]. In UC patients the effect of VSL#3 was similar to treatment with corticosteroids which also modulated cytokine production in colonic DC. Taken together these results suggest that this probiotic mixture mediates a positive effect in acute UC via modulation of colonic DC.
In one study where immune assays were not used as selection criteria there was an unfavourable outcome in the treatment of irritable bowel syndrom [67]. *L. plantarum* strain MF1298 was found to have the best *in vitro* probiotic properties of 22 strains of lactobacilli isolated from fermented food products. The selection was based on adherence to the human colon adenoma cell line CaCo2, increased epithelial barrier function, antimicrobial activity against potential pathogens, and good survival during passage through the human gastrointestinal tract. Nevertheless unfavourable effect on IBS symptoms was observed in subjects after intake of *L. plantarum* MF1298 compared to placebo.

Conclusions

Certain strains of probiotics have been shown to confer health benefits in clinical trials and experimental animal models but there have also been a number of inconclusive, negative and even contrasting outcomes from *in vivo* studies. It is becoming clear that the effects of probiotics are highly dependent on the strain properties and the study population or animal model. Furthermore, our lack of knowledge concerning the precise mechanisms of different probiotics is hampering the rational selection of strains and the correlation of *in vitro* data with the outcomes *in vivo*.
Many of the positive results obtained with probiotics *in vivo* are linked to effects on the immune system but comparison of *in vitro* data with efficacy *in vivo* is confounded by the lack of standardized assay methods and comparative studies. Despite these limitations there is evidence that the immune profile obtained in co-culture assays with bacteria and immune cells (especially for IL-10 and IL-12) can be predictive for their *in vivo* immunomodulatory activities [35, 37, 57]. This will depend on the assays and the strains used but also on the type of DCs or immune assays. Immune assays for screening the immunomodulatory effects of probiotics would benefit from using appropriate ratios of live bacteria to cells which most likely reflect the *in vivo* situation. Additionally, methods that use live whole bacteria, even if for short incubations with immune cells, are likely to be more predictable than the use of heat killed bacteria or bacterial fractions such as purified DNA. It should be taken in account that dead probiotic organisms may not have the same properties as live probiotics and further studies are needed to fully understand the effects of bacterial metabolites and secreted proteins on immune cells.
Results from mouse models provide growing evidence that the induction of Tregs by probiotics is mediated by interactions with LP DCs, but supporting data is lacking from human studies mainly due to the difficulty in obtaining cells from different compartments of the mucosal immune system. Other probiotic mechanisms are likely to in-

clude modulation of the T effector subsets and the CX3CR1+ LP cells that can sample bacteria through the epithelium. Further research is needed to identify host and bacterial target genes and molecules, which are involved in the effect of probiotics on the immune system. Additional interactions with dietary components could influence the efficiency of probiotic strains. Furthermore it is important to understand the effects of dose in humans and resolve how best to formulate probiotics. More understanding of the molecular mechanism of probiotics *in vivo* will help us to identify and select the right probiotics for prevention or treatment of certain inflammatory mediated diseases. Probiotics should be selected on strain by strain basis in comparative studies, because their effects on the immune system and other properties can vary considerably even within a species. Different probiotic species and even strains may also differ in their fate *in vivo*, and further research is needed to increase our knowledge about the effect of dose, and frequency of administration on sampling of different strains and species of probiotics by DCs and other APCs.

References

1. Miller, H., et al., Intestinal M cells: the fallible sentinels? World J Gastroenterol, 2007. 13(10): p. 1477-86.
2. Johansson, C. and B.L. Kelsall, Phenotype and function of intestinal dendritic cells. Semin Immunol, 2005. 17(4): p. 284-94.
3. Inaba, K., et al., High levels of a major histocompatibility complex II-self peptide complex on dendritic cells from the T cell areas of lymph nodes. J Exp Med, 1997. 186(5): p. 665-72.
4. Levin, D., et al., Role of dendritic cells in the priming of CD4+ T lymphocytes to peptide antigen *in vivo*. J Immunol, 1993. 151(12): p. 6742-50.
5. Macpherson, A.J. and T. Uhr, Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. Science, 2004. 303(5664): p. 1662-5.
6. Bogunovic, M., et al., Origin of the lamina propria dendritic cell network. Immunity, 2009. 31(3): p. 513-25.
7. Jaensson, E., et al., Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. J Exp Med, 2008. 205(9): p. 2139-49.
8. Sung, S.S., et al., A major lung CD103 (alphaE)-beta7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins. J Immunol, 2006. 176(4): p. 2161-72.
9. Coombes, J.L., et al., A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med, 2007. 204(8): p. 1757-64.
10. Schulz, O., et al., Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. J Exp Med, 2009. 206(13): p. 3101-14.
11. Chieppa, M., et al., Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. J Exp Med, 2006. 203(13): p. 2841-52.
12. Niess, J.H. and G. Adler, Enteric flora expands gut lamina propria CX3CR1+ dendritic cells supporting inflammatory immune responses under normal and inflammatory conditions. J Immunol, 2010. 184(4): p. 2026-37.
13. Varol, C., et al., Intestinal lamina propria dendritic cell subsets have different origin and functions. Immunity, 2009. 31(3): p. 502-12.
14. Iliev, I.D., et al., Human intestinal epithelial cells promote the differentiation of tolerogenic dendritic cells. Gut, 2009. 58(11): p. 1481-9.
15. Jarry, A., et al., Mucosal IL-10 and TGF-beta play crucial roles in preventing LPS-driven, IFN-gamma-mediated epithelial damage in human colon explants. J Clin Invest, 2008. 118(3): p. 1132-42.
16. Rimoldi, M., et al., Monocyte-derived dendritic cells activated by bacteria or by bacteria-stimulated epithelial cells are functionally different. Blood, 2005. 106(8): p. 2818-26.
17. Rimoldi, M., et al., Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. Nat Immunol, 2005. 6(5): p. 507-14.
18. Zeuthen, L.H., L.N. Fink, and H. Frokiaer, Epithelial cells prime the immune response to an array of gut-derived commensals towards a tolerogenic phenotype through distinct actions of thymic stromal lymphopoietin and transforming growth factor-beta. Immunology, 2008. 123(2): p. 197-208.
19. Allakhverdi, Z., et al., Thymic stromal lymphopoietin is released by human epithelial cells in response to microbes, trauma, or inflammation and potently activates mast cells. J Exp Med, 2007. 204(2): p. 253-8.

20. Kapsenberg, M.L., Dendritic-cell control of pathogen-driven T-cell polarization. Nat Rev Immunol, 2003. 3(12): p. 984-93.
21. Heufler, C., et al., Interleukin-12 is produced by dendritic cells and mediates T helper 1 development as well as interferon-gamma production by T helper 1 cells. Eur J Immunol, 1996. 26(3): p. 659-68.
22. De Smedt, T., et al., Regulation of dendritic cell numbers and maturation by lipopolysaccharide *in vivo*. J Exp Med, 1996. 184(4): p. 1413-24.
23. Depaolo, R.W., et al., Toll-like receptor 6 drives differentiation of tolerogenic dendritic cells and contributes to LcrV-mediated plague pathogenesis. Cell Host Microbe, 2008. 4(4): p. 350-61.
24. Volz, T., et al., Natural *Staphylococcus aureus*-derived peptidoglycan fragments activate NOD2 and act as potent costimulators of the innate immune system exclusively in the presence of TLR signals. Faseb J, 2010.
25. Konstantinov, S.R., et al., S layer protein A of *Lactobacillus acidophilus* NCFM regulates immature dendritic cell and T cell functions. Proc Natl Acad Sci U S A, 2008. 105(49): p. 19474-9.
26. Smits, H.H., 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(6): p. 1260-7.
27. Round, J.L. and S.K. Mazmanian, Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. Proc Natl Acad Sci U S A, 2010. 107(27): p. 12204-9.
28. Sherman, P.M., J.C. Ossa, and K. Johnson-Henry, Unraveling mechanisms of action of probiotics. Nutr Clin Pract, 2009. 24(1): p. 10-4.
29. Karczewski, J., et al., Regulation of human epithelial tight junction proteins by *Lactobacillus plantarum in vivo* and protective effects on the epithelial barrier. Am J Physiol Gastrointest Liver Physiol, 2010. 298(6): p. G851-9.
30. Wells, J.M., et al., Epithelial crosstalk at the microbiota-mucosal interface. Proc Natl Acad Sci U S A, 2011. 108 Suppl 1: p. 4607-14.
31. Di Giacinto, C., et al., Probiotics ameliorate recurrent Th1-mediated murine colitis by inducing IL-10 and IL-10-dependent TGF-beta-bearing regulatory cells. J Immunol, 2005. 174(6): p. 3237-46.
32. Pronio, A., et al., Probiotic administration in patients with ileal pouch-anal anastomosis for ulcerative colitis is associated with expansion of mucosal regulatory cells. Inflamm Bowel Dis, 2008. 14(5): p. 662-8.
33. O'Mahony, C., et al., Commensal-induced regulatory T cells mediate protection against pathogen-stimulated NF-kappaB activation. PLoS Pathog, 2008. 4(8): p. e1000112.
34. Foligne, B., et al., A key role of dendritic cells in probiotic functionality. PLoS One, 2007. 2(3): p. e313.
35. Kwon, H.K., et al., Generation of regulatory dendritic cells and CD4+Foxp3+ T cells by probiotics administration suppresses immune disorders. Proc Natl Acad Sci U S A, 2010. 107(5): p. 2159-64.
36. Meijerink, M., et al., Identification of genetic loci in *Lactobacillus plantarum* that modulate the immune response of dendritic cells using comparative genome hybridization. PLoS One, 2010. 5(5): p. e10632.
37. Foligne, B., et al., Correlation between *in vitro* and *in vivo* immunomodulatory properties of lactic acid bacteria. World J Gastroenterol, 2007. 13(2): p. 236-43.
38. Chen, T., et al., Human cytokine responses induced by gram-positive cell walls of normal

intestinal microbiota. Clin Exp Immunol, 1999. 118(2): p. 261-7.
39. Lammers, K.M., 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(2): p. 165-72.
40. Latvala, S., et al., Potentially probiotic bacteria induce efficient maturation but differential cytokine production in human monocyte-derived dendritic cells. World J Gastroenterol, 2008. 14(36): p. 5570-83; discussion 5581-2.
41. Rachmilewitz, D., et al., Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. Gastroenterology, 2004. 126(2):p. 520-8.
42. Pochard, P., et al., Lactic acid bacteria inhibit TH2 cytokine production by mononuclear cells from allergic patients. J Allergy Clin Immunol, 2002. 110(4): p. 617-23.
43. Fink, L.N., et al., Human antigen-presenting cells respond differently to gut-derived probiotic bacteria but mediate similar strain-dependent NK and T cell activation. FEMS Immunol Med Microbiol, 2007. 51(3): p. 535-46.
44. Zeuthen, L.H., H.R. Christensen, and H. Frokiaer, Lactic acid bacteria inducing a weak interleukin-12 and tumor necrosis factor alpha response in human dendritic cells inhibit strongly stimulating lactic acid bacteria but act synergistically with gram-negative bacteria. Clin Vaccine Immunol, 2006. 13(3): p. 365-75.
45. Ghadimi, D., et al., Effects of probiotic bacteria and their genomic DNA on TH1/TH2-cytokine production by peripheral blood mononuclear cells (PBMCs) of healthy and allergic subjects. Immunobiology, 2008. 213(8): p. 677-92.
46. Medina, M., et al., Differential immunomodulatory properties of *Bifidobacterium longum* strains: relevance to probiotic selection and clinical applications. Clin Exp Immunol, 2007. 150(3): p. 531-8.
47. Hall, J.A., et al., Commensal DNA limits regulatory T cell conversion and is a natural adjuvant of intestinal immune responses. Immunity, 2008. 29(4): p. 637-49.
48. van Hemert, S., et al., Identification of *Lactobacillus plantarum* genes modulating the cytokine response of human peripheral blood mononclear cells. BMC Microbiology, 2010. 10(1): p. 293.
49. Mileti, E., et al., Comparison of the immunomodulatory properties of three probiotic strains of Lactobacilli using complex culture systems: prediction for *in vivo* efficacy. PLoS One, 2009. 4(9): p. e7056.
50. Vissers, Y.M., et al., Differential effects of *Lactobacillus acidophilus* and *Lactobacillus plantarum* strains on cytokine induction in human peripheral blood mononuclear cells. FEMS Immunol Med Microbiol, 2010.
51. van Baarlen, P., et al., Differential NF-kappaB pathways induction by *Lactobacillus plantarum* in the duodenum of healthy humans correlating with immune tolerance. Proc Natl Acad Sci U S A, 2009. 106(7): p. 2371-6.
52. Christensen, H.R., H. Frokiaer, and J.J. Pestka, Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. J Immunol, 2002. 168(1): p. 171-8.
53. Miettinen, 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(12): p. 058-62.
54. Miettinen, M., J. Vuopio-Varkila, and K. Varkila, Production of human tumor necrosis factor alpha, interleukin-6, and interleukin-10 is induced by lactic acid bacteria. Infect Immun, 1996. 64(12): p. 5403-5.
55. Niers, L.E., et al., Selection of probiotic bacteria for prevention of allergic diseases: immunomodulation of neonatal dendritic cells. Clin Exp Immunol, 2007. 149(2): p. 344-52.

56. Lopez, P., et al., Distinct *Bifidobacterium* strains drive different immune responses *in vitro*. Int J Food Microbiol, 2010. 138(1-2): p. 157-65.
57. Peran, L., et al., Preventative effects of a probiotic, *Lactobacillus salivarius* ssp. *salivarius*, in the TNBS model of rat colitis. World J Gastroenterol, 2005. 11(33): p. 5185-92.
58. Zoumpopoulou, G., et al., *Lactobacillus fermentum* ACA-DC 179 displays probiotic potential *in vitro* and protects against trinitrobenzene sulfonic acid (TNBS)-induced colitis and Salmonella infection in murine models. Int J Food Microbiol, 2008. 121(1): p. 18-26.
59. Ibnou-Zekri, N., et al., Divergent patterns of colonization and immune response elicited from two intestinal *Lactobacillus* strains that display similar properties *in vitro*. Infect Immun, 2003. 71(1): p. 428-36.
60. Mastrangeli, G., et al., Effects of live and inactivated VSL#3 probiotic preparations in the modulation of *in vitro* and *in vivo* allergen-induced Th2 responses. Int Arch Allergy Immunol, 2009. 150(2): p. 133-43.
61. Shibolet, O., et al., Variable response to probiotics in two models of experimental colitis in rats. Inflamm Bowel Dis, 2002. 8(6): p. 399-406.
62. Gaudier, E., et al., The VSL# 3 probiotic mixture modifies microflora but does not heal chronic dextran-sodium sulfate-induced colitis or reinforce the mucus barrier in mice. J Nutr, 2005. 135(12): p. 2753-61.
63. Flinterman, A.E., et al., Probiotics have a different immunomodulatory potential *in vitro* versus *ex vivo* upon oral administration in children with food allergy. Int Arch Allergy Immunol, 2007. 143(3): p. 237-44.
64. Kopp, M.V., et al., *Lactobacillus* GG has *in vitro* effects on enhanced interleukin-10 and interferon-gamma release of mononuclear cells but no *in vivo* effects in supplemented mothers and their neonates. Clin Exp Allergy, 2008. 38(4): p. 602-10.
65. Ng, S.C., et al., Immunosuppressive effects via human intestinal dendritic cells of probiotic bacteria and steroids in the treatment of acute ulcerative colitis. Inflamm Bowel Dis, 2010.
66. Hart, A.L., et al., Modulation of human dendritic cell phenotype and function by probiotic bacteria. Gut, 2004. 53(11): p. 1602-9.
67. Ligaarden, S.C., et al., A candidate probiotic with unfavourable effects in subjects with irrita ble bowel syndrome: a randomised controlled trial. BMC Gastroenterol, 2010. 10: p. 16.

Chapter 3

Identification of genetic loci in *Lactobacillus plantarum* that modulate the immune response of dendritic cells using comparative genome hybridization

M. Meijerink, S. van Hemert, N. Taverne, M. Wels, P. de Vos, P. A. Bron, H. F. Savelkoul, J. van Bilsen, M. Kleerebezem, J. M. Wells

PLoS One (2010) 5, e10632.

Abstract

Background
Probiotics can be used to stimulate or regulate epithelial and immune cells of the intestinal mucosa and generate beneficial mucosal immunomodulatory effects. Beneficial effects of specific strains of probiotics have been established in the treatment and prevention of various intestinal disorders, including allergic diseases and diarrhea. However, the precise molecular mechanisms and the strain-dependent factors involved are poorly understood.

Methodology/Principal Findings
In this study, we aimed to identify gene loci in the model probiotic organism *Lactobacillus plantarum* WCFS1 that modulate the immune response of host dendritic cells. The amounts of IL-10 and IL-12 secreted by dendritic cells (DCs) after stimulation with 42 individual *L. plantarum* strains were measured and correlated with the strain-specific genomic composition using comparative genome hybridisation and the Random Forest algorithm. This *in silico* "gene-trait matching" approach led to the identification of eight candidate genes in the *L. plantarum* genome that might modulate the DC cytokine response to *L. plantarum*. Six of these genes were involved in bacteriocin production or secretion, one encoded a bile salt hydrolase and one encoded a transcription regulator of which the exact function is unknown. Subsequently, gene deletions mutants were constructed in *L. plantarum* WCFS1 and compared to the wild-type strain in DC stimulation assays. All three bacteriocin mutants as well as the transcription regulator (lp_2991) had the predicted effect on cytokine production confirming their immunomodulatory effect on the DC response to *L. plantarum*. Transcriptome analysis and qPCR data showed that the transcript level of *gtcA3*, which is predicted to be involved in glycosylation of cell wall teichoic acids, was substantially increased in the *lp_2991* deletion mutant (44 and 29 fold respectively).

Conclusion
Comparative genome hybridization led to the identification of gene loci in *L. plantarum* WCFS1 that modulate the immune response of DCs.

Introduction

Several species of *Lactobacillus* are naturally present in the human intestinal tract and several species and strains have been evaluated for their probiotic activity. Oral administration of certain probiotic strains has shown significant and promising results in human clinical trials and experimental animal models of inflammatory bowel disease, irritable bowel syndrome and allergy [1-8]. Conclusive evidence for the mechanisms underlying the beneficial properties of probiotics is lacking but results obtained from *in vitro* studies and animal intervention models indicate a strong role for immunomodulation and enhancement of the epithelial barrier functions [9, 10]. Proposed immunomodulatory mechanisms include down-regulation of inflammatory responses through the modulation of dendritic cell (DC) function and subsequent expansion or induction of regulatory T cells producing the anti-inflammatory cytokines transforming growth factor (TGF)-β and interleuking (IL)-10 [11, 12]. The capacity of lactobacilli to stimulate pro-inflammatory or anti-inflammatory cytokines in co-culture with DCs or peripheral blood mononuclear cells (PBMCs) indicates that different strains of *Lactobacillus plantarum* may differentially stimulate immune cells and could therefore exert opposite immunomodulatory effects.

Dendritic cells play a key role in mucosal immunity and tolerance and not surprisingly have been implicated as key players in the interaction between probiotics and the immune system. In the small intestine DCs are known to sample microbes that enter the Peyer's Patches via M-cells [13] but also directly across the epithelium by opening tight junctions and sending dendrites to the luminal side [14]. In the mucosa DCs are the main activators of naive T cells and their T cell polarising properties are largely governed by the nature of the microbial products encountered at mucosal sites. Additionally cellular cytokines such as thymic stromal lymphopoietin (TSLP) and TGF-β which are produced by epithelial cells can also modulate the function of resident DCs [15, 16]. Previous studies have shown that the DC responses to different probiotic bacteria are strain-specific and that this can have different outcomes on T cell polarisation [17, 18], [19], [20], [21]. Immature DCs are characterized by a high capacity for antigen uptake and following antigen capture in the presence of appropriate stimuli, they migrate to secondary lymphoid organs and mature. During maturation the major histocompatibility complex (MHC) molecules for antigen presentation and co-stimulatory molecules, such as CD40, CD54, CD83 and B7.1 and B7.2 (CD80 and CD86) are up regulated for an effective T-cell stimulation [22]. T helper(h) 1 immune responses critically depend on the ability of DCs to produce interleukin (IL)-12 and are characterized by the production of interferon (IFN)-γ and IL-2, which induce cell-mediated immunity. Th2 immune responses involve IL-4, IL-5, IL-6, and IL-13 and induce humoral immunity. IL-10 is a critical regulatory T cell cytokine which suppresses IL-12 production and consequently other Th1 cytokines such as IFN-γ and tumor necrosis factor alpha (TNF-α). It also prevents activation of antigen-presenting cells, inhibits the maturity of DCs, limits T-cell proliferation, and can induce a state of antigen-specific tolerance. In the gut, IL-10 is a key

molecule for the induction of regulatory T cells and prevention of mouse colitis [23-25]. The suppression of IL-12 in the gut by IL-10 prevents an inflammatory cascade of Th1 cytokines and cellular migration. The ability of different lactobacilli to induce a high ratio of IL-10/IL-12 production in human PBMCs has been shown to correlate with their capacity to provide significant protection from 2,4,6-trinitrobenzene sulfonic acid (TNBS) induced colitis in mice [26].

Lactobacilli have been shown to differ substantially in their ability to modulate DC responses and subsequent T cell induction but the factors responsible for these differences remain largely unknown. Comparative genomic analysis is one approach to identify genetic loci linked to certain phenotypes and has been applied to *Lactobacillus* spp. to identify genes involved in persistence in the intestine [27], mannose binding [28], and pilli formation [29]. Several innate pattern recognition receptors (PRR) recognizing evolutionary conserved microbe-associated molecular patterns (MAMPS) are potentially involved in immunomodulation by lactobacilli. The most well characterised family of innate receptors are the Toll-like receptors (TLR) of which TLR-2 is known to recognise bacterial cell wall components such as peptidoglycan (PG), and when present as a heterodimer with TLR-6 it can also recognize the fatty acid chains present on lipoteichoic acid (LTA) [30, 31]. There are considerable differences between the LTA molecules of different *Lactobacillus* species and strains, which may influence their immune stimulating activities, including variation of the quantity of LTA produced per cell, its chain length, and its degree of substitution with D-alanine and/or glucose [32-34]. For example, a D-alanyl transfer protein B (dltB) deletion mutant of *L. plantarum* induced higher levels of the anti-inflammatory cytokine IL-10 in PBMC co-culture experiments than the wild type strain thereby increasing its protective effect in a TNBS mouse model of intestinal colitis [30]. DltB is a putative transmembrane protein predicted to be involved in the passage of the activated D-alanyl-Dcp complex across the glycerol phosphate backbone of LTA. Thus, the altered phenotype of the dltB mutant was attributed to the loss of D-alanyl substitution in LTA although an increase in glucose substitution on LTA was also observed. In *L. rhamnosus* the DltD membrane protein facilitates the binding of Dcp for ligation with D-Ala and additionally has thioesterase activity for removing mischarged D-alanyl carrier proteins. In contrast to *L. plantarum*, deletion of the D-alanyl transfer protein in *L. rhamnosus* had no effect on cytokine production in co-culture with PBMCs [35]. These findings suggest the involvement of multiple interacting bacterial molecules in the observed phenomena. TLR-2 in combination with TLR-1 or TLR-6 can also recognise the fatty acid chains present on lipidated lipoproteins of bacteria although for *Lactobacillus* this topic remains relatively unexplored.

Extracellular polysaccharides (EPS) are potentially important probiotic effector molecules and they show a high level of diversity and complexity among different strains and species. EPS could have a shielding effect on the recognition of LTA by host receptors or a direct effect on immunomodulation by binding to specific lectin receptors such as the dendritic-specific intercellular adhesion molecule 3-grabbing non intergrin (DC-SIGN) [36]. Yasuda and colleagues have shown that in *Lactobacillus casei* the cell wall

associated polysaccharide PS-1 functions as a modulator that suppresses the immune response of macrophages and monocytes [37].
The NOD-like receptors NOD1 and NOD2 are intracellular receptors for muropeptides of the PG in the bacterial cell wall. NOD2 recognises muramyl dipeptide which is present in lactobacilli but for these peptides to be released PG would have to be hydrolysed by PG-hydrolases of either bacterial or host origin (lysozyme). An immune evasion strategy established by some pathogens involves the action of cell wall modifying enzymes to prevent cell wall degradation by lysozyme and thus the release of immunostimulatory muropeptides [38, 39].
The aim of this study was to identify genetic loci that encode, modify or regulate immunomodulating factors in *L. plantarum* by measurement of cytokine responses to 42 different *L. plantarum* strains and a comparative genome hybridization approach based on the genome sequence of *L. plantarum* WCFS1 and comparative genome hybridization datasets of different *L. plantarum* strains [40]. WCFS1 is a single colony isolate of strain NCIMB8826, which was originally isolated from human saliva and known to survive gastrointestinal passage after oral administration in healthy volunteers [41]. Host responses to *L. plantarum* WCFS1 were recently demonstrated in healthy human volunteers by transcriptome analyses of duodenal tissue samples [42, 43]. Mutation analysis of several of these candidate genes in *L. plantarum* WCFS1 allowed us to evaluate their immunomodulatory properties and had the predicted effect on cytokine production confirming their immunomodulatory effect on the DC response to *L. plantarum*.

Material and methods

Bacterial strains
42 different *L. plantarum* strains isolated from humans and different food resources were used for immunoassays and comparative genome hybridization studies (CGH) (Table S1). Genomic composition of the strains has been previously described [44, 45]. *L. plantarum* strains were grown overnight to stationary phase at 37°C in deMan, Rogosa Sharpe (MRS) broth (Merck, Darmstadt, Germany). The bacteria were recovered by centrifugation and washed twice in phosphate buffered saline (PBS, pH = 7.4) and resuspended at 2×10^8 CFU/ml in PBS containing 20% glycerol and stored at −80°C prior to use in the immunoassays. Colony forming units (CFU) were determined by plating serial dilutions of the cultures on MRS agar.

Blood donors
The study was approved by Wageningen University Ethical Committee and was performed according to the principles of the Declaration of Helsinki. Buffy coats from blood donors were obtained from the Sanquin Blood bank in Nijmegen (The Netherlands). A written informed consent was obtained before the sample collection.

Differentiation and maturation of dendritic cells
Human monocytes were isolated from blood using a combination of Ficoll density centrifugation and cell separation using CD14-specific antibody coated magnetic microbeads (Miltentyi Biotec, Bergisch Gladbach, Germany). The purity of isolated CD14+ cell fraction was greater than 90% in all experiments. To generate immature DC (iDCs), the purified CD14+ cells were cultured for 6 days in the presence of IL-4 (R&D systems) and GM-CSF (R&D systems) to differentiate then into myeloid dendritic cells. At day 6 the cells were left unstimulated (iDCs) or were stimulated with LPS (1 µg/mL) or with different *L. plantarum* strains or WCFS1 deletion mutants (1:1 bacteria to DC ratio) for 48 hours. As anticipated, as a consequence of the application of antibiotics, no bacterial growth was observed during this period.

Analysis of cell surface markers and measurement of cell death by flow cytometry
During the 8 day culture period of the CD14+ cells, cells were stained on days 3, 6 and 8 with fluorescence-conjugated monoclonal antibodies specific for CD83, CD86 or their isotype-matched controls (BD biosciences, San Diego, USA) and analyzed by flow cytometry (FACSCanto II, BD, San Diego, USA). To check the activation status of the cells (data from day 3 and 6 not shown), the CD86 expression on the cells were measured. CD83 was not expressed on immature dendritic cells (d3 and 6) but was highly expressed on DCs after activation with known maturation factors (e.g. LPS)[46]. Live, apoptotic and necrotic cells were discriminated by staining with Annexin V and propidium iodide (PI) on days 3, 6 and 8 according to the manufacturer's protocol. The cells were analyzed by flow cytometry. Cells that were negative for both Annexin V and PI are not apoptotic

or necrotic as translocation of the membrane phospholipid phosphatidylserine has not occurred and the plasma membrane is still intact. Therefore, Annexin V and PI double negative cells were considered as viable cells, whereas both single and double positive cells were regarded as non-viable [47]. The flow cytometry data was analyzed using the BD FACSDiva software. On days 3 to 8 the viability of the cells was between 50–80%.

Cytokine analysis
Supernatants from the DC stimulation assays were collected after stimulation for 48 hours and analyzed for the presence of cytokines (TNF-α, IL-12p70 and IL-10) using a cytometric bead-based immunoassay that enables multiplex measurements of soluble cytokines in the same sample [48], according to the manufacturer's protocol (BD biosciences). The limits of sensitivity for detection were as follows: TNF-α 0.7 pg/mL; IL-12p70 0.6 pg/mL and IL-10 0.13 pg/mL. The antibody used to measure IL-12 specifically recognizes the dimeric cytokine designated IL-12p70 whereas some antibodies recognize the p40 subunit which is also present in IL-23. The flow cytometry data were analysed using the BD FCAP software.

Identification of candidate genes involved in cytokine secretion by gene-trait matching
Candidate *L. plantarum* genes, that were potentially involved in modulation of the DC responses were identified by *in silico* gene-trait matching [28] using genotype information referenced from the *L. plantarum* WCFS1 genome [40, 44]. The significance of the gene-trait co-occurrence was assessed by assuming a discrete probability distribution of genes and traits in the context of a null hypothesis that co-occurrence is caused by a random process [49]. All *L. plantarum* genes were tested for their significant co-occurrence with each cytokine concentration or cytokine concentration ratio (i.e. IL-10/IL-12). *L. plantarum* WCFS1 genes with the highest variable importance measures as returned by the Random Forest method were selected for further characterization using a gene deletion approach in combination with immunoassays.

Construction of knock-out mutants
A previously described *L. plantarum lp_3536* deletion mutant was used in this study [50]. Gene deletion mutants of *lp_0419-0422* and *lp_0423-30* were constructed for a different study involving immune cytokine profiling of lactobacilli with PBMCs (van Hemert et al., personal communication) (Fig. 1). Construction of the *L. plantarum* gene deletion mutants for *lp_0423* and *lp_2991* was performed as previously described with several modifications [51]. The upstream and downstream flanking regions of the target genes, as well as the *lox-cat-lox* region of pNZ5319 were amplified by PCR. The resulting amplicons were used as templates in a SOEing PCR reaction [52] linking the flanking regions and lox-cat-lox together by means of complementary 5' regions in the primers (Table 1). Subsequently, PCR products were cloned into the non-replicating integration vector pNZ5319 [51] digested with SwaI and Ecl136II. Plasmids were transformed into competent cells of *E. coli* JM109 by electroporation as described by the manufac-

turer (Invitrogen, Groningen, The Netherlands). This resulted in a plasmid containing the complete gene replacement cassette. Plasmid DNA was isolated from E. coli by using Jetstar columns, following the manufacturer's instructions (Genomed GmbH, Bad Oeynhausen, Germany). The sequence of the cloned DNA was confirmed by sequence analysis (BaseClear, Leiden, The Netherlands).

A double cross-over gene replacement strategy was used to substitute the target gene(s) with a chloramphenicol gene (cat). The mutagenesis plasmids were transformed to *L. plantarum* WCFS1 and integrants were selected on MRS plates containing 10 µg/mL chloramphenicol. After growth at 37°C for 2 to 4 days, full-grown colonies were replica-plated to MRS with or without 30 µg/mL erythromycin. Colonies displaying an erythromycin sensitive phenotype represent candidate cat-gene replacements resulting from a simultaneous double cross-over event in both the 5'- and 3'-flanking regions of the target gene. The anticipated cat-replacement genotype was confirmed by PCR using primers flanking the sites of recombination (Table 1). One integrant displaying the anticipated phenotype and genotype was selected for all mutants and was utilized in the remainder of the study.

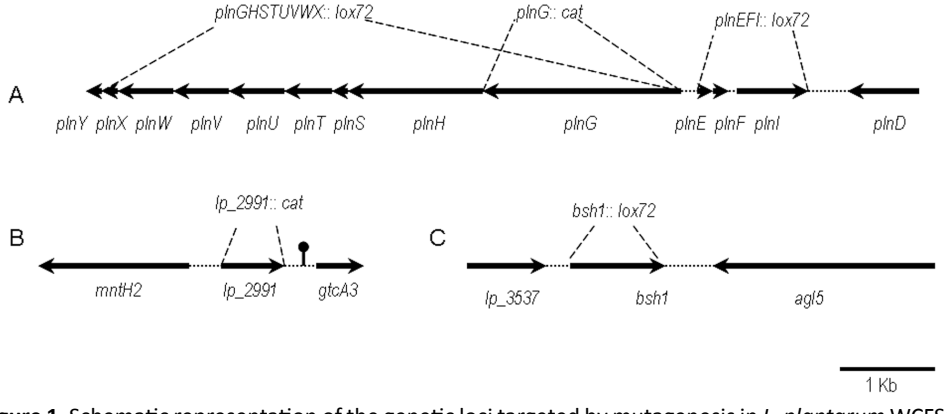

Figure 1. Schematic representation of the genetic loci targeted by mutagenesis in *L. plantarum* WCFS1.

RNA isolation

Bacteria (WCFS1 and delta-lp_2991) were grown in MRS to $OD_{600\ nm}$ of 1 and then 40 mL of quenching buffer (66.7 mM HEPES (pH 6.5) 60% methanol, −40°C) was added to 10 mL of bacterial suspension to quench cellular processes. Cells were harvested by centrifugation at 15500 g for 10 min at −20°C and then resuspended in 400 µL of ice-cold MRS. The cell suspensions were transferred to ice-cold tubes containing 0.5 g of glass beads, 400 µL of phenol, 100 µL chloroform, 30 µL 10% sodium dodecyl sulfate and 30 µL 3 M sodium acetate (pH 5.2). Cells were disrupted with three 40-s treatments in a Fastprep (Obiogene Inc., Illkirch, France) separated by 1 min intervals on ice. After centrifugation the aqueous phase was used for RNA isolation with a High Pure kit (Roche diagnostics, Mannheim, Germany). The RNA concentration was measured at

an OD of 260 nm with the ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, USA). The A_{260}/A_{280} ratio was measured to check purity of the RNA. Quality of the RNA obtained was analyzed with the 2100 Bioanalyser (Agilent Technologies, Amstelveen, The Netherlands). Samples whereby the 23S/16S RNA ratio was superior or equal to 1.6 were taken for further labeling.

Table 1. Primers used for deletion mutants in *L. plantarum* WCFS1 and RT-PCR.

Primer	Sequence[a]
LF2991F	5′- CCGTTTACTGAACGACTTGTCG-3′
LF2991R	5′- **CGAACGGTAGATTTAAATTGTTT** TGAAAAATTCATTTTCACACCTCC -3′
RF2991F	5′- **GTACAGCCCGGGCATGAG** AAGACTTCAGATTAGGTGTTCAG -3′
RF2991R	5′- TACTCGTCATTCTAACTACCGC-3′
Ecl-loxR	5′- AAACAATTTAAATCTACCGTTCG -3′
Pml-loxF	5′- CTCATGCCCGGGCTGTAC -3′
LF2991F2	5′-TGGCACCGATAATCCCTAAAGC-3′
RF2991R2	5′-TGTAATCTTAATCCGCTTTCACAC-3′
LF423F	5′-AATTGATACATGTGGTTTCGAAAG-3′
LF423R	5′- **CGAACGGTAGATTTAAATTGTTT** CCAATGCATACTTGTACTCCC -3′
RF423F	5′- **GTACAGCCCGGGCATGAG** CGACTTGATCAATAGCTGAGGG-3′
RF423R	5′-TTGGTTGCCTTGATCGTGTAAG-3′
Tr-F (transcription regulator lp_2991)	5′-CAAACTGACATCGCGACAC-3′
Tr-R	5′-CGTAATAACCCCCACTAATTG-3′
mntH2-F (manganese transport protein)	5′-TTGTGGATTTTAACCGAATTAG-3′
mntH2-R	5′-TCTTCCGCGTTTGTGAGA-3′
gtcA3-F (teichoic acid glycosylation protein)	5′-ATGAGTAAGCCAGATTCGATTA-3′
gtcA3-R	5′-GCCGAGCTCCCCAATA-3′
recA-F (recombination protein RecA)	5′-TGGATCGTTGGCCTTAGA-3′
recA-R	5′-CCGGAGCGCTTGTGA-3′

[a]Bold and underlined nucleotides indicate overlapping ends with the Ecl-loxR and Pml-loxF primers.
doi:10.1371/journal.pone.0010632.t001

cDNA synthesis and quantitative reverse transcription-PCR

cDNA was synthesized from 1 µg of total purified bacterial RNA and random oligonucleotide hexamers using Superscript III reverse transcriptase (Invitrogen). Reverse transcription was performed at 55°C for 60 min, after which the reverse transcriptase was inactivated at 85°C for 5 min. Primers were designed using Oligo Program version 6 (MedProbe, Oslo, Norway) to have melting temperatures between 60 and 62°C and an amplicon size of approximately 400 bp (Table 1). Quantitative PCR was performed with the synthesized cDNAs by using Absolute SYBR Green QPCR (Westburg, Leusden, The Netherlands). Each reaction mixture contained 1x SYBR Green master mix, 70 nM of each primer and 5 ng of reverse-transcribed RNA. After activation of Thermoprime Plus DNA polymerase at 95°C for 15 minutes, PCR was carried out for 40 cycles of 95°C for 15 s, 55°C for 30 s and 72°C for 30 s. The identities of the amplicons resulting from the reactions were checked after amplification by melting curve analysis and 1.5% agarose gel electrophoresis. Reaction mixtures containing no template were included in each real-time PCR experiment to control for contamination. Relative transcript levels were calculated by using the comparative $\Delta\Delta Ct$ method described by Pfaffl [53]. The average

Ct values observed for the target gene transcripts (lp_2991, mntH2 and gtcA3) were normalized to the average *Ct* values obtained for the reference gene recA from the same RNA sample. Two replicates of all samples and primer pairs were included and the experiment was performed in duplicate.

Transcriptome analysis

A two-color microarray-based gene expression analysis was performed on a custom-made 60-mer oligonucleotide array (Agilent Biotechnologies, submitted in GEO under platform GPL9359) to determine the global gene transcription levels of WCFS1 and the lp_2991 deletion mutant. Cy3- and Cy5-labeled cDNAs were prepared using a Cyscribe post labeling kit (GE Healthcare, Bucks, United Kingdom). Slides were prehybridized for 45 min at 42°C in 20 ml prehybridization solution (1% bovine serum albumin, 5× SSC, 0.1% sodium dodecyl sulfate; filtered) (1× SSC is 0.15 NaCl plus 0.015 M sodium citrate), washed in filtered deionized water, and dried. Co-hybridization with Cy5- and Cy3-labeled cDNA probes was performed overnight at 42°C for 16 h in Slidehyb#1 (Ambion, Austin, TX). The slides were then washed twice in 1× SSC-0.1% sodium dodecyl sulfate and twice in 1× SSC before they were scanned. The experiment was repeated with Cy5/Cy3 dye swaps.

Slides were scanned with a ScanArray Express 4000 scanner (Perkin Elmer, Wellesley, USA), and image analysis and processing were performed using the ImaGene Version 7.5 software (BioDiscovery Inc., Marina Del Rey, CA, USA). The microarrays were scanned at different intensities. For each of the individual microarrays the best scan was selected on the basis of signal distribution (combination of a low number of saturated spots and a low number of low signal spots). The data were normalized using Lowess normalization as available in MicroPrep [54]. The data were corrected for inter-slide differences on the basis of total signal intensity per slide using Postprep [54]. The median intensity of the different probes per gene was selected as the gene expression intensity. CyberT was used to compare the different transcriptomes, taking into account the duplicates (dye swaps) of each of the conditions [55]. This analysis resulted in a gene expression ratio and false discovery rate (FDR) for each gene. Genes with FDR values <0.05 were considered to be statistically significant. All microarray data is MIAME compliant and are available in GEO.

Statistical analysis

Mixed general linear model using restricted maximum likelihood (REML) was used to determine the statistical differences within donors between cytokines produced by DCs stimulated with the constructed deletion mutants compared to the wild type *L. plantarum* WCFS1. A two-sided p-value of 0.05 or lower was considered to be significant. The statistical analysis was performed by using SAS software (version 9.1, SAS Institute Inc., Cary, NC, USA).

Results

DC cytokine responses to different L. plantarum strains

Monocyte derived immature dendritic cells were cultured in the presence of 42 different *L. plantarum* strains. Based on the variation in CD83 and CD86 expression and cytokine secretion, 20 strains were selected to repeat the culturing with monocyte derived DCs from 5 different donors. The strains differed considerably in their ability to modulate DC responses (Fig. S1 and Fig. 2). For example, the amounts of IL-10 induced by the strains ranged from 28 pg/mL to 1095 pg/mL (39 fold) and for IL-12 the values ranged from 20 to 11996 pg/mL (600 fold). For TNF-α some strains induced very low amounts (close to the detection limit of 0.7 pg/mL) whereas others induced 8.4 to 12 ng/mL (Fig. 2 and Table 2). The large variation in strain immune profiles suggested that there could be some underlying strain-dependent genetic differences influencing the innate response to *L. plantarum*.

Some strains such as NIZO2766, NIZO2801 and NIZO2897 were clearly strong inducers of pro-inflammatory cytokines IL-12 and TNF-α while others were considerably less potent (e.g. strains NIZO1839, NIZO2494 and NIZO2831; Fig. 2 and Table 2).

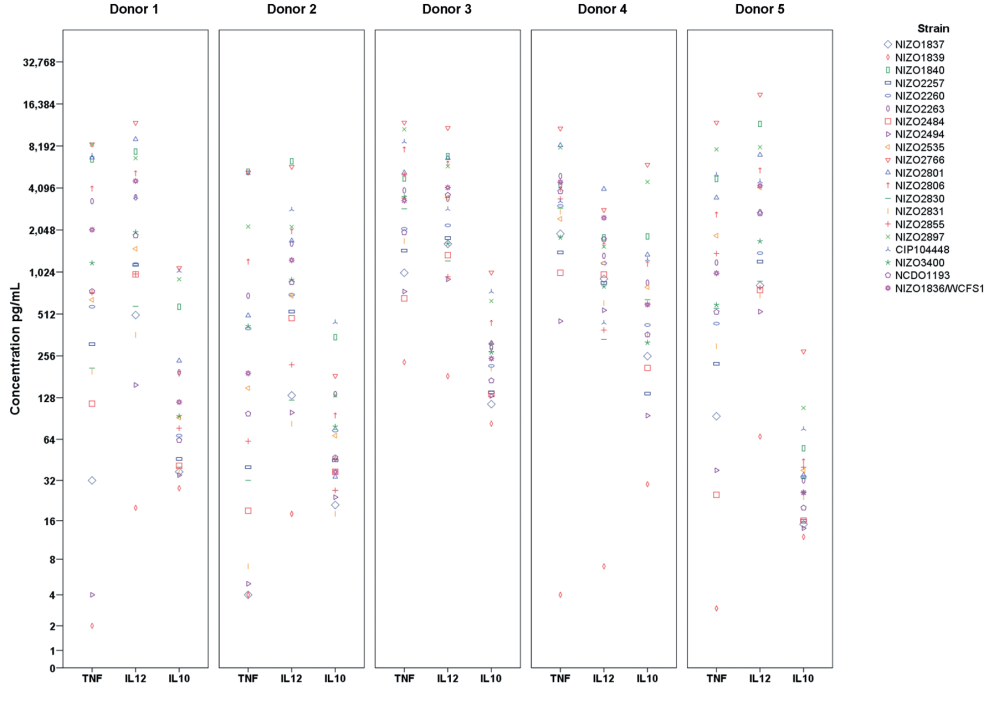

Figure 2. Cytokine secretion by DCs. IL-10, TNF-α and IL-12p70 production by monocyte-derived dendritic cells derived from blood of five different donors after stimulation with 20 different *L. plantarum* strains. Each symbol represents a different *L. plantarum* strain.

Similarly, the strains showed strikingly different capacities to induce the anti-inflammatory cytokine IL-10. From a comparison of IL-12 to IL-10 ratios it is clear that these cytokines can vary independently of each other allowing the possibility for strains with distinct pro-inflammatory (e.g. strain NIZO1840 and NIZO2257) and anti-inflammatory profiles (e.g. strain CIP104448). As expected levels of cytokines induced by *L. plantarum* strains differed between donors but the ranking of the strains was highly consistent for each cytokine showing that the strain immunoprofiles were reproducible (Fig. 2).

Table 2. Ranges of DC cytokine induction by *L. plantarum* strains.

		Range*	Fold difference*	Highest strain	Lowest strain
Donor 1	**IL-10**	(35–1095)	31	NIZO2766	NIZO2494
	IL-12	(158–11996)	76	NIZO2766	NIZO2494
	TNF-alpha	(4–8412)	2103	NIZO2897	NIZO2494
Donor 2	**IL-10**	(18–447)	25	CIP104448	NIZO2831
	IL-12	(83–6358)	77	LMG18021	NIZO2831
	TNF-alpha	(24–5352)	223	NIZO1840	NIZO1837
Donor 3	**IL-10**	(115–1017)	9	NIZO2766	NIZO1837
	IL-12	(909–11026)	12	NIZO2766	NIZO2494
	TNF-alpha	(663–12052)	18	NIZO2766	NIZO2484
Donor 4	**IL-10**	(95–6011)	63	NIZO2766	NIZO2494
	IL-12	(336–4026)	12	NIZO2801	NIZO2830
	TNF-alpha	(455–10945)	24	NIZO2766	NIZO2494
Donor 5	**IL-10**	(14–276)	20	NIZO2766	NIZO2494
	IL-12	(532–19114)	36	NIZO2766	NIZO2494
	TNF-alpha	(25–12052)	482	NIZO2766	NIZO2484

*NIZO1839 was excluded, since DC cytokine induction by strain NIZO1839 were strikingly lower than for all other strains.
doi:10.1371/journal.pone.0010632.t002

Identification of candidate gene loci by in silico *gene trait matching*

L. plantarum genes potentially involved in the production of pro- and anti-inflammatory cytokines were identified by *in silico* gene-trait matching by correlating measurements of cytokines induced by the different strains with their genotypic information based on comparative hybridisation to a whole genome microarray [44-45]. Seven genes displayed a match with lower levels of IL-10 concentration in the co-culture system. One of these genes, *lp_2991* is annotated as a transcription regulator (Table 3) which is present in 90% of the strains tested. The other six genes (*lp_0422*, *lp_0423*, *lp_0424a*, *lp_0424*, *lp_0425* and *lp_0429*) lie within the plantaricin locus (*lp_0403* to *lp_0431*) involved in plantaricin biosynthesis and secretion. The *plnEFI* operon (*lp_0419* to *lp_0422*) is encoding two bacteriocin-like peptides and a bacteriocin immunity protein. Homologues of the gene loci in this operon are present in 81-85% of the tested strains. *Lp_0423* is distal to *lp_0422* and located in another operon and encodes an ABC transporter involved in the transport of bacteriocins [56, 57]. *Lp_0423* (*plnG*) is present in 88% of the tested strains [58].

Three genes (*lp_2991*, *lp_0422* and *lp_3536*) had a gene-trait match with a lower concentration of TNF-α produced in the supernatant of *L. plantarum* DC co-culture (Table

3). There was a co-occurrence of low TNF-α secretion and the presence of *lp_2991* and *lp_3536*. *Lp_3536* is predicted to encode a bile salt hydrolase capable of removing the amino acid moiety from the steroid nucleus of conjugate bile salts by hydrolysis and is present in 81% of the tested strains.

Table 3. Candidate genes identified by gene trait matching.

Gene name	Gene nr	Description	Importance[a]	Predicted k.o. phenotype[b]
Lp_2991	lp_2991	Transcription regulator	6.4E06 (IL-10) 1.5E08 (TNF-alpha)	IL-10 and TNF-alpha ↑
plnEFI	lp_0419-lp_0422	Bacteriocin like peptide E Bacteriocin like peptide F Immunity protein plnI	1.1E07	IL-10 ↑
plnG	lp_0423	ABC transporter	2.4E06	IL-10 ↑
plnGHSTUVWX	lp_0423-30	Bacteriocin ABC-transporter, ATP-binding and permease protein plnG	3.6E06	IL-10 ↑
bsh1	lp_3536	choloylglycine hydrolase	3.4E07	TNF-alpha ↑

[a]Estimated importance values as given by Random Forest.
[b]Phenotype in DC assay affected by the presence of the gene.
↑ indicated a higher effect when the gene is absent.
doi:10.1371/journal.pone.0010632.t003

Validation of the role of the candidate genes in cytokine secretion

To further investigate the role of the genes identified by gene-trait matching in modulating DC cytokine secretion, specific gene-replacement mutants of genes *lp_2991* and *lp_0423* were constructed in *L. plantarum* strain WCFS1, to yield strains *lp_2991::cat* and *plnG::cat*. Construction of the *bsh1::lox72*, *plnGHSTUVWX::lox72* and *plnEFI:lox72* mutant is described in the Material and Methods section above. The capacity of the deletion mutants to induce IL-10, IL-12p70 and TNF-α production by DCs was higher than compared to the wild-type strain *L. plantarum* WCFS1. As predicted on basis of the gene-trait correlation, deletion mutants lacking genes involved in plantaricin secretion and immunity induced significantly higher amounts of IL-10 in DC co-culture compared to the wild type strain WCFS1 (Fig. 3 and Table 4). For mutant *plnEFI::lox72* in which the two bacteriocin-like peptides and a bacteriocin immunity protein were deleted, IL-10 was significantly increased 3.3 fold ($p<0.05$). Deletion of the pheromone and bacteriocin transport operon (*plnGHSTUVWX*), in strain *plnGHSTUVWX::lox72* also significantly increased IL-10 3.1-fold ($p<0.05$) compared to the wild type strain WCFS1. Similar increases (3.2 –fold; $p<0.05$) were also observed for *plnG::cat* (Fig. 3 and Table 4). In the *plnEFI*, *plnGHSTUVWX* and *plnG* mutants TNF-α secretion was significantly increased by 4.2-fold ($p<0.05$), 4.1-fold ($p<0.05$) and 7.4 fold ($p<0.05$) respectively. In all plantaricin associated mutants IL-12p70 secretion was also significantly ($p<0.05$) increased between 1.9 and 2.4 fold.

The presence of the *lp_2991* gene in strains was associated with induction of lower amounts of IL-10 and TNF-α secretion compared to strains lacking this gene. As predicted by gene-trait correlation analysis, deletion of this gene in wild type strain WCFS1 significantly increased IL-10 and TNF-α secretion compared to the wild type strain. IL-10

secretion was increased 6.3-fold ($p<0.05$) and TNF-α secretion was increased 17.2-fold ($p<0.05$). Additionally, IL-12p70 secretion was induced 3.2-fold ($p<0.05$) (Fig. 3 and Table 4). Deletion of *lp_3536* (strain *lp_3536::loxp72*) had no significant effect on cytokine production compared to the wt strain (Fig. 3 and Table 4). In conclusion four of the five deletion mutants showed the predicted effect on DC immune responses.

Table 4. Validation of the candidate genes knock out mutants.

Gene name	Gene nr	Predicted k.o. phenotype[a]	p-value IL-10[b]	p-value TNF-alpha[b]	p-value IL-12p70[b]
lp_2991	lp_2991	IL-10 and TNF-alpha ↑	0.0016	0.0058	0.0003
plnEFI	lp_0419-lp_0422	IL-10 ↑	0.0064	0.0095	0.0001
plnG	lp_0423	IL-10 ↑	0.0024	0.0144	0.0117
plnGHSTUVWX	lp_0423-30	IL-10 ↑	0.0003	0.1041	0.0016
bsh1	lp_3536	TNF-alpha ↑	0.2449	0.6648	0.6431

[a]Phenotype in DC assay affected by the presence of the gene.
↑ indicated a higher effect when the gene is absent.
[b]Difference between wild-type and mutant according mixed general linear model using REML. A two-sided *p*-value of 0.05 or lower was considered to be significant.
doi:10.1371/journal.pone.0010632.t004

Transcriptome analysis

Transcriptomes of the WCFS1 and lp_2991 deletion mutant were compared to obtain an expression ratio for each gene. Genes with FDR values <0.05 were considered significant. Of the 3100 genes present on the microarray 33 genes were significantly upregulated in the lp_2991 deletion mutant and 25 genes were significantly downregulated (Table S2).

In the lp_2991 deletion mutant, the gene lp_2991 signal was not significantly different to the wild type due to the very low expression (signal) level. In the mutant the most highly increased transcript (44.7-fold) was the *gtcA3* gene which is predicted to be involved in glycosylation of cell wall teichoic acids including LTA which is an agonist of TLR-2. Another gene associated with cell envelope structures that showed increased expression in the mutant was tagO (4.4-fold), encoding an enzyme involved teichoic acid synthesis. A group of 6 genes involved in nucleoside and nucleotide synthesis were 4.1 to 17 fold higher in the mutant as was the *purR1* pyrimidine operon regulator (2.6 fold). The reasons for this are not clear but these genes are regulated by several factors and are often differentially expressed in microarray experiments (unpublished results Michiel Kleerebezem). Transcript levels of several genes encoding hypothetical proteins and transporters were either increased or decreased in the mutant compared to the wild type. In the mutant, the largest decrease in expression was for *lp_2531* which is predicted to be a PTS transporter for N-acetylglucosamine and glucose.

RT-PCR results

Quantitative reverse transcription-PCR (RT-PCR) was performed to validate the results of the transcriptome analysis for *mntH2* and *gctA3* using RNA isolated from the WCFS1 and delta-lp_2991 strains. The specificity of the PCR products was confirmed by

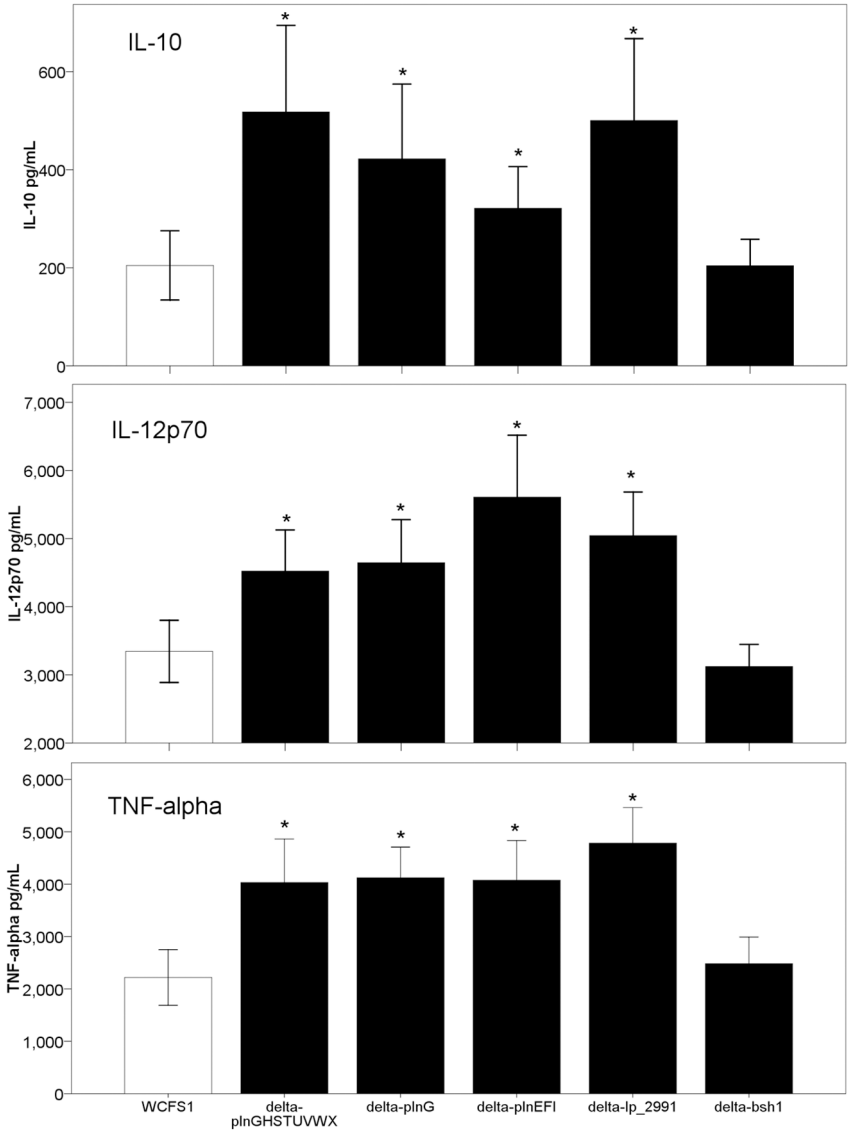

Figure 3. Validation of the candidate genes in different deletion mutants of *L. plantarum* WCFS1. IL-10, IL-12p70, and TNF-α secretion by monocyte derived dendritic cells after stimulation with different *L. plantarum* deletion mutants and the wild type strain WCFS1. Data are presented as the mean +/− standard error of the mean from five donors of duplicate wells. Differences between wild-type and mutant were calculated according mixed general linear model using REML. A two-sided p-value of 0.05 or lower was considered to be significant, indicated by *.

a combination of melting curve analysis and 1.5% agarose gel electrophoresis (data not shown). The *gtcA3* gene which lies directly downstream of *lp_2991* was indeed strongly up-regulated (29.6 fold) in the mutant strain suggesting that it is normally repressed by *lp_2991* under these growth conditions (Fig. 4). In contrast transcript levels of *mntH2*, which is orientated in the opposite direction to *lp_2991* were only modestly altered in the deletion mutant (1.8 fold) suggesting that this gene is not directly regulated by the putative transcription regulator. The *lp_2991* gene was expressed in strain WCFS1 but no specific PCR product was amplified for in the lp_2991 deletion mutant as expected.

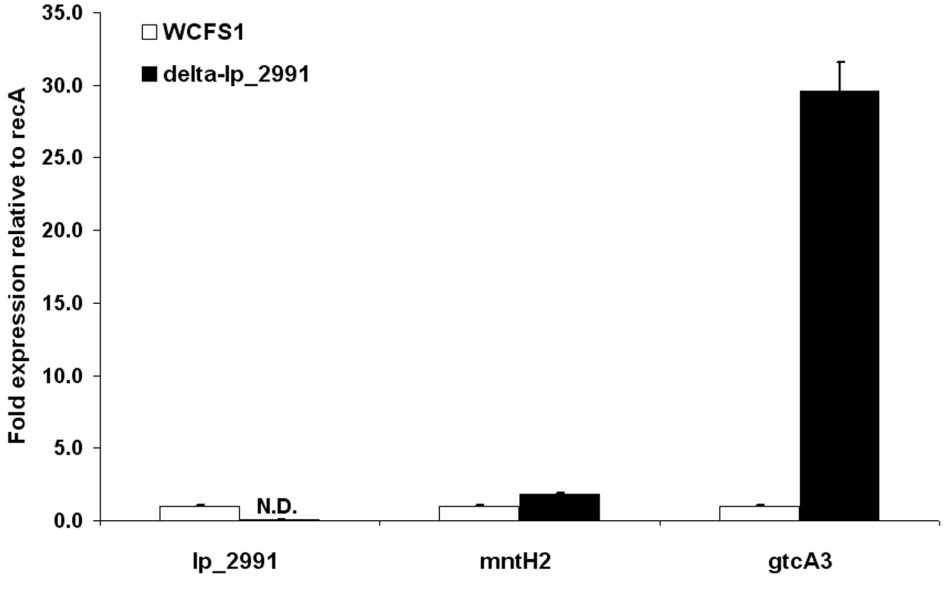

Figure 4. Relative gene expression levels determined by quantitative RT-PCR. RNA prepared from the wild type WCFS1 strain and lp-2991 deletion mutant was used for quantitative RT-PCR. The average Ct values observed for the target gene transcripts (*lp_2991*, *mntH2* and *gtcA3*) were normalized to the average Ct values obtained for the reference gene recA from the same RNA sample. Two replicates of all samples and primer pairs were included and the experiment was performed in duplicate. Fold changes in transcript level were calculated by using the comparative ΔΔCt method described by Pfaffl [53].

Discussion

In this study we aimed to determine whether 42 different strains of *L. plantarum*, several strains of which are currently marketed as probiotics, possess different immunomodulatory properties in co-culture with DCs. Additionally, we aimed to identify genetic loci of *L. plantarum* WCFS1, that influence the immune response using comparative genome hybridization data and a gene-trait matching approach. The *L. plantarum* strains differed considerably in their ability to induce pro- and anti-inflammatory cytokines. Immune responses to strain NIZO1839 were strikingly lower than for all other strains suggesting that it might directly attenuate immune responses, possess non-typical MAMPs or produce a capsule-polysaccharides that interferes with innate recognition [59]. The amounts of IL-10 induced by the 42 strains ranged from 28 pg/mL to 1095 pg/mL (39 fold) and IL-12 measurements ranged from 20 to 11996 pg/mL (600 fold). The amounts of TNF-α induced by the strains ranged from very low (close to the detection limit of 0.7 pg/mL) to more than 8.4 ng/mL. These ranges are higher than those reported for different *Bifidobacterium longum* strains (8-fold) [60] and for multiple *Lactobacillus* and *Bifidobacterium* species in PBMC co-culture assays (10–15 fold) [26], [60-63]. The highest IL-10 secreting cells in PBMCs are the monocytes which typically represent about 8% of the total leucocytes. This could explain why a larger range of IL-10 measurements were obtained in our experiments using a pure dendritic cell population. The strain differences observed in our experiments were not due to variation in CFU of bacteria as the samples used in the assays were checked twice by plating on solid medium and measurements of optical density ($OD_{600\ nm}$).

Probiotics might affect the immune system by the induction of regulatory T cells [64, 65]. Recently a study was published suggesting that probiotics may not directly generate regulatory T cells but induce regulatory DCs via the differentiation of naïve T cells into regulatory T cells in the mesenteric lymph nodes [12]. Regulatory T cells are important in immunological tolerance by the suppression of effector T cells at inflammatory sites. The study by Kwon et al. [12] underlines the important role of dendritic cells in the induction of regulatory T cells and how *in vitro* assays (as described in this study) can be used for the selection of probiotic strains and species that have therapeutic effects in models of inflammatory bowel disease, atopic dermatitis and rheumatoid arthritis.

The large variation in strain immune profiles suggested that there could be some underlying strain-dependent genetic differences influencing the innate response to *L. plantarum*. Therefore results from the cytokine secretion by the DCs or concentration ratios were correlated with the gene presence/absence patterns in the *L. plantarum* strains by regression using the Random Forest algorithm. The output of the algorithm is a model (forest) consisting of many different decision trees. As an output of the algorithm importance measures for each individual gene of the *L. plantarum* WCFS1 genome are given. Genes that score with high importance show a high correlation with the tested immune response and are therefore the most likely candidates to cause the change in immune response. The Random Forest Algorithm model has been used for many appli-

cations in bioinformatics, for example to identify single nucleotide polymorphisms predictive of a certain phenotype [66], and to select disease marker genes from microarray gene expression datasets relevant for the prediction of a certain disease [67].
This approach allowed us to identify eight variable *L. plantarum* genes which influence the immune response of DCs to *L. plantarum*. Other genes influencing the immune response of DC may also exist but were not picked up in this screen because a natural mutant was not present in the strain collection. For example, this would include essential genes involved in the production of MAMPs such as peptidoglycan and LTA that form key structural elements of the bacterial the cell wall. Nevertheless, differences in these structures are known to occur due to modifying enzymes which can affect recognition by innate receptors. Point mutations or small deletions that alter bacterial gene expression or protein activity could also modulate the immune response but these would not be detected using this approach. As the genome comparison is based on strain WCFS1, genes present in the other strains but not in WCFS1 may also be involved in immunomodulation. Six of the identified genes were located in operons linked to bacteriocin production or secretion, the other two encode a bile salt hydrolase and a predicted transcriptional regulator of which the exact function is unknown. Deletion mutants of the candidate genes were constructed in the WCFS1 strain in order to validate their anticipated effect on cytokine induction and all but one (the bile salt hydrolase) affected the immune response as predicted by the direction of the correlation. The identification of false positive candidate genes has been reported previously, e.g. only one of the two candidate mannose-specific adhesin genes identified by gene trait matching was shown to be correct [28]. As far as we know this is the first time a comparative genome hybridization approach has been used to identify bacterial gene loci that modulate host immune responses.
Most of the candidate genes influencing the immune response were involved in bacteriocin production and secretion. Bacteriocins are antimicrobial peptides secreted by bacteria that inhibit the growth of closely related micro-organisms. Bacteriocins produced by lactic acid bacteria have received special attention due to their potential use as food preservatives [68]. For example, the bacteriocin called nisin A and nisin-producing strains of *Lactococcus lactis* are used commercially worldwide in dairy products, as a bio-preservative [69, 70]. Among the different bacteriocins described in *L. plantarum* strains [56, 71-73], the complex *pln* regulon is the best known. This system is organized into five operons (see Fig. 1). The regulatory operon (*plnABCD*) encodes an inducing bacteriocin-like peptide (*plnA*), a histidine protein kinase (HPK) (*plnB*), and two cytoplasmic response regulators (RR) (*plnC* and *plnD*). Another operon (*plnGHSTUVWX*) is associated with plantaricin transport and the operons (*plnJKLR*, *plnMNOP*, *plnEFI*) are related to plantaricin production and immunity [56, 74]. Genes *plnW*, *plnX* and *plnY* are related to membrane integral proteins and other plantaricin biosynthesis proteins. Deletion mutants were constructed for *plnG* and the operons *plnEFI* and *plnGHSTUVWX*. All the deletion mutants of these gene loci induced significantly increased levels of IL-10 secretion (3.3-fold), IL-12p70 (2.4-fold) and TNF-α (7.4 fold) compared to the

wild-type strain WCFS1. It is possible that the bacteriocin secreted by *L.plantarum* affects the DC immune response because some human antimicrobial peptides have also been shown to activate Toll-like receptors and modulate immune functions [56, 75, 76]. Three independent approaches, namely (*in vivo* expression technology (R-IVET, [77]), qRT-PCR [78] and transcriptomics [79] showed that the expression of the *L. plantarum* plantaricin immunity protein PlnI is induced in the mouse intestine, suggesting that it may be important *in vivo*.

Lp_2991 was one of the candidate genes identified during the *in silico* gene-trait matching. DC stimulation with the deletion mutant of *lp_2991* led to a significantly higher secretion of IL-10, IL-12p70 and TNF-α compared to the wild-type control (Table 4 and Fig. 3). *Lp_2991* encodes for a predicted transcriptional regulator gene upstream of *gtcA3*, a putative teichoic acid glycosylation protein. Upstream of *lp_2991* is a manganese transport gene *mntH2* which is orientated in the opposite direction.

Transcriptome analysis and qPCR data showed that transcript level of *gtcA3* was substantially increased in the lp_2991 deletion mutant (44 and 29 fold respectively). This supports the idea that *lp_2991* is a repressor of *gtcA3* transcription and points to this enzyme as being a prime candidate for the altered immune response. GtcA3 is predicted to glycosylate teichoic acid including lipoteichoic acid (LTA) which is a known TLR-2 agonist capable of modulating immune cell responses. Modification of LTA e.g. by substitution can have striking effects on the immune response as shown by Grangette et al., using a mutant in which *dltB* was deleted [30]. DltB is a putative transmembrane protein predicted to be involved in the passage of the activated D-alanyl-Dcp complex across the glycerol phosphate backbone of LTA. The *dltB* mutant of *L. plantarum* increased IL-10 secretion and dramatically raised the IL-10/IL-12 ratio in PBMC co-culture assays. In our experiments the lp_2991 deletion mutant also led to an increased IL-10 secretion in immune assays (6.3 fold) compared to wild type strain but the IL-10/IL-12 ratio was only slightly elevated due a corresponding increase in the induced levels of IL-12 (3.2-fold).

As the transcript levels of *gtcA3* were increased 44 fold in the lp_2991 deletion mutant and this enzyme is predicted to glycosylate TA or LTA we consider it most likely to be responsible for the altered immune response. However, we cannot rule out possible effects due to changes in *mntH2* gene expression which was modestly affected in the mutant (1.8 and 2.4 fold increase by qPCR and transcriptome analysis respectively). Manganese is an important element involved in the protection of *L. plantarum* against oxidative stress [80]. One uptake mechanism described is the Mn (2+) and Cd (2+)-specific P-type ATPase MntA. Besides *mntA*, WCFS1 encodes an ABC transporter system (*mtsCBA*) and three genes encoding Nramp transporters (*mntH1*, *mntH2* and *mntH3*) [80]. Studies conducted in pathogenic bacteria have shown that Nramp transporter mutants are less virulent [81] due to the role of Nramp in sequestering of Mn(2+) which is a cofactor for enzymes that protect against host oxidative killing mechanisms. The mutation of *lp_2991* may increase manganese transport and therefore enhance survival of lactobacilli in the phagolysosome. This may alter the kinetics and magnitude of the im-

mune response due to altered release of ligands for host pattern recognition receptors. This study emphasizes the usefulness of *in silico* gene-trait matching in assessing the role of specific bacterial genes in the interaction with the host immune system, an approach that is fully supported by the recent availability of full genome sequences for some lactobacilli. Screening with another *L. plantarum* than WCFS1 may also lead to the identification of other genes influencing the immune response. In the future this knowledge may be useful to select probiotic strains with anti-inflammatory or immune stimulatory properties. Future work is aimed at understanding the role of the genes we have identified in modulating the immune response to *L. plantarum*.

Acknowledgement

The authors are grateful to Roger Bongers for assistance with the purification of bacterial RNA and microarray analysis, and Germano Orru for advice on primer design. We thank Joost van Neerven and Mary-Léne Zeeuw-Brouwer for useful discussions about the DC methods.

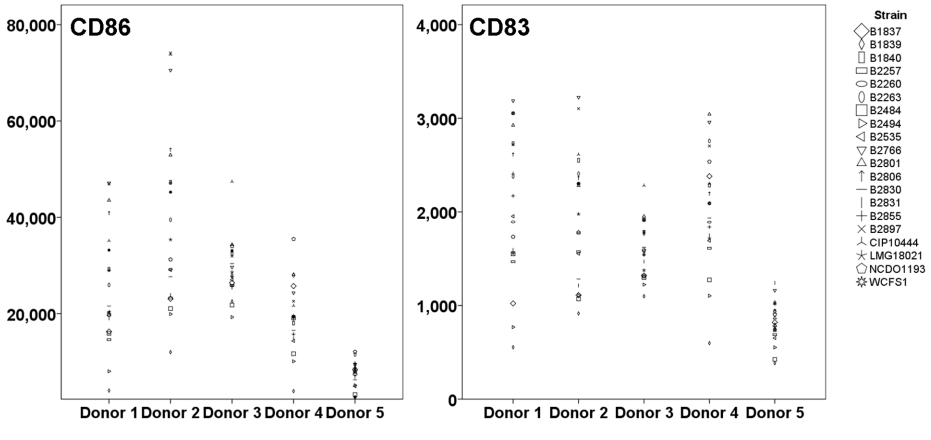

Figure S1. CD83 and CD86 expression by monocyte-derived dendritic cells derived from blood of five different donors after stimulation with 20 different *L. plantarum* strains. Each symbol represents a different *L. plantarum* strain.

Table S1. Origin of bacterial strains used in this study.

Strain #	Received as	Isolation source	Geographical origin
NIZO1836	**WCFS1**	Human saliva	England
NIZO2263	**LP80**	Silage	n.a.
NIZO2814	Lp95	Wine red grapes	Italy
CIP102359	**CIP102359**	Human spinal fluid	France
NIZO2726	**ATCC8014**	Maize ensilage	n.a.
NIZO2891	LD3	Radish pickled	Vietnam
NIZO2457	CHEO3	Pork pickled sour sausage	Vietnam
NIZO2535	LD2	Orange fermented	Vietnam
NIZO2830	BLL(EI31)	n.a.	n.a.
NIZO2259	**CIP104452**	Human tooth abscess	France
NIZO2831	CECT221(24Ab04)	Grass silage	United States
NIZO2262	**LM3**	Silage	n.a.
NIZO2494	NCTH27	Pork pickled sour sausage	Vietnam
NCDO1193	**NCDO1193**	Vegetables	n.a.
NIZO2806	LMG9208	Sauerkraut	United Kingdom
NIZO2896	**ATCC14917**[a]	Cabbage pickled	Denmark
NIZO2741	NOS140	Cabbage kimchi	Japan
NIZO1837	**299**	Human colon	United Kingdom
NIZO2855	N58	Pork pickled sour sausage	Vietnam
NIZO2877	X17	Hot dog	Vietnam
NIZO2260	**299v/DSM9843**	Human intestine	United Kingdom
NIZO2029	MLC43	Raw cheese with rennet	Italy
NIZO2889	LAC7	Banana fermented	Vietnam
NIZO2264	**LP85-2**[b]	Silage	France
NIZO2484	NCTH19-1	Pork pickled sour sausage	Vietnam
NIZO2485	NCTH19-2	Pork pickled sour sausage	Vietnam
NIZO2261	**NC8**	Grass silage	Sweden
NIZO2802	KOG24	Cheese	Japan
NIZO2801	KOG18	Turnip pickled	Japan
NIZO3400	**LMG18021**	Milk	Senegal
NIZO2753	Q2	Sourdough fermented	Italy
NIZO1839	**SF2A35B**[b]	Sour cassava	South America
NIZO2258	**CIP104451**	Human urine	France
NIZO2257	**CIP104450**	Human stool	France
CIP104448	**CIP104448**	Human stool	France
NIZO2897	DKO22[b]	Sour cassava	Nigeria
NIZO2766	H14	Sourdough fermented	Italy
NIZO2757	H4	Sourdough fermented	Italy
NIZO2776	CECT4645	Cheese	n.a..
NIZO2256	**CIP104441**	Human stool	France
NIZO1838	**CIP104440**	Human stool	France
NIZO1840	**NCIMB12120**[b]	Cereal fermented (Ogi)	Nigeria

n.a. not available

Strains in bold were also compared in Molenaar et al (2005). The other strains were new in this study.

[a] Draft genome sequence available April 2009 (NZ_ACGZ00000000.1)

[b] Putative subspecies *argentoratensis*

Table S2 Transcriptome analysis of WCFS1 and lp_2991 deletion mutant.

Gene no. (gene name)	Fold change	Description of proposed function
Genes with increased relative transcript levels in lp_2991 deletion mutant compared to WCFS1		
Cell envelope		
lp_2988 (zmp3)	2.2	extracellular zinc metalloproteinase, M10 family (putative)
lp_2809	2.5	extracellular protein (putative)
lp_0730 (tagO)	4.4	undecaprenyl-phosphate N-acetyl-glucosaminyl transferase
lp_2989 (gtcA3)	45	teichoic acid glycosylation protein (putative)
Cellular processes		
lp_0214	3.1	chromosome condensation protein (putative)
Central intermediary metabolism		
lp_1173	5.6	UDP-N-acetylglucosamine 2-epimerase
lp_0181 (mapB)	5.9	maltose phosphorylase
Energy metabolism		
lp_2659 (xpkA)	2.1	xylulose-5-P phosphoketolase & fructose-6-P phosphoketolase
lp_3490	8.6	FMN-binding protein
Hypothetical proteins		
lp_1726	2.1	unknown
lp_0311	2.2	acetyltransferase (putative)
lp_2066	2.2	unknown
lp_3346	2.9	unknown
lp_3002	3.2	integral membrane protein
lp_2230	16.7	unknown
lp_2093	18.1	unknown
lp_3348	19.1	unknown
Protein synthesis		
lp_2807 (tyrS)	2.8	tyrosine--tRNA ligase
Purines, pyrimidines, nucleosides and nucleotides		
lp_2702 (pyrC)	4.1	dihydroorotase
lp_2699 (pyrD)	4.9	dihydroorotate oxidase
lp_2701 (pyrAA)	5.6	carbamoyl-phosphate synthase, pyrimidine-specific, small chain
lp_2700 (pyrAB)	6.8	carbamoyl-phosphate synthase, pyrimidine-specific, large chain
lp_2698 (pyrF)	7	orotidine-5'-phosphate decarboxylase
lp_2697 (pyrE)	17	orotate phosphoribosyltransferase
Regulatory functions		
lp_1938	2.2	transcription regulator, LysR family
lp_2704 (purR1)	2.6	pyrimidine operon regulator
Transport and binding proteins		
lp_2992 (mntH2)	2.4	manganese transport protein
lp_0092	2.5	ABC transporter, substrate binding protein, oligopeptide
lp_2371 (pyrP)	2.6	uracil transport protein
lp_0286 (pts6C)	5.1	cellobiose PTS, EIIC
lp_1792	19.7	ABC transporter, permease protein
lp_p3_38	3.2	
mtf_9	14.7	

Genes with decreased relative transcript levels in lp_2991 deletion mutant compared to WCFS1		
Biosynthesis of cofactors, prosthetic groups, and carriers		
lp_0369 (gshR1)	-5.5	glutathione reductase
Cellular processes		
lp_2210 (ftsK2)	-5.8	cell division protein FtsK
DNA metabolism		
lp_1839 (parC)	-2.3	topoisomerase IV, subunit A
Energy metabolism		
lp_3595 (rhaB)	-5.2	rhamnulokinase
lp_3449 (nox5)	-2.5	NADH oxidase
Hypothetical proteins		
lp_1533	-2.9	methyltransferase (putative)
lp_0753	-2.8	integral membrane protein
lp_0967	-2.8	unknown
lp_2058	-2.4	endonuclease (putative)
lp_1390	-2.2	acetyltransferase, GNAT family (putative)
lp_1136	-2	oxidoreductase, NAD(P)-dependent
lp_2114	-2	NTP pyrophosphohydrolase (putative)
Protein synthesis		
lp_0443 (dus1)	-2.3	tRNA-dihydrouridine synthase
Regulatory functions		
lp_0188 (scrR)	-3.4	oligosucrose operon repressor
lp_0319	-2.3	transcription regulator, spermidine/putrescine transport operon
Transport and binding proteins		
lp_2531 (pts18CBA)	-13.1	N-acetylglucosamine and glucose PTS, EIICBA
lp_3686	-9	ABC transporter, substrate binding protein
lp_0317 (potB)	-5.1	spermidine/putrescine ABC transporter, permease protein
lp_0218	-4.9	ABC transporter, ATP-binding protein
lp_2352	-3	ABC transporter, ATP binding binding protein, D-Methionine -like precursor
lp_3279 (kup2)	-2.5	potassium uptake protein
lp_2351	-2.4	ABC transporter, permease protein, D-Methionine -like precursor
lp_0367 (choS)	-2.3	glycine betaine/carnitine/choline ABC transporter, substrate binding and permease protein
lp_0368 (choQ)	-2.3	glycine betaine/carnitine/choline ABC transporter, ATP-binding protein

References

1. Fabia R, Ar'Rajab A, Johansson ML, Willen R, Andersson R, et al. (1993) The effect of exogenous administration of *Lactobacillus reuteri* R2LC and oat fiber on acetic acid-induced colitis in the rat. Scand J Gastroenterol 28: 155-162.
2. Gionchetti P, Rizzello F, Venturi A, Brigidi P, Matteuzzi D, et al. (2000) Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: a double-blind, placebo-controlled trial. Gastroenterology 119: 305-309.
3. Madsen KL, Doyle JS, Jewell LD, Tavernini MM, Fedorak RN (1999) *Lactobacillus* species prevents colitis in interleukin 10 gene-deficient mice. Gastroenterology 116: 1107-1114.
4. Mao Y, Nobaek S, Kasravi B, Adawi D, Stenram U, et al. (1996) The effects of *Lactobacillus* strains and oat fiber on methotrexate-induced enterocolitis in rats. Gastroenterology 111: 334-344.
5. Mimura T, Rizzello F, Helwig U, Poggioli G, Schreiber S, et al. (2004) Once daily high dose probiotic therapy (VSL#3) for maintaining remission in recurrent or refractory pouchitis. Gut 53: 108-114.
6. Rembacken BJ, Snelling AM, Hawkey PM, Chalmers DM, Axon AT (1999) Non-pathogenic Escherichia coli versus mesalazine for the treatment of ulcerative colitis: a randomised trial. Lancet 354: 635-639.
7. Kalliomaki M, Antoine JM, Herz U, Rijkers GT, Wells JM, et al. Guidance for Substantiating the Evidence for Beneficial Effects of Probiotics: Prevention and Management of Allergic Diseases by Probiotics. J Nutr.
8. Rijkers GT, Bengmark S, Enck P, Haller D, Herz U, et al. Guidance for Substantiating the Evidence for Beneficial Effects of Probiotics: Current Status and Recommendations for Future Research. J Nutr.
9. Resta-Lenert SC, Barrett KE (2009) Modulation of intestinal barrier properties by probiotics: role in reversing colitis. Ann N Y Acad Sci 1165: 175-182.
10. Wells JM, Loonen LM, Karczewski JM (2009) The role of innate signaling in the homeostasis of tolerance and immunity in the intestine. Int J Med Microbiol.
11. Delcenserie V, Martel D, Lamoureux M, Amiot J, Boutin Y, et al. (2008) Immunomodulatory effects of probiotics in the intestinal tract. Curr Issues Mol Biol 10: 37-54.
12. Kwon HK, Lee CG, So JS, Chae CS, Hwang JS, et al. Generation of regulatory dendritic cells and CD4+Foxp3+ T cells by probiotics administration suppresses immune disorders. Proc Natl Acad Sci USA 107: 2159-2164.
13. Stagg AJ, Hart AL, Knight SC, Kamm MA (2003) The dendritic cell: its role in intestinal inflammation and relationship with gut bacteria. Gut 52: 1522-1529.
14. Rescigno M, Urbano M, Valzasina B, Francolini M, Rotta G, et al. (2001) Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat Immunol 2: 361-367.
15. Rimoldi M, Chieppa M, Salucci V, Avogadri F, Sonzogni A, et al. (2005) Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. Nat Immunol 6: 507-514.
16. Zeuthen LH, Fink LN, Frokiaer H (2008) Epithelial cells prime the immune response to an array of gut-derived commensals towards a tolerogenic phenotype through distinct actions of thymic stromal lymphopoietin and transforming growth factor-beta. Immunology 123: 197-208.
17. Hart AL, Lammers K, Brigidi P, Vitali B, Rizzello F, et al. (2004) Modulation of human dendritic cell phenotype and function by probiotic bacteria. Gut 53: 1602-1609.
18. Braat H, van den Brande J, van Tol E, Hommes D, Peppelenbosch M, et al. (2004)

Lactobacillus rhamnosus induces peripheral hyporesponsiveness in stimulated CD4+ T cells via modulation of dendritic cell function. Am J Clin Nutr 80: 1618-1625.
19. Christensen HR, Frokiaer H, Pestka JJ (2002) Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. J Immunol 168: 171-178.
20. Drakes M, Blanchard T, Czinn S (2004) Bacterial probiotic modulation of dendritic cells. Infect Immun 72: 3299-3309.
21. Niers LE, Hoekstra MO, Timmerman HM, van Uden NO, de Graaf PM, et al. (2007) Selection of probiotic bacteria for prevention of allergic diseases: immunomodulation of neonatal dendritic cells. Clin Exp Immunol 149: 344-352.
22. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, et al. (2000) Immunobiology of dendritic cells. Annu Rev Immunol 18: 767-811.
23. Asseman C, Mauze S, Leach MW, Coffman RL, Powrie F (1999) An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. J Exp Med 190: 995-1004.
24. Powrie F, Leach MW, Mauze S, Caddle LB, Coffman RL (1993) Phenotypically distinct subsets of CD4+ T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice. Int Immunol 5: 1461-1471.
25. Van Montfrans C, Hooijberg E, Rodriguez Pena MS, De Jong EC, Spits H, et al. (2002) Generation of regulatory gut-homing human T lymphocytes using *ex vivo* interleukin 10 gene transfer. Gastroenterology 123: 1877-1888.
26. Foligne B, Nutten S, Grangette C, Dennin V, Goudercourt D, et al. (2007) Correlation between *in vitro* and *in vivo* immunomodulatory properties of lactic acid bacteria. World J Gastroenterol 13: 236-243.
27. Denou E, Pridmore RD, Berger B, Panoff JM, Arigoni F, et al. (2008) Identification of genes associated with the long-gut-persistence phenotype of the probiotic *Lactobacillus johnsonii* strain NCC533 using a combination of genomics and transcriptome analysis. J Bacteriol 190: 3161-3168.
28. Pretzer G, Snel J, Molenaar D, Wiersma A, Bron PA, et al. (2005) Biodiversity-based identification and functional characterization of the mannose-specific adhesin of *Lactobacillus plantarum*. J Bacteriol 187: 6128-6136.
29. Kankainen M, Paulin L, Tynkkynen S, von Ossowski I, Reunanen J, et al. (2009) Comparative genomic analysis of *Lactobacillus rhamnosus* GG reveals pili containing a human- mucus binding protein. Proc Natl Acad Sci U S A 106: 17193-17198.
30. Grangette C, Nutten S, Palumbo E, Morath S, Hermann C, et al. (2005) Enhanced antiinflammatory capacity of a *Lactobacillus plantarum* mutant synthesizing modified teichoic acids. Proc Natl Acad Sci U S A 102: 10321-10326.
31. Henneke P, Morath S, Uematsu S, Weichert S, Pfitzenmaier M, et al. (2005) Role of lipoteichoic acid in the phagocyte response to group B streptococcus. J Immunol 174: 6449-6455.
32. Kim HG, Gim MG, Kim JY, Hwang HJ, Ham MS, et al. (2007) Lipoteichoic acid from *Lactobacillus plantarum* elicits both the production of interleukin-23p19 and suppression of pathogen-mediated interleukin-10 in THP-1 cells. FEMS Immunol Med Microbiol 49: 205-214.
33. Matsuguchi T, Takagi A, Matsuzaki T, Nagaoka M, Ishikawa K, et al. (2003) Lipoteichoic acids from *Lactobacillus* strains elicit strong tumor necrosis factor alpha-inducing activities in macrophages through Toll-like receptor 2. Clin Diagn Lab Immunol 10: 259-266.
34. Ryu YH, Baik JE, Yang JS, Kang SS, Im J, et al. (2009) Differential immunostimulatory effects of Gram-positive bacteria due to their lipoteichoic acids. Int Immunopharmacol 9: 127-133.

35. Perea Velez M, Verhoeven TL, Draing C, Von Aulock S, Pfitzenmaier M, et al. (2007) Functional analysis of D-alanylation of lipoteichoic acid in the probiotic strain *Lactobacillus rhamnosus* GG. Appl Environ Microbiol 73: 3595-3604.
36. Smits HH, Engering A, van der Kleij D, de Jong EC, Schipper K, et al. (2005) 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 115: 1260-1267.
37. Yasuda E, Serata M, Sako T (2008) Suppressive effect on activation of macrophages by *Lactobacillus casei* strain Shirota genes determining the synthesis of cell wall-associated polysaccharides. Appl Environ Microbiol 74: 4746-4755.
38. Boneca IG, Dussurget O, Cabanes D, Nahori MA, Sousa S, et al. (2007) A critical role for peptidoglycan N-deacetylation in Listeria evasion from the host innate immune system. Proc Natl Acad Sci U S A 104: 997-1002.
39. Wolfert MA, Roychowdhury A, Boons GJ (2007) Modification of the structure of peptidoglycan is a strategy to avoid detection by nucleotide-binding oligomerization domain protein 1. Infect Immun 75: 706-713.
40. Kleerebezem M, Boekhorst J, van Kranenburg R, Molenaar D, Kuipers OP, et al. (2003) Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc Natl Acad Sci U S A 100: 1990-1995.
41. Vesa T, Pochart P, Marteau P (2000) Pharmacokinetics of *Lactobacillus plantarum* NCIMB 8826, *Lactobacillus fermentum* KLD, and *Lactococcus lactis* MG 1363 in the human gastrointestinal tract. Aliment Pharmacol Ther 14: 823-828.
42. Troost FJ, van Baarlen P, Lindsey P, Kodde A, de Vos WM, et al. (2008) Identification of the transcriptional response of human intestinal mucosa to *Lactobacillus plantarum* WCFS1 in vivo. BMC Genomics 9: 374.
43. van Baarlen P, Troost FJ, van Hemert S, van der Meer C, de Vos WM, et al. (2009) Differential NF-kappaB pathways induction by *Lactobacillus plantarum* in the duodenum of healthy humans correlating with immune tolerance. Proc Natl Acad Sci U S A 106: 2371-2376.
44. Molenaar D, Bringel F, Schuren FH, de Vos WM, Siezen RJ, et al. (2005) Exploring *Lactobacillus plantarum* genome diversity by using microarrays. J Bacteriol 187: 6119-6127.
45. Siezen RJ, Tzeneva VA, Castioni A, Wels M, Phan HTK, et al. (2009) Phenotypic and genomic diversity of *Lactobacillus plantarum* strains isolated from various environmental niches. Environmental Microbiology in revision.
46. Zhou LJ, Tedder TF (1995) Human blood dendritic cells selectively express CD83, a member of the immunoglobulin superfamily. J Immunol 154: 3821-3835.
47. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C (1995) A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods 184: 39-51.
48. Morgan E, Varro R, Sepulveda H, Ember JA, Apgar J, et al. (2004) Cytometric bead array: a multiplexed assay platform with applications in various areas of biology. Clin Immunol 110: 252-266.
49. Jim K, Parmar K, Singh M, Tavazoie S (2004) A cross-genomic approach for systematic mapping of phenotypic traits to genes. Genome Res 14: 109-115.
50. Lambert JM, Bongers RS, de Vos WM, Kleerebezem M (2008) Functional analysis of four bile salt hydrolase and penicillin acylase family members in *Lactobacillus plantarum* WCFS1. Appl Environ Microbiol 74: 4719-4726.
51. Lambert JM, Bongers RS, Kleerebezem M (2007) Cre-lox-based system for multiple gene deletions and selectable-marker removal in *Lactobacillus plantarum*.

Appl Environ Microbiol 73: 1126-1135.
52. Horton RM, Cai ZL, Ho SN, Pease LR (1990) Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. Biotechniques 8: 528-535.
53. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e45.
54. van Hijum SA, Garcia de la Nava J, Trelles O, Kok J, Kuipers OP (2003) MicroPreP: a cDNA microarray data pre-processing framework. Appl Bioinformatics 2: 241-244.
55. Baldi P, Long AD (2001) A Bayesian framework for the analysis of microarray expression data: regularized t -test and statistical inferences of gene changes. Bioinformatics 17: 509-519.
56. Diep DB, Havarstein LS, Nes IF (1996) Characterization of the locus responsible for the bacteriocin production in *Lactobacillus plantarum* C11. J Bacteriol 178: 4472-4483.
57. Rojo-Bezares B, Saenz Y, Navarro L, Jimenez-Diaz R, Zarazaga M, et al. (2008) Characterization of a new organization of the plantaricin locus in the inducible bacteriocin-producing *Lactobacillus plantarum* J23 of grape must origin. Arch Microbiol 189: 491-499.
58. Diep DB, Straume D, Kjos M, Torres C, Nes IF (2009) An overview of the mosaic bacteriocin pln loci from *Lactobacillus plantarum*. Peptides 30: 1562-1574.
59. Lebeer S, Verhoeven TL, Francius G, Schoofs G, Lambrichts I, et al. (2009) Identification of a Gene Cluster for the Biosynthesis of a Long, Galactose-Rich Exopolysaccharide in *Lactobacillus rhamnosus* GG and Functional Analysis of the Priming Glycosyltransferase. Appl Environ Microbiol 75: 3554-3563.
60. Medina M, Izquierdo E, Ennahar S, Sanz Y (2007) Differential immunomodulatory properties of *Bifidobacterium longum* strains: relevance to probiotic selection and clinical applications. Clin Exp Immunol 150: 531-538.
61. Miettinen M, Matikainen S, Vuopio-Varkila J, Pirhonen J, Varkila K, et al. (1998) Lactobacilli and streptococci induce interleukin-12 (IL-12), IL-18, and gamma interferon production in human peripheral blood mononuclear cells. Infect Immun 66: 6058-6062.
62. Miettinen M, Vuopio-Varkila J, Varkila K (1996) Production of human tumor necrosis factor alpha, interleukin-6, and interleukin-10 is induced by lactic acid bacteria. Infect Immun 64: 5403-5405.
63. Niers LE, Timmerman HM, Rijkers GT, van Bleek GM, van Uden NO, et al. (2005) Identification of strong interleukin-10 inducing lactic acid bacteria which down-regulate T helper type 2 cytokines. Clin Exp Allergy 35:1481-1489.
64. Feleszko W, Jaworska J, Rha RD, Steinhausen S, Avagyan A, et al. (2007) 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 37: 498-505.
65. Karimi K, Inman MD, Bienenstock J, Forsythe P (2009) *Lactobacillus reuteri*-induced regulatory T cells protect against an allergic airway response in mice. Am J Respir Crit Care Med 179: 186-193.
66. Bureau A, Dupuis J, Falls K, Lunetta KL, Hayward B, et al. (2005) Identifying SNPs predictive of phenotype using random forests. Genet Epidemiol 28: 171-182.
67. Jiang H, Deng Y, Chen HS, Tao L, Sha Q, et al. (2004) Joint analysis of two microarray gene-expression data sets to select lung adenocarcinoma marker genes.BMC Bioinformatics 5: 81.
68. Cotter PD, Hill C, Ross RP (2005) Bacteriocins: developing innate immunity for food. Nat Rev Microbiol 3: 777-788.
69. Twomey D, Ross RP, Ryan M, Meaney B, Hill C (2002) Lantibiotics produced by lactic acid bacteria: structure, function and applications. Antonie Van Leeuwenhoek 82: 165-185.
70. Ross RP, Galvin M, McAuliffe O, Morgan SM, Ryan MP, et al. (1999) Developing applications

for lactococcal bacteriocins. Antonie Van Leeuwenhoek 76: 337-346.
71. Holo H, Jeknic Z, Daeschel M, Stevanovic S, Nes IF (2001) Plantaricin W from *Lactobacillus plantarum* belongs to a new family of two-peptide lantibiotics. Microbiology 147: 643-651.
72. Maldonado A, Ruiz-Barba JL, Floriano B, Jimenez-Diaz R (2002) The locus responsible for production of plantaricin S, a class IIb bacteriocin produced by *Lactobacillus plantarum* LPCO10, is widely distributed among wild-type *Lact. plantarum* strains isolated from olive fermentations. Int J Food Microbiol 77: 117-124.
73. Remiger A, Eijsink VG, Ehrmann MA, Sletten K, Nes IF, et al. (1999) Purification and partial amino acid sequence of plantaricin 1.25 alpha and 1.25 beta, two bacteriocins produced by *Lactobacillus plantarum* TMW1.25. J Appl Microbiol 86: 1053-1058.
74. Anderssen EL, Diep DB, Nes IF, Eijsink VG, Nissen-Meyer J (1998) Antagonistic activity of *Lactobacillus plantarum* C11: two new two-peptide bacteriocins, plantaricins EF and JK, and the induction factor plantaricin A. Appl Environ Microbiol 64: 2269-2272.
75. Funderburg N, Lederman MM, Feng Z, Drage MG, Jadlowsky J, et al. (2007) Human -defensin-3 activates professional antigen-presenting cells via Toll-like receptors 1 and 2. Proc Natl Acad Sci U S A 104: 18631-18635.
76. Yang D, Biragyn A, Hoover DM, Lubkowski J, Oppenheim JJ (2004) Multiple roles of antimicrobial defensins, cathelicidins, and eosinophil-derived neurotoxin in host defense. Annu Rev Immunol 22: 181-215.
77. Bron PA, Grangette C, Mercenier A, de Vos WM, Kleerebezem M (2004) Identification of *Lactobacillus plantarum* genes that are induced in the gastrointestinal tract of mice. J Bacteriol 186: 5721-5729.
78. Marco ML, Bongers RS, de Vos WM, Kleerebezem M (2007) Spatial and temporal expression of *Lactobacillus plantarum* genes in the gastrointestinal tracts of mice. Appl Environ Microbiol 73: 124-132.
79. Marco ML, Peters TH, Bongers RS, Molenaar D, van Hemert S, et al. (2009) Lifestyle of *Lactobacillus plantarum* in the mouse caecum. Environ Microbiol 11: 2747-2757.
80. Groot MN, Klaassens E, de Vos WM, Delcour J, Hols P, et al. (2005) Genome-based in silico detection of putative manganese transport systems in *Lactobacillus plantarum* and their genetic analysis.Microbiology 151: 1229-1238.
81. Papp-Wallace KM, Maguire ME (2006) Manganese transport and the role of manganese in virulence. Annu Rev Microbiol 60: 187-209.

Chapter 4

Identification of *Lactobacillus plantarum* genes modulating the cytokine response of human peripheral blood mononuclear cells

S. van Hemert, M. Meijerink, D. Molenaar, P. A. Bron, P. de Vos, M. Kleerebezem, J. M. Wells and M. L. Marco

BMC Microbiology (2010) 10, 293

Abstract

Background
Modulation of the immune system is one of the most plausible mechanisms underlying the beneficial effects of probiotic bacteria on human health. Presently, the specific probiotic cell products responsible for immunomodulation are largely unknown. In this study, the genetic and phenotypic diversity of strains of the *Lactobacillus plantarum* species were investigated to identify genes of *L. plantarum* with the potential to influence the amounts of cytokines interleukin 10 (IL-10) and IL-12 and the ratio of IL-10/IL-12 produced by peripheral blood mononuclear cells (PBMCs).

Results
A total of 42 *Lactobacillus plantarum* strains isolated from diverse environmental and human sources were evaluated for their capacity to stimulate cytokine production in PBMCs. The *L. plantarum* strains induced the secretion of the anti-inflammatory cytokine IL-10 over an average 14-fold range and secretion of the pro-inflammatory cytokine IL-12 over an average 16-fold range. Comparisons of the strain-specific cytokine responses of PBMCs to comparative genome hybridization profiles obtained with *L. plantarum* WCFS1 DNA microarrays (also termed gene-trait matching) resulted in the identification of 6 candidate genetic loci with immunomodulatory capacities. These loci included genes encoding an N-acetyl-glucosamine/galactosamine phosphotransferase system, the LamBDCA quorum sensing system, and components of the plantaricin (bacteriocin) biosynthesis and transport pathway. Deletion of these genes in *L. plantarum* WCFS1 resulted in growth phase-dependent changes in the PBMC IL-10 and IL-12 cytokine profiles compared with wild-type cells.

Conclusions
The altered PBMC cytokine profiles obtained with the *L. plantarum* WCFS1 mutants were in good agreement with the predictions made by gene-trait matching for the 42 *L. plantarum* strains. This study therefore resulted in the identification of genes present in certain strains of *L. plantarum* which might be responsible for the stimulation of anti- or pro-inflammatory immune responses in the gut.

Introduction

Metagenomics and host-microbe molecular interaction studies are rapidly expanding our understanding of the indigenous gut microbiota and the contributions of microbes to human health [1,2]. These efforts are complementary to the numerous reports describing health benefits associated with the ingestion of probiotic bacteria [3,4]. Probiotics are living microorganisms which confer health effects on the host when administered in sufficient amounts [5]. Strains of *Lactobacillus* and *Bifidobacterium* are the most commonly applied probiotics in food products. Members of these genera are residents of the human intestine and have a long history of safe use in foods and beverages. Health benefits conferred by probiotics can be specific to the gastrointestinal tract (e.g. protection against intestinal inflammation or enteric pathogens) or occur at peripheral mucosal sites in the human body (e.g. prevention of allergy or dermatitis) [6].

There is substantial evidence that an important mechanism by which probiotics provide health benefits is through modulation of immune functions [7-11]. Differences among probiotic strains to stimulate immune cells towards pro- and anti-inflammatory responses have been shown in studies measuring cytokine production *in vitro* [7-11]. These comparisons have resulted in the identification of strains inducing similar responses *in vivo*. For example, ratios of IL-10 to IL-12 produced by peripheral blood mononuclear cells (PBMCs) in response to different probiotics *in vitro* were correlated to their protective capacity in a mouse model of colitis [10]. Similarly, recent studies on the mechanisms of probiotics highlight their effects on epithelial barrier function via Toll-like receptor 2 signaling and the generation of regulatory dendritic cells and regulatory CD4+Foxp3+ T cells in peripheral tissues [12,13]. The latter mechanism is linked to the administration of a collection of five strains which induced a high IL-10/IL-12 ratio in co-culture with immune cells [12]. Administration of these strains was shown to have a therapeutic effect in experimental mouse models of inflammatory bowel disease, atopic dermatitis, and rheumatoid arthritis and was associated with enrichment of CD4+ Foxp3+ Tregs in the inflamed regions [12].

The cell products of probiotics that are responsible for modulation of cytokine induction are largely unknown but might involve modifications of some of the known microbe associated molecular patterns (MAMPs) such lipoteichoic acids (LTA) [14-16] and (lipo)proteins localized on the bacterial cell surface [17] which interact with Toll-like receptors. Additionally cell-surface associated bacterial glycosylated proteins or exopolysaccharides [18] may interact with other host pattern recognition receptors including the C-type lectins and scavenger receptors found on antigen presenting cells [19]. These extracellular and secreted products produced by probiotic cells are the likely targets for strain-dependent interactions with host cells and have been the focus of several recent reviews [6,20,21].

Certain strains of *Lactobacillus plantarum* are marketed as probiotics and reported to confer various health effects including immunomodulation [22]. The genome sequence of *L. plantarum* strain WCFS1 is known [23] and extensive bioinformatics tools [24,25],

molecular models [26], and a database of genome hybridization profiles [27,28] are available for this organism. It is a single colony isolate of strain NCIMB8826, which was shown to survive gastrointestinal passage after oral administration to healthy volunteers [29]. Global gene expression profiling of *L. plantarum* WCFS1 in the intestinal contents of the human gut and conventionally-raised and germ-free mice has shown that this organism adapts for growth *in vivo* by modification of its cell-surface composition and metabolism in a diet-dependent manner [30-34]. Human duodenal transcriptional response profiles have also been obtained in response to ingestion of *L. plantarum* WCFS1 [35,36]. Notably, exponential phase and stationary phase *L. plantarum* WCFS1 cells elicited distinct human duodenal transcript profiles which appeared to mainly result from differential modulation of canonical NF-κB-dependent signaling pathways associated with immune tolerance [35]. The aim of the present study was to identify genes involved in immunomodulation by *L. plantarum* WCFS1. The IL-10 and IL-12 cytokine levels elicited by PBMCs upon stimulation with *L. plantarum* WCFS1 and 41 other *Lactobacillus plantarum* strains were determined. We compared the IL-10 and IL-12 stimulating phenotypes of each strain to its genome composition determined by comparative genome hybridization (CGH) to identify candidate *L. plantarum* genes with the capacity to affect cytokine production in PBMCs. The immunomodulatory potential of these gene products was confirmed for *L. plantarum* WCFS1 gene deletion mutants and found to be dependent on the growth-phase of the *L. plantarum* cultures.

Results

Immunomodulation of PBMCs is a variable phenotype in L. plantarum

A total of 42 *L. plantarum* strains from distinct (fermented) food, environmental, and gastrointestinal sources (Table 1 and [27,28]) were investigated for their capacities to stimulate PBMCs to produce the cytokines IL-10 and IL-12. Comparisons of cytokine amounts induced among different donors in response to the *L. plantarum* strains showed that the *L. plantarum* cultures induced a similar range of IL-10 but up to 10-fold different levels of IL-12 (Figure 1). This result is in agreement with previous studies showing that PBMCs respond differently depending on the donor from which the cells were isolated [37]. However, the capacity of individual *L. plantarum* strains to induce cytokines production in PBMCs was similar among the different donors relative to the other strains tested. For example, *L. plantarum* KOG18 consistently induced the highest amounts of IL-12 whereas strain CIP104448 induced the highest ratios of IL-10 to IL-12. Collectively, the 42 *L. plantarum* strains induced, on average, IL-10 and IL-12 in PBMCs over a 14- and 16 - fold range, respectively, and IL-10/IL-12 ratios over a 13.5 - fold range (Figure 1). Strain WCFS1 induced relatively low IL-10 amounts (between 440 and 780 pg/ml), moderate amounts of IL-12 (between 20 and 260 pg/ml), and consequently a moderate to low IL-10/IL-12 ratio (bottom quartiles) compared with the other strains (Figure 1).

Table 1. *L. plantarum* strains selected for genotyping and screening for immunomodulatory capacity.

Strain	Strain ID[a]	Isolation source	Geographical origin
WCFS1	NIZO1836	Human saliva	n.a.
LP80	NIZO2263	Silage	n.a.
Lp95	NIZO2814	Wine red grapes	Italy
CIP102359	CIP102359	Human spinal fluid	France
ATCC8014	NIZO2726	Maize ensilage	n.a.
LD3	NIZO2891	Radish pickled	Vietnam
CHEO3	NIZO2457	Pork pickled sour sausage	Vietnam
LD2	NIZO2535	Orange fermented	Vietnam
BLL(EI31)	NIZO2830	n.a.	Not known
CIP104452	NIZO2259	Human tooth abscess	France
CECT221(24Ab04)	NIZO2831	Grass silage	United States
LM3	NIZO2262	Silage	n.a.
NCTH27	NIZO2494	Pork pickled sour sausage	Vietnam
NCDO1193	NCDO1193	Vegetables	n.a.
LMG9208	NIZO2806	Sauerkraut	United Kingdom
ATCC14917	NIZO2896	Cabbage pickled	Denmark
NOS140	NIZO2741	Cabbage kimchi	Japan
299	NIZO1837	Human colon	United Kingdom
N58	NIZO2855	Pork pickled sour sausage	Vietnam
X17	NIZO2877	Hot dog	Vietnam
299v/DSM9843	NIZO2260	Human intestine	United Kingdom
MLC43	NIZO2029	Raw cheese with rennet	Italy
LAC7	NIZO2889	Banana fermented	Vietnam
LP85-2	NIZO2264	Silage	France
NCTH19-1	NIZO2484	Pork pickled sour sausage	Vietnam
NCTH19-2	NIZO2485	Pork pickled sour sausage	Vietnam
NC8	NIZO2261	Grass silage	Sweden
KOG24	NIZO2802	Cheese	Japan
KOG18	NIZO2801	Turnip pickled	Japan
LMG18021	NIZO3400	Milk	Senegal
Q2	NIZO2753	Sourdough fermented	Italy
SF2A35B	NIZO1839	Sour cassava	South America
CIP104451	NIZO2258	Human urine	France
CIP104450	NIZO2257	Human stool	France
CIP104448	CIP104448	Human stool	France
DKO22	NIZO2897	Sour cassava	Nigeria
H14	NIZO2766	Sourdough fermented	Italy
H4	NIZO2757	Sourdough fermented	Italy
CECT4645	NIZO2776	Cheese	n.a.
CIP104441	NIZO2256	Human stool	France
CIP104440	NIZO1838	Human stool	France
NCIMB12120	NIZO1840	Cereal fermented (Ogi)	Nigeria

n.a. not available

[a] See references [27,28] for comparative genome hybridization analyses of these strains.

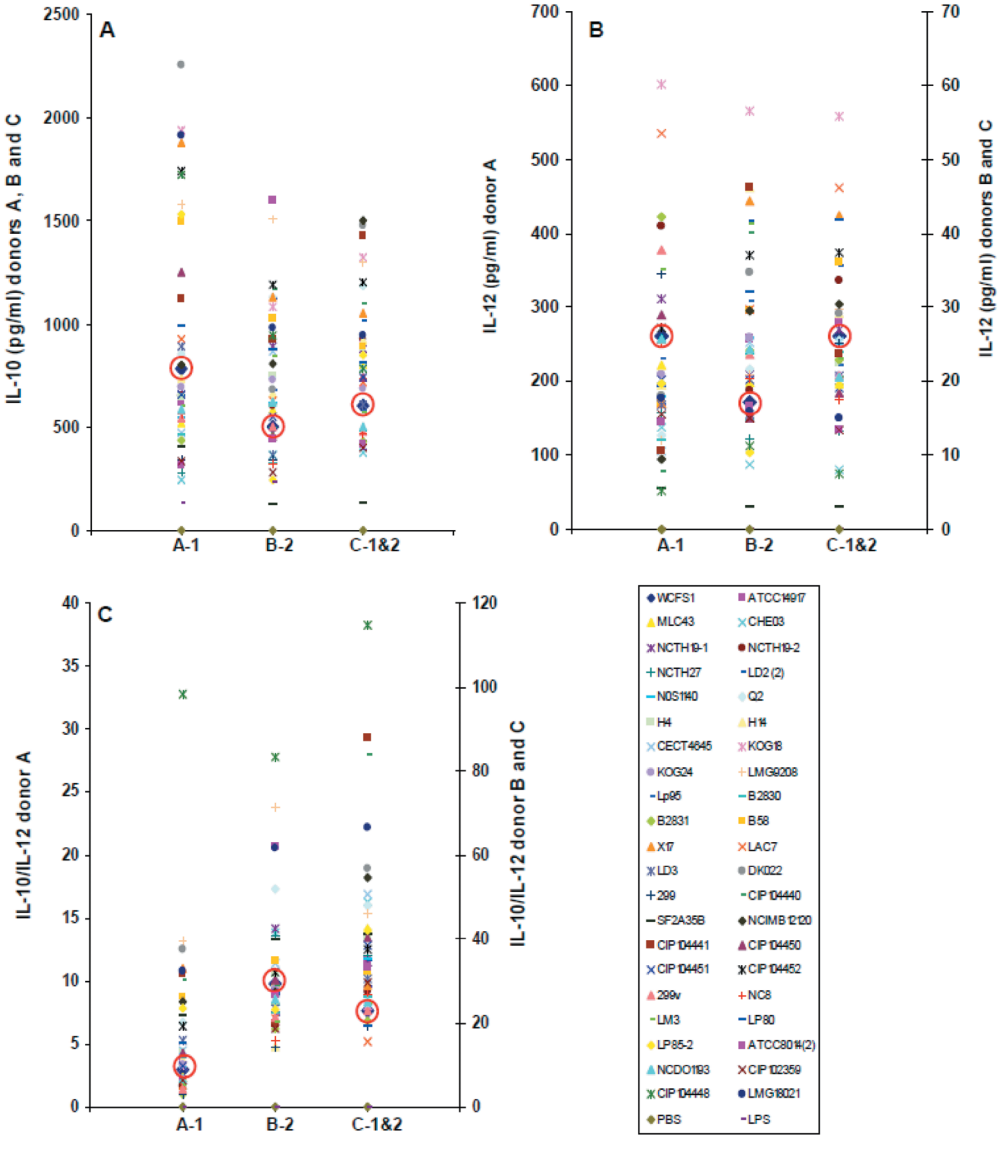

Figure 1. Cytokine secretion by PBMCs after 24 h co-culture with *L. plantarum* strains. IL-10 (A) and IL-12 (B) production and the IL-10/IL-12 ratio (C) by peripheral blood mononuclear cells (PBMCs) derived from blood of 3 different healthy donors after stimulation with 42 different *L. plantarum* strains harvested in stationary-phase. The *L. plantarum* strains grown and prepared on separate days constitute set 1 and set 2. PBMCs isolated from donor A were inoculated with *L. plantarum* culture set 1 (A-1) and PBMCs from donor B were inoculated with the *L. plantarum* replicate set 2 (B-2). PBMCs from Donor C received both sets of cultures and the mean of the IL-10 and IL-12 amounts induced by these cultures is shown. Each symbol represents a different *L. plantarum* strain or the PBS or LPS controls.

Identification of candidate genes involved in immunomodulation

To identify candidate *L. plantarum* genes involved in the modulation of the immune response, Random Forest models [38] were used to compare *L. plantarum* CGH profiles with the relative amounts of IL-10 and IL-12 and IL-10/IL-12 ratios induced by the strains in co-culture with PBMCs (Figure 1). PBMCs from different donors incubated with replicate *L. plantarum* cultures were used for these models to take into account the levels of variation in cytokine production. Comparisons of *L. plantarum* strain genotype to the IL-10-stimulating capacities resulted in the identification of 6 different chromosomal loci and a total of 13 genes that might influence IL-10 production or hte IL-10/IL-12 ratio (Table 2). In comparison, concise correlations between *L. plantarum* CGH profiles and IL-12 amounts were not found. One of the genes correlated with IL-10 amounts was *L. plantarum* WCFS1 lp_1953. *L. plantarum* strains harboring this gene stimulated the production of IL-10 in 1.6-fold higher amounts, on average, compared to *L. plantarum* strains for which this gene was absent. Lp_1953 encodes a hypothetical intracellular protein of unknown function [25]. The remaining five genes with putative roles in IL-10 modulation comprise a putative 5 gene operon (lp_2647 to lp_2651) encoding Pts19ADCBR, an N-acetyl-galactosamine/glucosamine phosphotransferase system (PTS). Strains harboring these genes were associated with induction of lower amounts of IL-10 by PBMCs.

Table 2. *L. plantarum* genes with putative roles in modulating PBMC cytokine production.

Gene(s)	Gene number [a]	Product	Percent of strains with the gene(s) [b]	Gene-dependent contribution to cytokine stimulation [c]	
lp_1953	lp_1953	Hypothetical protein	48	IL-10 (0.29)	1.6-fold ⩚
pts19ADCBR	lp_2647-2651	N-galactosamine PTS, EIIADCB and transcription regulator, GntR family	33	IL-10 (0.16)	1.7-fold ⩛
plnEFI	lp_0419-0422	Immunity protein plnI Bacteriocin like peptide plnF Bacteriocin like peptide pln E	81-85	IL-10 / IL-12 (0.35)	1.7-fold ⩛
plnG	lp_0423	ABC transporter	88	IL-10 / IL-12 (0.13)	1.8-fold ⩛
lamB	lp_3582	Accessory gene regulator protein	43	IL-10 / IL-12 (0.14)	1.3-fold ⩛
Prophage P2b 1 and 21	lp_2460 lp_2480	Prophage P2b protein 21 Prophage P2b protein 1, integrase	38	IL-10 / IL-12 (0.17)	1.5-fold ⩚

a Gene number on the L. plantarum WCFS1 chromosome [23].
b Percentage of L. plantarum strains containing the gene according to CGH [27,28].
c Gene-trait matching importance measures (in parentheses) and predicted effects of the gene(s) on the variable and average magnitude and direction (higher or lower) of IL-10 and IL-10/IL-12 amounts.

Comparisons between *L. plantarum* strain-specific CGH profiles and IL-10/IL-12 ratios from PBMCs resulted in the identification of four *L. plantarum* WCFS1 loci which correlated with IL-10/IL-12 values (Table 2). *L. plantarum* WCFS1 plnEFI and plnG (lp_419-423) and lamB (lp_3582) were most commonly present in strains stimulating low IL-10/IL-12 ratios. These genes are under the control of the auto-inducing peptide (AIP)-based quorum sensing (QS) two-component regulatory systems (QS-TCSs) found in *L. plantarum* [39,40]. The genes plnEFI and plnG encode two bacteriocin peptides, a bacteriocin

immunity protein, and an ATP - Binding Cassette (ABC) transporter [23,41]. The lamB is the first gene in the *L. plantarum* lamBDCA operon and shows 30% amino acid identity to the S. aureus AgrD-processing protein AgrB required for AIP modification and export [39]. The other *L. plantarum* genes associated with specific IL-10/IL-12 ratios are lp_2460 and lp_2480 coding for prophage R-Lp3 remnant proteins P2b protein 21 and 1, respectively [23]. These genes are conserved among *L. plantarum* strains stimulating high IL-10/IL-12 ratios in PBMCs. The functions of prophage R-Lp3 and other complete prophages in the *L. plantarum* WCFS1 genome are not known [42]. Because the different prophages found in *L. plantarum* WCFS1 share high levels of sequence homology and potential functional redundancy [42], these genes were not examined further.

Verification of the roles of the candidate genes in immunomodulation

To validate the influence of the candidate *L. plantarum* genes on PBMC cytokine responses, *lp_1953*, *pts19ADCBR*, *plnEFI*, and *plnG* deletion mutants were constructed for *L. plantarum* WCFS1. A previously constructed *L. plantarum* WCFS1 *lamA* (*lp_3580*) *lamR* (*lp_3087*) double mutant was used to examine the potential roles of the *lamBCDA QS-TCS* on PBMCs. This strain was selected because *lamA* and *lamR* encode the response regulators of the 2 TCS (*lamBCDA* and *lamKR*) regulating the expression of the *LamD* AIP in *L. plantarum* WCFS1 [40]. In the ΔlamA ΔlamR mutant, expression levels of lamB and the other genes in this operon were at 5% of the levels found in wild-type cells [40].

Wild-type and mutant *L. plantarum* WCFS1 cells harvested in the stationary- and exponential phases of growth were examined for their capacity to stimulate IL-10 and IL-12 in PBMCs. Overall, among the donors examined, IL-10 and IL-12 were produced in response to *L. plantarum* at levels between 500 to 4500 pg/ml and 3 to 68 pg/ml, respectively (shown as log2 values in Figure 2 and 3). Notably, exponential cultures of wild-type *L. plantarum* WCFS1 and most mutant strains stimulated PBMCs to secrete higher amounts of IL-10 and IL-12 than stationary-phase cells (Figure 2 and 3).

L. plantarum strains harboring the *plnEFI*, *plnG* or *lamB* loci were associated with the stimulation of lower IL-10/IL-12 ratios by *L. plantarum* in the PBMC assay (Table 2). In agreement with the gene-trait correlations, the *plnEFI*, *plnG*, and *lamA lamR* deletion mutants of strain WCFS1 induced higher IL-10/IL-12 ratios than the wild-type strain (Figure 4 and Table 3). However, the effects of the *plnEFI* deletion on cytokine induction in different donors was not highly significant compared to wild-type *L. plantarum* when the p value was adjusted for multiple hypothesis testing (adjusted (adj.) p value = 0.071) (Figure 4 and Table 3). Mutants deficient in the ABC- transporter *plnG* induced significantly higher cytokine ratios compared with *L. plantarum* wild-type cells (Figure 4 and Table 3). These differences were observed only for wild-type and mutant cells harvested during exponential phase growth (adj. p value = 0.005). Immunomodulation of the ΔlamA ΔlamR mutant was also substantially different compared to wild-type *L. plantarum* WCFS1. The ΔlamA ΔlamR mutant induced significantly higher IL-10/IL-12 ratios (adj. p value = 0.016) and IL-12 (adj. p value < 0.001) and IL-10 (adj. p value

< 0.001) amounts in PBMCs (Table 3). These effects were partially dependent on the growth-phase of the *L. plantarum* cells. IL-10/IL-12 ratios and IL-10 amounts induced by wild-type and mutant cells were significantly different when exponential phase cultures were used in the PBMC assay, whereas IL-10 and IL-12 amounts also differed when stationary-phase cells were examined (Figure 2, 3, 4 and Table 3).

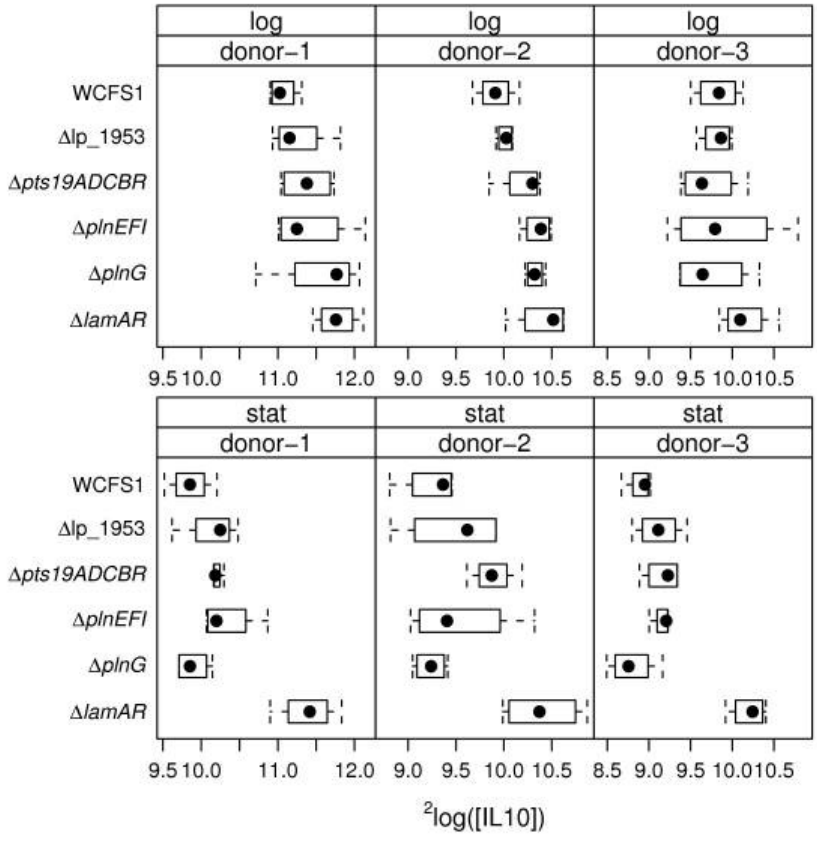

Figure 2. Boxplots of IL-10 amounts produced by PBMCs in response to *L. plantarum* wild-type and mutant cells. 2Log transformed IL-10 amounts induced by exponential and stationary phase *L. plantarum* cells are shown. The dots indicate the median value, the boxes indicate first and third quartile, and the whiskers extend to outlying data points for a total of 12 measurements (3 PBMC donors were measured using 4 replicate cultures of each *L. plantarum* strain).

In agreement with the gene trait matching correlations, the Δpst19ADCBR mutant induced significantly higher amounts of IL-10 than wild-type *L. plantarum* (adj. p value = 0.031) (Figure 2 and Table 3). Similarly, the IL-10/IL-12 ratio was significantly higher (p < 0.001) upon stimulation with *L. plantarum* Δpst19ADCBR compared with the parental

strain (Figure 4 and Table 3). *L. plantarum* strains harboring *lp_1953* were also predicted to induce higher IL-10 production levels compared with strains lacking this gene. However, the *L. plantarum lp_1953* deletion mutant stimulated equivalent amounts of IL-10 and somewhat higher IL-10/IL -12 ratios (adj. p value = 0.024) relative to wild-type *L. plantarum* WCFS1 (Figure 4 and Table 3). Although the *lp_1953* mutant induces a modest, yet significantly different, IL-10/IL-12 response relative to the parental strain, these results are not in agreement with the immunomodulatory effects predicted for this gene.

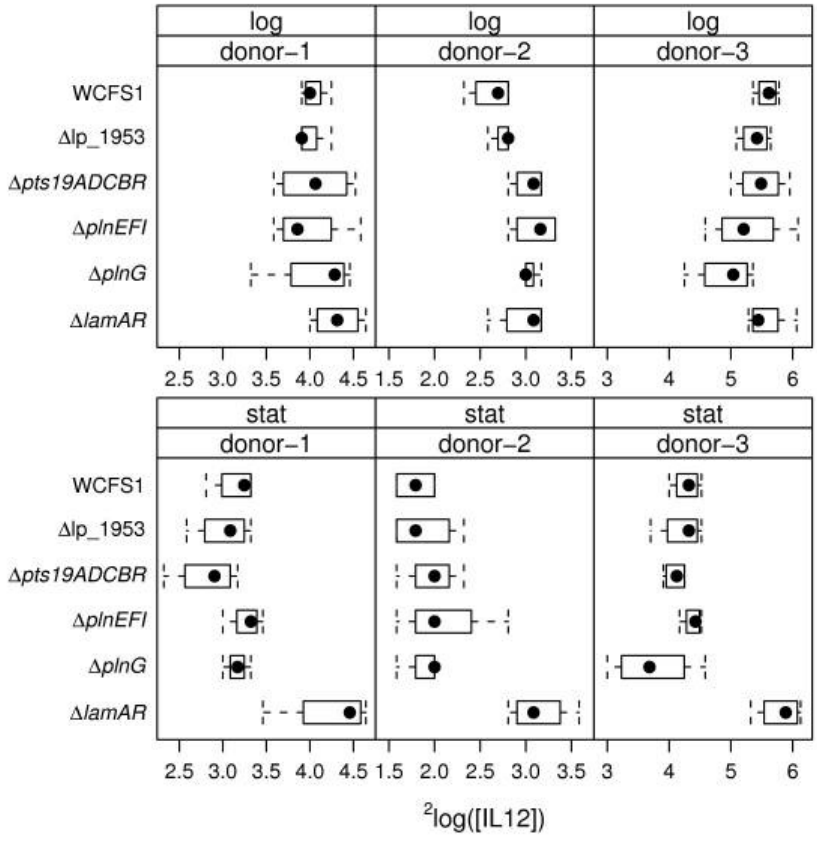

Figure 3. Boxplots of IL-12 amounts produced by PBMCs in response to *L. plantarum* wild-type and mutant cells. 2Log transformed IL-12 amounts induced by exponential and stationary phase *L. plantarum* cells are shown. The dots indicate the median value, the boxes indicate first and third quartile, and the whiskers extend to outlying data points for a total of 12 measurements (3 PBMC donors were measured using 4 replicate cultures of each *L. plantarum* strain).

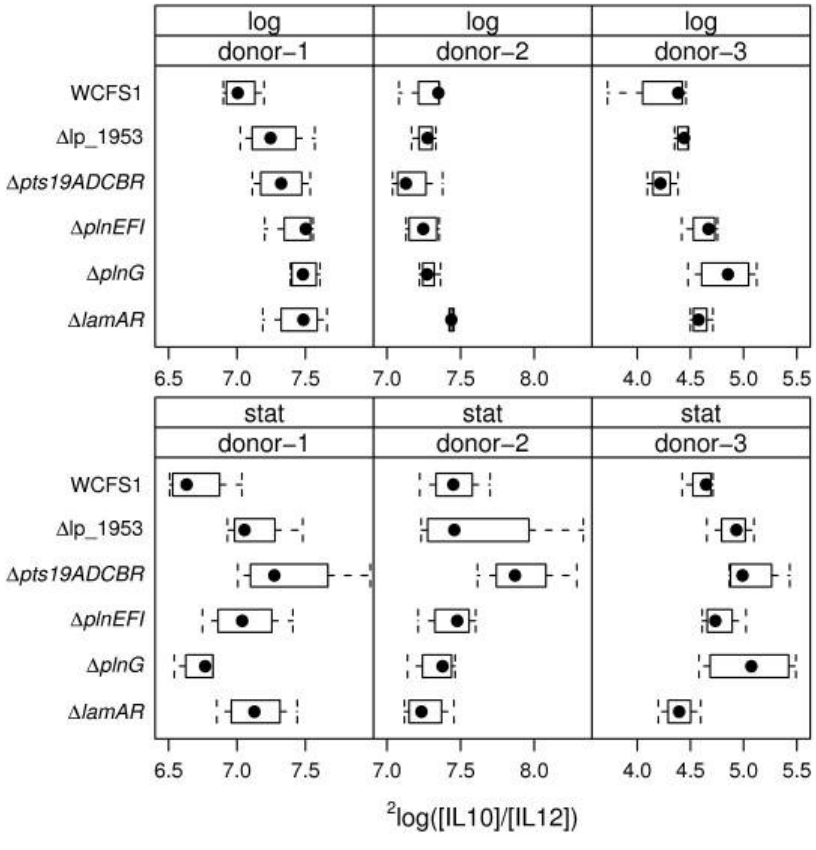

Figure 4. Boxplots of IL-10/IL-12 amounts produced by PBMCs in response to *L. plantarum* wild-type and mutant cells. 2Log transformed IL-10/IL -12 ratios induced by exponential and stationary phase *L. plantarum* cells are shown. The dots indicate the median value, the boxes indicate first and third quartile, and the whiskers extend to outlying data points for a total of 12 measurements (3 PBMC donors were measured using 4 replicate cultures of each *L. plantarum* strain).

Table 3. Relative differences in cytokine amounts between *L. plantarum* WCFS1 wild-type and deletion mutants.

mutant		IL-10 [c]				IL1-2				IL-10/IL-12			
comparison [a]	Growth phase [b]	value	p-value	adj. p-value		value	p-value	adj. p-value		value	p-value	adj. p-value	
lp_1953	log	0.097	0.461	0.830		-0.041	0.775	0.825		0.138	0.161	0.803	
	stat	0.253	0.057	0.228		-0.043	0.761	0.825		0.296	0.003	0.024	*
pts19ADCBR	log	0.164	0.216	0.647		0.106	0.458	0.825		0.058	0.556	0.923	
	stat	0.396	0.004	0.031	*	-0.131	0.371	0.825		0.529	0.000	0.000	***
plnEFI	log	0.287	0.031	0.176		0.032	0.825	0.825		0.255	0.010	0.071	
	stat	0.344	0.010	0.071		0.174	0.225	0.825		0.170	0.084	0.507	
plnG	log	0.280	0.035	0.176		-0.070	0.625	0.825		0.350	0.000	0.005	**
	stat	-0.028	0.830	0.830		-0.146	0.307	0.825		0.118	0.230	0.921	
lamA lam R	log	0.511	0.000	0.001	***	0.199	0.165	0.825		0.312	0.002	0.016	*
	stat	1.331	0.000	0.000	***	1.321	0.000	0.000	***	0.009	0.923	0.923	

a *L. plantarum* WCFS1 deletion mutant measured in the PBMC assay.
b Phase of growth from which *L. plantarum* cells were harvested (log = exponential phase; stat = stationary phase).
c The value is the average difference in 2Log cytokine amounts induced by wild-type *L. plantarum* and mutant cells harvested in the same phase of growth (log or stat). A positive value indicates an increase in IL-10 levels produced by PBMCs in response to mutant *L. plantarum* compared to the wild-type cells. Calculations of t-test p-values and adjusted (adj.) p-values are described in the text (Materials and Methods). * ($0.01 < p < 0.05$); ** ($0.002 < p < 0.01$); *** ($p < 0.002$) for the adj. p-values.

In summary, of the 5 mutants tested here, three (Δ*lamA* Δ*lamR*, Δ*pst19ADCBR*, and Δ*plnG*) significantly affected the immune response of PBMCs in different donors according to the phenotypes predicted from the gene-trait matching data (Table 2). The *plnEFI* mutant also affected the immune response in the predicted manner but this was not significant considering the adjusted p value. The Δ*lamA* Δ*lamR* mutant conferred the largest differences on the induction of IL-10 and IL-12 and the IL-10/IL-12 ratio by *L. plantarum* (Table 3).

Discussion

This study demonstrated the diverse capacities of *L. plantarum* strains to stimulate cytokine production in human PBMCs and confirmed the contributions of specific *L. plantarum* genes to modulate these responses. Forty-two *L. plantarum* strains induced PBMCs to secrete IL-10 over an average 14-fold range. This range was similar to IL-10 amounts stimulated by 7 *Bifidobacterium longum* strains [43] and the 10 to 15-fold differences in cytokine amounts induced in PBMCs by multiple *Lactobacillus* and *Bifidobacterium* species [7-11]. Moreover, we found that variation in IL-10 and IL-12 amounts and IL-10/IL-12 ratios induced by the distinct *L. plantarum* strains was higher than reported previously [44]. This result was probably due to the analysis of more strains in the present study (42 versus 3), which were isolated from diverse environmental niches encompassing a greater genetic and phenotypic diversity of the *L. plantarum* species. Such strain-specific differences should therefore be taken into consideration when selecting a probiotic *Lactobacillus* culture for health conditions which are dependent on modulating immunity such as in the prevention of allergy, eczema, or inflammatory bowel disease.

To identify *L. plantarum* genes with roles in modulating immune cell responses, *L. plantarum* genetic diversity was correlated with strain-specific capacities to induce cytokines in PBMCs. Genes with putative contributions to the observed PBMC responses were further investigated in *L. plantarum* WCFS1. A similar gene-trait matching approach previously resulted in the identification of a *L. plantarum* mannose-specific adhesin (Msa) [45] and genes which modulate dendritic cell responses [46]. Although the gene-trait matching approach has been successful, it should be recognized that only a subset of immunomodulatory cell components produced by *L. plantarum* was likely identified here. Firstly, the identified immunomodulatory genetic loci were restricted to genes in the *L. plantarum* WCFS1 reference strain genome. Secondly, genes with high levels of sequence conservation such that they are not distinguished by CGH (presence versus absence, rather than minor sequence variations) might be excluded from detection. For example, *L. plantarum* has highly conserved LTA biosynthesis and modification genes known to have established effects on mammalian immunity were not found in this biodiversity-based gene-trait matching approach. Finally, genetic assessments do not take into account strain-specific variations in gene expression, translation, or post-translational modification of proteins with immunomodulatory effects.

Despite these limitations and the considerable variation in the production of cytokines by PBMCs from different donors, the present study demonstrated that gene-trait matching is also suitable for the identification of genes that affect cytokine levels in the mixture of immune cells collectively termed PBMCs. The products of AIP-based QS-TCSs and the N-acetyl-galactosamine/glucosamine phosphotransferase system identified here might constitute a new class of bacterial cell products which are recognized by host receptors. The findings are significant because these genes were identified using intact cells which likely have multiple interactions with immune cells such that single genes only confer incremental effects.

L. plantarum WCFS1 LamB, a processing/export protein of the AIP-based QS-TCS LamBDCA [47], was correlated with immunomodulation of PBMCs. LamB, a transmembrane protein, is under the control of two response regulators LamA and LamR [40]. A *L. plantarum ΔlamA ΔlamR* mutant investigated in this study was found to induce PBMCs to secrete significantly higher amounts of the cytokines IL-10 and IL-12. In a previous report, global transcript profiling of the *lamA lamR* deletion mutant showed that the *lamBDCA* system is auto-regulated and controls the production of several surface-associated proteins, stress-associated functions, and surface polysaccharides [40]. Higher amounts of surface polysaccharides produced by *L. plantarum ΔlamA ΔlamR* decreased the biofilm-forming capacity of the mutant strain [40]. Polysaccharides produced by some *Lactobacillus* species are known for their immunomodulatory effects either by direct interactions with immune cells or by shielding MAMPs on the bacterial cell surface from detection by the immune system [18,48,49]. Therefore the observed PBMC IL-10/IL-12 ratios for *L. plantarum* might either be mediated directly through the LamBDCA system and the cognate secreted peptide, or indirectly through cell products (e.g., polysaccharides) under the control of this regulatory system. The latter is supported by the genetic similarities between LamBDCA and the *Staphylococcus aureus* agr system, an AIP-based QS-TCS which controls the evasion of innate host defenses by S. aureus through the production of secreted peptides and proteases [50].

The plantaricin biosynthesis pathway of *L. plantarum* WCFS1 is also controlled by an AIP-based QS-TCS [47] and genes required for plantaricin production and transport contributed to *L. plantarum* effects on PBMCs. Plantaricin is a bacteriocin composed of two small secreted peptides (PlnE and PlnF) which destabilize the integrity of the plasma membrane of susceptible cells [51]. *L. plantarum* strains harboring *plnEF* and *plnI* encoding a plantaricin immunity protein, and/or *plnG* encoding a membrane bound ABC-transporter induced PBMCs to secrete IL-10 and IL-12 in amounts that yielded lower IL -10/IL-12 ratios (Table 2). Similarly, wild-type *L. plantarum* WCFS1 conferred lower IL-10/IL-12 ratios compared to the *plnEFI* and *plnG* deletion mutants, although this was significant only for the *plnG* mutant ($p = 0.005$) and not the mutant lacking *plnEFI* ($p = 0.071$). The identification of the AIP plantaricin is intriguing because human antimicrobial peptides such as defensins secreted in the gut are known to modulate immune responses [52,53] and suggest that antimicrobial peptides of bacterial origin might have similar capacities. These findings are also compatible with a recent study showing that plantaricins can modulate dendritic cell responses [46]. Moreover, several independent studies showed that *L. plantarum* WCFS1 genes involved in plantaricin biosynthesis and activity, including *plnI* and *plnF*, are induced in the mouse gut [30-32], thereby indicating that plantaricin production is active in the intestine where it might come into contact with mucosal immune cells.

Another of the confirmed genes with immunomodulatory capacities was the *pts19ADCBR* locus coding for a cell membrane-associated N-acetyl-galactosamine/glucosamine phosphotransferase system. The relevance of the *pts19ADCBR* genes in adaptation to the intestinal ecosystem was also demonstrated by their higher expression levels in the

intestine of conventionally-raised and germ-free mice [31,32]. Moreover, in *Lactobacillus johnsonii*, a putative mannose phosphotransferase gene locus with 43% amino acid identity to the *L. plantarum* WCFS1 *pts19ADCBR* cluster was found to be important for long term persistence *in vivo* [54]. Although the regulatory signals for expression of these genes are unknown, immunomodulatory effects conferred by Pts19ADCBR might influence the ability of *L. plantarum* to modify the intestinal environment for survival in the gut.

Cytokine profiles of the *lp_1953* deletion mutant were not in agreement with the IL-10 stimulating capacity predicted for this gene by gene-trait matching. This result exemplifies the need for mutation analysis to confirm gene-trait predictions, which are likely to encompass false-positive associations. A similar conclusion was drawn during the identification of the *L. plantarum* Msa gene [45]. Moreover, the product of *lp_1953* is predicted to be intracellular, which contrasts the predicted subcellular location of all other genes examined here (secreted or cell envelope associated) [24,25]. This finding supports the notion that surface-localized proteins or components are the most likely candidate-participants in host-microbe interactions [49,55]. Thus far, the majority of the known immunomodulating MAMPs known for lactobacilli are extracellular or cell surface associated products such as LTA, exopolysaccharides, and peptidoglycan, although intracellular CpG-containing oligodeoxynucleotides (ODNs) produced by some lactobacilli are able to induce IL-10 production in immune cells [21,49]. These MAMPs are recognized by specific pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD)-like receptors [21]. To identify the mechanisms underlying the effects of AIP-based QS-TCSs and the N-acetylgalactosamine/glucosamine phosphotransferase system on immune cells, the cellular products encoded by the genes in these pathways should be investigated to identify the specific cell types among the PBMCs, which include lymphocytes, monocytes and macrophages, that recognize these compounds as well as the specific mechanisms leading to altered cytokine production.

Comparisons of mutant and wild-type *L. plantarum* WCFS1 cells included examination of the effects of culture growth phase on the stimulation of PBMCs. Exponential- and stationary-phase *L. plantarum* WCFS1 cultures were evaluated because the growth phase of probiotic cells was previously shown to influence the immune responses to probiotic bacteria *in vitro* [56-59] and *in vivo* [35]. Using human PBMCs, we found significant growth-phase dependent differences in the immunomodulatory capacities of the wild-type and mutant *L. plantarum* cultures. Collectively, the exponential-phase *L. plantarum* WCFS1 cultures stimulated higher absolute amounts of IL-10 and IL-12 and hence appear to induce heightened immune responses by PBMCs compared with stationary-phase cells. Notably, this result was not due to extensive *L. plantarum* growth because antibiotics were added to the PBMC growth medium to prevent bacterial overgrowth which would generate artifacts from acidification of the medium causing PBMC cell stress or death. Moreover, intact and lysed *L. plantarum* strains cells collected from the exponential and stationary phase of growth do not show striking differences in their

TLR-9 signaling activity and there was not a clear trend among all strains tested (personal observation, M. Meijerink and J. M. Wells). Therefore the higher amounts of cytokines induced by exponential phase bacteria are unlikely to be caused by differential cell lysis resulting in the release of intracellular CpG DNA, a known MAMP recognized by TLR-9.

Comparisons of wild-type and mutant *L. plantarum* cultures also showed growth-phase dependent effects. The IL-10 amounts and IL-10/IL-12 ratios induced by the *pts19ADCBR* deletion mutant were significantly different from wild-type *L. plantarum* WCFS1 for only the stationary-phase cultures. Stationary-phase cells of the *ΔlamA ΔlamR* mutant also induced significantly higher amounts of IL-10 and IL-12 in compared with *L. plantarum* WCFS1 harvested at the same growth phase. However, differences between IL-10/IL-12 ratios induced by *ΔlamA ΔlamR* and wild-type cell differed only for exponential phase cultures. This result might have been partially due to the extensive alterations in expression of *L. plantarum ΔlamA ΔlamR* in actively growing cultures [39], such that differences in expression of *lamBDCA* and *lamKR* regulated genes might have influenced the ability of the exponential-phase *L. plantarum* cells to stimulate different PBMC IL -10/IL-12 ratios. A similar result was found for the comparisons of *L. plantarum plnG* (and *plnEFI*), the other 2 TCS system examined, although the specific growth-phase-dependent modifications of the plantaricin system on cytokine production in PBMCs is not presently known.

Conclusions

The present study compared the genetic and phenotypic diversity of *L. plantarum* WCFS1 to identify cell components of this species with the capacity to modulate human PBMC responses. We successfully identified several *L. plantarum* WCFS1 genes that are associated with the production of IL-10 and the IL-10/IL-12 ratio by PBMCs and established that the immune response to *L. plantarum* can be significantly altered by the deletion of specific *L. plantarum* cell surface proteins. The increased IL-10/IL-12 ratios of the *L. plantarum* mutants indicate that these cultures would be more protective against intestinal inflammation compared with wild-type cells. These effects might be mediated by the down-regulation of local inflammatory responses through various subsets of T cells producing a collection anti-inflammatory cytokines. As a result of this study, strain selection for protection against intestinal inflammation might include screening for strains lacking the LamB, PlnG, or Pts19 homologs or by modifying culture growth conditions or food delivery matrices to minimize the expression of these genes *in vivo*. Such studies are required to distinguish between health effects conferred by individual probiotic strains and to develop methods to ensure that probiotic cells express host-modulatory cell products at the appropriate level and time in food products and the human gut.

Methods

Bacterial strains

Immune assays and genetic analysis was performed on a total of 42 *L. plantarum* strains with distinct phenotypic profiles [27,28] (Table 1). Comparative genome hybridization (CGH) of these strains was performed previously [27,28]. For immunoprofiling, the *L. plantarum* strains were grown at 37°C in de Man Rogosa Sharpe (MRS) broth (Merck, Darmstadt, Germany) until mid-exponential (optical density (OD) 600 nm = 1) phase or stationary phase (24 h after the start of the culture, OD600 nm = 7.6 ± 1.1) [60]. The exponential and stationary phase cells were washed twice in phosphate buffered saline (PBS) at pH 7.4, suspended at 2×10^8 cells/ml in PBS containing 20% glycerol, and stored at -80°C until co-culturing with human immune cells. Quantification of the exponential and stationary phase viable cells before and after freezing showed no significant losses in cell viability (data not shown). Colony forming units (CFUs) were determined by plating serial dilutions of the cultures on MRS agar (data not shown).

Peripheral blood mononuclear cells assay

This study was approved by Wageningen University Ethical Committee and was performed according to the principles of the Declaration of Helsinki. Peripheral blood of healthy donors was from the Sanquin Blood Bank, Nijmegen, The Netherlands. Before sample collection, a written informed consent was provided. Peripheral blood mononuclear cells (PBMCs) were separated from the blood of healthy donors using Ficoll-Paque Plus gradient centrifugation according to the manufacturer's protocol (Amersham biosciences, Uppsala, Sweden). After centrifugation the mononuclear cells were collected, washed in Iscove's Modified Dulbecco's Medium (IMDM) + glutamax (Invitrogen, Breda, The Netherlands) and adjusted to 1×10^6 cells/ml in IMDM + glutamax supplemented with penicillin (100 U/ml) (Invitrogen), streptomycin (100 µg/ml) (Invitrogen), and 1% human AB serum (Lonza, Basel, Switzerland). PBMCs (1×10^6 cells/well) were seeded in 48-well tissue culture plates. After an overnight rest at 37°C in 5% CO^2, 5 µl aliquots of thawed bacterial suspensions at 2×10^8 CFU/ml were added to the PBMCs (*L. plantarum*: PBMC ratio of 1:1). PBS (5 µl) and LPS (1 µg) served as negative (PBS) and positive (LPS, TLR-4 ligand) controls for the stimulation of PBMCs. IL-10 was produced in sufficient amounts for quantification in response to LPS but not to PBS. Similarly, neither LPS nor the PBS buffer stimulated the production of IL-12. To test the capacity of the 42 *L. plantarum* strains to stimulate PBMC cytokine production, PBMCs from 3 different donors were examined (donors A, B, and C). For donors A and B, separate stationary-phase cultures of each *L. plantarum* strain were used. For donor C, both replicate cultures of each *L. plantarum* strain were examined. In PBMC assays comparing responses to *L. plantarum* WCFS1 wild-type and mutant strains, PBMCs from 3 different donors were examined using 4 independent replicate wild-type and mutant *L. plantarum* cultures harvested during exponential-phase and stationary-phase of growth.

Following 24 hr incubation at 37°C in 5% CO^2, culture supernatants were collected and stored at -20°C until cytokine analysis. This time point was selected for analysis because previous studies showed that IL-12 levels remain unaltered after 4 days of *L. plantarum* incubation with PBMCs. Although IL-10 was shown to increase 2- fold after 4 days of co-incubation with *L. plantarum*, sufficient cytokine amounts were produced after 24 h to permit flow cytometric measurements [61]. No viable bacteria could be cultured and medium acidification was not observed after incubation of *L. plantarum* strains with the PBMCs for 24 h (data not shown). Cytokines were measured using a FACS CantoII flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and BD Cytometric Bead Array Flex-sets (BD Biosciences) for IL-10 and IL-12p70 (in chapter 4 referred to as IL-12) according to the manufacturer's recommendations. Detection limits were 0.13 and 0.6 pg/ml for IL-10 and IL-12, respectively. Concentrations of analytes were calculated with the use of known standards and plotting the sample values against a standard curve in the BD Biosciences FCAP software. Donor-specific variation in cytokine production capacities was taken into account by dividing the cytokine amounts induced by individual *L. plantarum* strains against average cytokine quantities induced by all *L. plantarum* strains for the same donor. These values were then compared to amounts induced by *L. plantarum* WCFS1 and used for gene-trait matching.

Identification of candidate genes involved in cytokine secretion by gene-trait matching

L. plantarum genes with potential roles in modulation of PBMC cytokine production were identified by *in silico* matching using genotype information referenced from the *L. plantarum* WCFS1 genome (also termed gene-trait matching) [45]. Individual *L. plantarum* WCFS1 gene presence or absence scores for the 42 strains were used as putative predictor variables for PBMC induced IL-10, IL-12 and IL-10/IL-12 amounts by regression using the Random Forest algorithm [38]. The "RandomForest" package for R [62] was used with standard parameter settings. *L. plantarum* WCFS1 genes with the highest variable importance measures by the Random Forest method were selected for deletion analysis.

Construction of L. plantarum WCFS1 gene deletion mutants

A previously described *L. plantarum ΔlamA ΔlamR* mutant was used in this study [40]. Construction of *L. plantarum lp_1953*, *lp_2647-2651*, *lp_0419-0422* and *lp_0423* gene deletion mutants was performed as previously described [63] with several modifications. The mutagenesis vectors were generated by a splicing by overlap extension (SOE) procedure [64]. This procedure was designed to expedite mutagenesis vector construction for *L. plantarum* using a single step, blunt-ended cloning and positive selection for transformants based on chloramphenicol resistance. PCR was used to amplify approximately 1 kb of the 5' and 3' regions flanking the genes targeted for deletion (for primer sequences see Table 4). In addition, the *loxP-cat-loxP* region of pNZ5319 was amplified using primers Ecl-loxR and Pml-loxF (Table 4). For each mutagenesis vector,

the amplicons representing the corresponding 2 flanking regions and the *loxP-cat-loxP* region were mixed in a 1:1:1 molar ratio and used as template in a second PCR reaction with the 5'forward and 3'reverse flanking primers. These PCR reactions resulted in 3 kb amplicons which were cloned into the integration vector pNZ5319 [63] after prior digestion of the vector with SwaI and Ecl136II. Plasmids were transformed into competent cells of *E. coli* JM109 by electroporation as recommended by the manufacturer (Invitrogen). Plasmid DNA was isolated from *E. coli* using Jetstar columns (Genomed GmbH, Bad Oeynhausen, Germany) using the manufacturer's recommended protocol. DNA sequencing (BaseClear, Leiden, The Netherlands) was performed to confirm the integrity of the cloned genes. The resulting plasmids containing the complete gene replacement cassettes were used for mutagenesis [63].

Primer	Sequence[a]
LF1953F	5'- TGCCGCATACCGAGTGAGTAG-3'
LF1953R	5'-**CGAACGGTAGATTTAAATTGTTT**ATCAAAAAACACCGTTAATTTGCATC-3'
RF1953F	5'-**GTACAGCCCGGGCATGAG**CGTGGCCATTAGTTGACGAGAC-3'
RF1953R	5'-AACGCCATCGCACTGATGCATC-3'
EcI-loxR	5'-AAACAATTTAAATCTACCGTTCG-3'
PmI-loxF	5'-CTCATGCCCGGGCTGTAC-3'
LF1953F2	5'-GCAACGGCTGTCAGTAACCTGCCTTC-3'
RF1953R2	5'-TCAAATCTCGAAGCGGTTCAAAACTG-3'
LF2647F	5'-**GTACAGCCCGGGCATGAG**GGTATTTAGCGAAATATACAGATTG-3'
LF2647R	5'-CTTTAGCCGTCTCATTAGTCG-3'
RF2651F	5'-GGATTACCAAAACGAACATGG-3'
RF2651R	5'-**CGAACGGTAGATTTAAATTGTTT**ACTAGCCATTTTGTTTTTATCTCC-3'
LF2647R2	5'-TGACATGACTATCCTGACTTGC-3'
RF2651F2	5'-AACGTTCAACGGCAGATAAGCC-3'
LF423F	5'-AATTGATACATGTGGTTTCGAAAG-3'
LF423R	5'-**CGAACGGTAGATTTAAATTGTTT**CCAATGCATACTTGTACTCCC-3'
RF423F	5'-**GTACAGCCCGGGCATGAG** CGACTTGATCAATAGCTGAGGG-3'
RF423R	5'-TTGGTTGCCTTGATCGTGTAAG-3'
LF423F2	5'-CTTCAGTTATCGCTACAATCAACG-3'
RF423R2	5'-ACTAACGTACTTTGCACCACGG-3'
LF419F	5'-**GTACAGCCCGGGCATGAG**GACGAGTAATCATCCATTCTGA-3'
LF419R	5'-ATGAGTTTGCAATGGAGCTTAGG-3'
RF422F	5'-CAAAGACGTGCCGAATATAGCC-3'
RF422R	5'-**CGAACGGTAGATTTAAATTGTTT**AAACTGTAGCATAAATAATCCCC-3'
LF419R2	5'-GAGATAATTATTGTAAGACCGTC-3'
RF422F2	5'-CTAACGCATCAATAATCTTACTGG-3'

[a] Bold and underlined nucleotides signify overlapping ends with the EcI-loxR and PmI-loxF primers.

Statistical analysis

Linear mixed effect models using restricted maximum likelihood (REML) were used to statistically compare the mean cytokine values of IL-10, IL-12, and IL-10/IL-12 produced in response to *L. plantarum* wild-type and mutant cells. The effect of the donor on the response variable was modeled as a random effect. The fixed effects in the model were the strains (WCFS1 [wild type], *Δpts19ADCBR*, *Δlp_1953*, *ΔplnG*, *ΔplnEFI*, and *ΔlamA ΔlamR*) and the growth phase at the time of harvest (exponential phase and station-

ary phase). Logarithmic transformations of [IL-10], [IL-12] and [IL-10]/[IL-12] yielded residuals that showed approximately normal distributions (data not shown) and, hence, were used as the response variables in the fitting procedure. Statistical analysis was performed using R http://www.r-project.org webcite, with the package "nlme" [65] for mixed effect modeling. The donor random effect was modeled as a constant offset relative to the average level of the response variable ("model 1"), or alternatively, as a donor-dependent offset plus a donor-dependent variation in the effect of the bacterial growth phase on the response variable ("model 2"). Model 2 yielded better fits for 2log([IL-10]) and 2log([IL-10]/[IL-12]) response variables whereas, indications of a donor dependent variation in growth phase effects were not found for the 2log([IL-12]) response, and hence model 2 was applied for comparison of these cytokine amounts. The resulting relative difference coefficients and t tests were calculated from the fixed effects (mutation, growth phase, and their interaction) using analysis of variance in R. The p-values were adjusted for multiple hypothesis testing using the correction procedures by Hochberg [66].

Acknowledgements
We would like to thank Nico Taverne for his assistance with the immune assays. This work was funded by TI Food & Nutrition, Wageningen, The Netherlands.

References

1. Neish AS: Microbes in gastrointestinal health and disease. Gastroenterology 2009, 136(1):65-80.
2. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, Sogin ML, Jones WJ, Roe BA, Affourtit JP, et al.: A core gut microbiome in obese and lean twins. Nature 2009, 457(7228):480-484.
3. Sanders ME, Marco ML: Food formats for effective delivery of probiotics. Ann Rev Food Sci Technol 2010, 1:65-85.
4. Floch MH, Walker WA, Guandalini S, Hibberd P, Gorbach S, Surawicz C, Sanders ME, Garcia-Tsao G, Quigley EM, Isolauri E, et al.: Recommendations for probiotic use--2008. J Clin Gastroenterol 2008, 42(Suppl 2):S104-108.
5. Sanders ME: Probiotics: Considerations for human health. Nut Rev 2003, 61(3):91-99.
6. Marco ML, Pavan S, Kleerebezem M: Towards understanding molecular modes of probiotic action. Curr Opin Biotechnol 2006, 17(2):204-210.
7. Borchers AT, Selmi C, Meyers FJ, Keen CL, Gershwin ME: Probiotics and immunity. J Gastroenterol 2009, 44(1):26-46.
8. Niers LEM, Timmerman HM, Rijkers GT, van Bleek GM, van Uden NOP, Knol EF, Kapsenberg ML, Kimpen JLL, Hoekstra MO: Identification of strong interleukin-10 inducing lactic acid bacteria which down-regulate T helper type 2 cytokines. Clin Exp Allergy 2005, 35(11):1481-1489.
9. Miettinen M, VuopioVarkila 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(12):5403-5405.
10. Foligne B, Nutten S, Grangette C, Dennin V, Goudercourt D, Poiret S, Dewulf J, Brassart D, Mercenier A, Pot B: Correlation between *in vitro* and *in vivo* immunomodulatory properties of lactic acid bacteria. World J Gastroenterol 2007, 13(2):236-243.
11. Miettinen M, Matikainen S, Vuopio-Varkila J, Pirhonen J, Varkila K, Kurimoto M, Julkunen I: Lactobacilli and streptococci induce interleukin-12 (IL-12), IL-18, and gamma interferon production in human peripheral blood mononuclear cells. Infect Immun 1998, 66(12):6058-6062.
12. Kwon HK, Lee CG, So JS, Chae CS, Hwang JS, Sahoo A, Nam JH, Rhee JH, Hwang KC, Im SH: Generation of regulatory dendritic cells and CD4+Foxp3+ T cells by probiotics administration suppresses immune disorders. Proc Natl Acad Sci USA 2010, 107(5):2159-2164.
13. Karczewski J, Troost FJ, Konings I, Dekker J, Kleerebezem M, Brummer RJ, Wells JM: Regulation of human epithelial tight junction proteins by *Lactobacillus plantarum in vivo* and protective effects on the epithelial barrier. Am J Physiol Gastrointest Liver Physiol 2010, 298(6):G851-859.
14. Kim HG, Gim MG, Kim JY, Hwang HJ, Ham MS, Lee JM, Hartung T, Park JW, Han SH, Chung DK: Lipoteichoic acid from *Lactobacillus plantarum* elicits both the production of interleukin-23p19 and suppression of pathogen-mediated interleukin-10 in THP-1 cells. FEMS Immunol Med Microbiol 2007, 49(2):205-214.
15. Ryu YH, Baik JE, Yang JS, Kang SS, Im J, Yun CH, Kim DW, Lee K, Chung DK, Ju HR, et al.: Differential immunostimulatory effects of Gram-positive bacteria due to their lipoteichoic acids. Int Immunopharmacol 2009, 9(1):127-133.
16. Matsuguchi T, Takagi A, Matsuzaki T, Nagaoka M, Ishikawa K, Yokokura T, Yoshikai Y:

Lipoteichoic acids from *Lactobacillus* strains elicit strong tumor necrosis factor alpha-inducing activities in macrophages through Toll-like receptor 2. Clin Diagn Lab Immunol 2003, 10(2):259-266.
17. 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(2):562-575.
18. Yasuda E, Serata M, Sako T: Suppressive effect on activation of macrophages by *Lactobacillus casei* strain Shirota genes determining the synthesis of cell wall-associated polysaccharides. Appl Environ Microbiol 2008, 74(15):4746-4755.
19. Konstantinov SR, Smidt H, de Vos WM, Bruijns SC, Singh SK, Valence F, Molle D, Lortal S, Altermann E, Klaenhammer TR, et al.: S layer protein A of *Lactobacillus acidophilus* NCFM regulates immature dendritic cell and T cell functions. Proc Natl Acad Sci USA 2008, 105(49):19474-19479.
20. Kleerebezem M, Hols P, Bernard E, Rolain T, Zhou M, Siezen RJ, Bron PA: The extracellular biology of the lactobacilli. FEMS Microbiol Rev 2010, 34(2):199-230.
21. Lebeer S, Vanderleyden J, De Keersmaecker SC: Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. Nat Rev Microbiol 2010, 8(3):171-184.
22. de Vries MC, Vaughan EE, Kleerebezem M, de Vos WM: *Lactobacillus plantarum*-survival, functional and potential probiotic properties in the human intestinal tract. Int Dairy J 2006, 16(9):1018-1028.
23. Kleerebezem M, Boekhorst J, van Kranenburg R, Molenaar D, Kuipers OP, Leer R, Tarchini R, Peters SA, Sandbrink HM, Fiers M, et al.: Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc Natl Acad Sci USA 2003, 100(4):1990-1995.
24. Boekhorst J, Wels M, Kleerebezem M, Siezen RJ: The predicted secretome of *Lactobacillus plantarum* WCFS1 sheds light on interactions with its environment. Microbiology 2006, 152(11):3175-3183.
25. Zhou M, Boekhorst J, Francke C, Siezen RJ: LocateP: genome-scale subcellular-location predictor for bacterial proteins. BMC Bioinformatics 2008, 9:173.
26. Teusink B, van Enckevort FHJ, Francke C, Wiersma A, Wegkamp A, Smid EJ, Siezen RJ: In silico reconstruction of the metabolic pathways of *Lactobacillus plantarum*: Comparing predictions of nutrient requirements with those from growth experiments. Appl Environ Microbiol 2005, 71(11):7253-7262.
27. Molenaar D, Bringel F, Schuren FH, de Vos WM, Siezen RJ, Kleerebezem M: Exploring *Lactobacillus plantarum* genome diversity by using microarrays. J Bacteriol 2005, 187(17):6119-6127.
28. Siezen RJ, Tzeneva VA, Castioni A, Wels M, Phan HTK, Rademaker JLW, Starrenburg MJC, Kleerebezem M, Molenaar D, van Hylckama Vlieg JET: Phenotypic and genomic diversity of *Lactobacillus plantarum* strains isolated from various environmental niches. Environmental Microbiology 2010, 12(3):758-773.
29. Vesa T, Pochart P, Marteau P: Pharmacokinetics of *Lactobacillus plantarum* NCIMB 8826, *Lactobacillus fermentum* KLD and *Lactococcus lactis* MG 1363 in the human gastrointestinal tract. Aliment Pharmacol Ther 2000, 14(6):823-828.
30. Marco ML, Bongers RS, de Vos WM, Kleerebezem M: Spatial and temporal expression of *Lactobacillus plantarum* genes in the gastrointestinal tracts of mice. Appl Environ Microbiol 2007, 73(1):124-132.
31. Bron PA, Grangette C, Mercenier A, de Vos WM, Kleerebezem M: Identification of *Lactobacillus plantarum* genes that are induced in the gastrointestinal tract of mice. J Bacteriol 2004, 186(17):5721-5729.

32. Marco ML, Peters THF, Bongers RS, Molenaar D, Van Hemert S, Sonnenburg JL, Gordon JI, Kleerbezem M: Lifestyle of *Lactobacillus plantarum* in the mouse cecum. Environ Microbiol 2009, 11(10):2747-2757.
33. Bron PA, Meijer M, Bongers RS, de Vos WM, Kleerebezem M: Dynamics of competitive population abundance of *Lactobacillus plantarum* ivi gene mutants in faecal samples after passage through the gastrointestinal tract of mice. J Appl Microbiol 2007, 103(5): 1424-1434.
34. Marco ML, de Vries MC, Wels M, Molenaar D, Mangell P, Ahrne S, de Vos WM, Vaughan EE, Kleerebezem M: Convergence in probiotic *Lactobacillus* gut-adaptive responses in humans and mice. ISME J 2010, 4(11):1481-4.
35. van Baarlen P, Troost FJ, van Hemert S, van der Meer C, de Vos WM, de Groot PJ, Hooiveld GJ, Brummer RJ, Kleerebezem M: Differential NF-kappaB pathways induction by *Lactobacillus plantarum* in the duodenum of healthy humans correlating with immune tolerance. Proc Natl Acad Sci USA 2009, 106(7):2371-2376.
36. Troost FJ, van Baarlen P, Lindsey P, Kodde A, de Vos WM, Kleerebezem M, Brummer RJ: Identification of the transcriptional response of human intestinal mucosa to *Lactobacillus plantarum* WCFS1 *in vivo*. BMC Genomics 2008, 9:374.
37. Yaqoob P, Newsholme EA, Calder PC: Comparison of cytokine production in cultures of whole human blood and purified mononuclear cells. Cytokine 1999, 11(8):600-605.
38. Breiman L: Random forests. Machine Learning 2001, 45(1):5-32.
39. Sturme MH, Nakayama J, Molenaar D, Murakami Y, Kunugi R, Fujii T, Vaughan EE, Kleerebezem M, de Vos WM: An agr-like two-component regulatory system in *Lactobacillus plantarum* is involved in production of a novel cyclic peptide and regulation of adherence. J Bacteriol 2005, 187(15):5224-5235.
40. Fujii T, Ingham C, Nakayama J, Beerthuyzen M, Kunuki R, Molenaar D, Sturme M, Vaughan E, Kleerbezem M, de Vos W: Two homologous agr-like quorum sensing systems co-operatively control adherence, cell morphology, and cell viability properties in *Lactobacillus plantarum* WCFS1. J Bacteriol 2008, 190(23):7655-7665.
41. Diep DB, Havarstein LS, Nes IF: Characterization of the locus responsible for the bacteriocin production in *Lactobacillus plantarum* C11. J Bacteriol 1996, 178(15):4472-4483.
42. Ventura M, Canchaya C, Kleerebezem M, de Vos WM, Siezen RJ, Brussow H: The prophage sequences of *Lactobacillus plantarum* strain WCFS1. Virology 2003, 316(2):245-255.
43. 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(3):531-538.
44. Wang B, Li J, Li Q, Zhang H, Li N: Isolation of adhesive strains and evaluation of the colonization and immune response by *Lactobacillus plantarum* L2 in the rat gastrointestinal tract. Int J Food Microbiol 2009, 132(1):59-66.
45. Pretzer G, Snel J, Molenaar D, Wiersma A, Bron PA, Lambert J, de Vos WM, van der Meer R, Smits MA, Kleerebezem M: Biodiversity-based identification and functional characterization of the mannose-specific adhesin of *Lactobacillus plantarum*. Journal of Bacteriology 2005, 187(17):6128-6136.
46. Meijerink M, van Hemert S, Taverne N, Wels M, de Vos P, Bron PA, Savelkoul HF, van Bilsen J, Kleerebezem M, Wells JM: Identification of genetic loci in *Lactobacillus plantarum* that modulate the immune response of dendritic cells using comparative genome hybridization. PLoS One 2010, 5(5):e10632.
47. Sturme MH, Francke C, Siezen RJ, de Vos WM, Kleerebezem M: Making sense of quorum sensing in lactobacilli: a special focus on *Lactobacillus plantarum* WCFS1. Microbiology 2007, 153(Pt 12):3939-3947.

48. Vinderola G, Perdigon G, Duarte J, Farnworth E, Matar C: Effects of the oral administration of the exopolysaccharide produced by *Lactobacillus kefiranofaciens* on the gut mucosal immunity. Cytokine 2006, 36(5-6):254- 260.
49. Lebeer S, Vanderleyden J, De Keersmaecker SC: Genes and molecules of lactobacilli supporting probiotic action. Microbiol Mol Biol Rev 2008, 72(4):728-764.
50. Kong KF, Vuong C, Otto M: *Staphylococcus* quorum sensing in biofilm formation and infection. Int J Med Microbiol 2006, 296(2-3):133-139.
51. Diep DB, Straume D, Kjos M, Torres C, Nes IF: An overview of the mosaic bacteriocin pln loci from *Lactobacillus plantarum*. Peptides 2009, 30(8):1562-1574.
52. Yang D, Biragyn A, Hoover DM, Lubkowski J, Oppenheim JJ: Multiple roles of antimicrobial defensins, cathelicidins, and eosinophil-derived neurotoxin in host defense. Annu Rev Immunol 2004, 22:181-215.
53. Funderburg N, Lederman MM, Feng Z, Drage MG, Jadlowsky J, Harding CV, Weinberg A, Sieg SF: Human-defensin-3 activates professional antigen-presenting cells via Toll-like receptors 1 and 2. Proc Natl Acad Sci USA 2007, 104(47):18631-18635.
54. Denou E, Pridmore RD, Berger B, Panoff JM, Arigoni F, Brussow H: Identification of genes associated with the long-gut-persistence phenotype of the probiotic *Lactobacillus johnsonii* strain NCC533 using a combination of genomics and transcriptome analysis. J Bacteriol 2008, 190(9):3161-3168.
55. Sanchez B, Bressollier P, Urdaci MC: Exported proteins in probiotic bacteria: adhesion to intestinal surfaces, host immunomodulation and molecular cross-talking with the host. FEMS Immunol Med Microbiol 2008, 54(1):1-17.
56. Maassen CBM, Boersma WJA, van Holten-Neelen C, Claassen E, Laman JD: Growth phase of orally administered *Lactobacillus* strains differentially affects IgG1/IgG2a ratio for soluble antigens: implications for vaccine development. Vaccine 2003, 21(21-22):2751-2757.
57. Foligne B, Dewulf J, Breton J, Claisse O, Lonvaud-Funel A, Pot B: Probiotic properties of non-conventional lactic acid bacteria: immunomodulation by Oenococcus oeni. Int J Food Microbiol 2010, 140(2-3):136-145.
58. Haller D, Bode C, Hammes WP: Cytokine secretion by stimulated monocytes depends on the growth phase and heat treatment of bacteria: a comparative study between lactic acid bacteria and invasive pathogens. Microbiol Immunol 1999, 43(10):925-935.
59. Sashihara T, Sueki N, Furuichi K, Ikegami S: Effect of growth conditions of *Lactobacillus gasseri* OLL2809 on the immunostimulatory activity for production of interleukin-12 (p70) by murine splenocytes. Int J Food Microbiol 2007, 210(3):274-281.
60. Stevens MJA, Wiersma A, de Vos WM, Kuipers OP, Smid EJ, Molenaar D, Kleerebezem M: Improvement of *Lactobacillus plantarum* aerobic growth as directed by comprehensive transcriptome analysis. Appl Environ Microbiol 2008, 74(15):4776-4778.
61. Vissers YM, Snel J, Zuurendonk PF, Smit BA, Wichers HJ, Savelkoul HF: Differential effects of *Lactobacillus acidophilus* and *Lactobacillus plantarum* strains on cytokine induction in human peripheral blood mononuclear cells.FEMS ImmunolMedMicrobiol 2010,59(1):60-70.
62. Liaw A, Wiener M: Classification and regression by randomForest. R news 2002, 2:18-22.
63. Lambert JM, Bongers RS, Kleerebezem M: Cre-lox-based system for multiple gene deletions and selectable-marker removal in *Lactobacillus plantarum*. Appl Environ Microbiol 2007, 73(4):1126-1135.
64. Horton RM, Cai ZL, Ho SN, Pease LR: Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. Biotechniques 1990, 8(5):528-535.
65. Pinheiro J, Bates D: Mixed-effects models in S and S-plus. New York: Springer-Verlag; 2000.
66. Hochberg Y: A sharper Bonferroni procedure for multiple tests of significance. Biometrika 1988, 75(4):800-802.

Chapter 5

Cryopreservation of monocytes or differentiated immature DCs leads to an altered cytokine response to TLR agonists and microbial stimulation

M. Meijerink, D. Ulluwishewa, R. C. Anderson and J. M. Wells

Journal of Immunological Methods (2011) in press

Abstract

Literature on the effects of cryopreservation and thawing of monocytes or monocyte-derived immature dendritic cells (iDCs) on the subsequent functional capacities of the DCs is limited to a few specific maturation stimuli and is focused on applications in clinical immunotherapy. Given the cardinal role of DCs in regulating tolerance and immunity at mucosal surfaces there is a growing interest in understanding the effect of stromal, microbial and probiotic signals on DC function. Therefore our aim was to investigate the effects of cryopreservation on the functional properties of DCs stimulated with bacteria or bacterial components using a standardized method. Surface markers CD83 and CD86 were expressed at similar levels on iDCs generated from cryopreserved or freshly isolated monocytes. Cryopreservation of iDCs led to slightly decreased expression of CD86 and CD83 compared to freshly generated iDCs prepared from unfrozen cells but this did not affect the capacity of DCs to acquire fully mature characteristics after stimulation. In contrast the cytokine response to lipoteichoic acid and bacterial stimulation was altered by cryopreservation of monocytes or iDCs, particularly for IL-12p70 which was decreased up to 250 fold or not detected. Cryopreservation also decreased TNF-α and IL-1β production in stimulated iDCs but to a lesser extent than for IL-12p70, depending on the maturation factors used. The amounts of IL-10 produced by stimulated iDCs were increased up to 3.6 fold when iDCs were cryopreserved, but decreased up to 90 fold when generated from cryopreserved monocytes. Immature DCs are often used to investigate the immunomodulatory properties of probiotics and here we show for the first time that cryopreserved monocytes and cryopreserved iDCs have a skewed cytokine response to microbial stimulation. These findings have implications for the methods used in bacterial-DC immune assays and highlight the importance of comparing different cytokines and stimuli in immune cell cryopreservation protocols.

Introduction

Dendritic cells (DCs) play a key role in mucosal immunity and tolerance, and are present throughout the intestine including the follicular tissue of the mucosal associated lymphoid tissue as well as the lamina propria (LP). In the Peyer's patches and isolated lymphoid follicles DCs are thought to prime Th2 responses leading to the induction of antigen specific IgA which is then transported into the lumen via the epithelial polymeric IgA receptor [1]. Direct communication can also occur between intestinal microbiota and a subset of DCs have been shown to open the epithelial tight junctions and send protrusions to the luminal side [2]. Epithelial cells can also transport antigens from the lumen to the underlying DCs although with much less efficiency than M cells [3].
Maturation is triggered by ligand binding to pattern recognition receptors such as Toll-like receptors (TLRs) that bind conserved microbial-associated molecular patterns (MAMPs) such as lipoteichoic acid (LTA) and lipopolysaccharide (LPS). During maturation, the major histocompatibility complex molecules for antigen presentation and co-stimulatory molecules, such as CD40, CD54, CD83 and CD80 (B7.1) and CD86 (B7.2), are up regulated for an effective T-cell stimulation [4]. The nature of the signals encountered by DCs including epithelial derived factors are known to shape the course of the immune response [5].
Given the important role of DCs in intestinal homeostasis and disease there is a growing interest in understanding the effect of stromal and microbial signals on DC function and the potential to manipulate DCs through nutrition and the microbiota. Furthermore recent therapeutic and mechanistic studies have highlighted the importance of determining the *in vitro* DC response to probiotic strains as a prerequisite to therapeutic studies [6, 7]. Several studies have shown that probiotic species of bacteria, and even different strains of the same species, differ markedly in their ability to induce DC maturation and cytokine production [8, 9]. Apart from the growing interest in the role of mucosal DCs and immunomodulation by food, enormous efforts have been given to DC-based vaccination against malignant diseases [10]. These approaches would benefit greatly from the ability to cryopreserve a single large batch of monocytes or immature DCs (iDCs) with the same viability, phenotype, and function as those generated from fresh blood. Few studies have examined the influence of cryopreservation/ thawing on the subsequent expansion of culture-generated DCs, the expression of surface molecules, and the functional capacities of immature and mature DCs [11-14]. Previous studies focused on the application of DCs in clinical immunotherapy and were limited to stimulations with synthetic ligands, cytokines or antibodies including LPS, tumor necrosis factor alpha (TNF-α), interferon-gamma (IFN-γ), and CD40L. For example, Lewalle et al. studied the effect of cryopreservation on DC maturation and antigen specific responses using CD40L and IFN-γ as maturation factors and concluded that freezing did not alter the capacity of iDCs to capture, process and generate antigen-specific reactions after thawing. Additionally, cryopreservation did not alter the capacity of DCs to acquire fully mature characteristics, as shown by the immunophenotype and bioactive interleu-

kin-12 (IL-12) secretion. A systematic comparison between fresh monocyte-derived DCs with cryopreserved DCs was also reported by John et al., who found that freezing led to semi-matured DCs, reduced endocytic activity and reduced transduction efficiency [12]. Hori et al. assessed the effect of cryopreservation on cell viability, maturation and the capacity of DCs for chemotactic migration and antigen presentation [14]. They concluded that regardless of the stage of maturation at which the DCs were freeze-thawed, cryopreservation did not have an effect on cell phenotype, function or viability. Hayden et al., performed an extensive study to assess the effect of cryopreservation on recovery, viability, phenotype, and function of monocytes and DCs at various developmental stages [11]. Cryopreserved cells were cultured with TNF-α, IFN-γ and LPS to obtain mature DCs. They found that the properties of the DCs derived from cryopreserved monocytes were equivalent to DCs generated from freshly isolated monocytes, but the cryopreservation of differentiated DCs led to a moderate increase in CD40, CD80 and CD83, a decreased capacity to stimulate expression of CD69, IFN-γ, and granzyme B and a reduced T-cell proliferation in co-culture with T-cells. As the previous studies have not included bacteria as maturation stimuli or do not provide data on cytokine responses, we have investigated the effects of cryopreservation and thawing of precursor monocytes and iDCs on the maturation and immune response of DCs to probiotic strains and bacterial components.

Our results demonstrate that cryopreservation of monocytes and/or iDCs can influence cytokine responses depending on the stimulus for maturation.

Material and methods

Bacterial strains
L. plantarum WCFS1, a human isolate from saliva obtained from the NIZO culture collection (Ede, The Netherlands) and *L. fermentum* AGR1485 a human oral isolate obtained from the AgResearch culture collection (Palmerston North, New Zealand) were grown overnight to stationary phase at 37°C in MRS. The bacteria were recovered by centrifugation and washed twice in phosphate buffered saline (PBS, pH=7.4) and resuspended at $2*10^8$ colony forming units (CFU)/ml in PBS containing 20% glycerol and stored at -80°C prior to use in the immunoassays. CFU were determined by plating serial dilutions of the cultures on MRS agar.

Blood donors
Buffy coats from blood donors were obtained from the Sanquin Blood bank Nijmegen (The Netherlands). An informed consent was obtained before the sample collection and the performed experiments were approved by the Local Ethical Committee.

Differentiation and maturation of dendritic cells
Human peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coat using a combination of Ficoll density centrifugation and cell separation using antibody coated microbeads. The blood was diluted 1:1 with Iscove's Modified Dulbecco's Medium (IMDM) containing GlutaMAX (Invitrogen, Breda, The Netherlands). The PBMCs were isolated by density gradient centrifugation on Ficoll-Plaque PLUS (GE Healthcare, Bucks, United Kingdom). The diluted plasma was removed and the layer of white blood cells was recovered and washed twice with IMDM. The CD14+ monocytes were then purified using magnetic cell sorting with CD14+ microbeads according to the manufacturer's recommended protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). The proportion of CD14+ cells was routinely determined using flow cytometry (BD FACSCanto II, BD, San Diego, USA) and antibody staining for CD14 following the same procedure as on days 3, 6 and 8 (described below). In all experiments the proportion of CD14+ cells was greater than 90%. To generate iDCs, approximately 10^6 CD14+ cells/ well were cultivated in RPMI 1640 containing 10% FBS gold (PAA), 1% penicillin, streptomycin (v/v) (Invitrogen), 50 ng/mL IL-4 (R&D systems, Minneapolis, USA) and 50 ng/mL granulocyte macrophage-colony stimulating factor (GM-CSF) (R&D systems) in a 24 well plates. GM-CSF combined with IL-4 induces monocytes to become myeloid dendritic cells *in vitro* in 6 days. At day 3 and day 6 half of the medium was refreshed. At day 6 the cells were left unstimulated iDCs or were stimulated with LPS (1 µg/mL), LTA (5 µg/mL), *L. plantarum* or *L. fermentum* (1:1 and 10:1 bacteria to DC ratio) for 48 hours. LPS the major component of the outer membrane of Gram-negative bacteria, was used as a TLR-4 agonist. LTA, a component of Gram-positive bacteria cell walls, was used as a TLR-2 agonist, as it is known to be recognised by TLR-2/ TLR-6 heterodimers. During the period of incubation with bacteria no acidification of the cell culture medium was observed.

Freezing and thawing of monocytes or day 6 iDCs
Cells for cryopreservation were resuspended at $2x10^7$ cells/mL in cold freezing media (10% DMSO in FBS). Samples were placed in a freezing container (Cryo 1 °C freezing container, Nalgene Nunc Int., Rochester, NY, USA) and stored overnight at -80°C. After freezing, the cryopreserved samples were rapidly thawed by immersing them in a water bath at 37°C until they could be decanted. The cell suspension was transferred to a 50 mL polypropylene tube containing pre-warmed IMDM containing 20% FBS and then washed twice with IMDM containing 20% FCS, to remove the DMSO. Cells were counted and checked for viability by tryphan blue staining. Cell viability was >95%.

Analysis of cell surface markers and measurement of cell death by flow cytometry
Monocyte-derived DCs were harvested at days 3, 6 and 8 and stained with specific monoclonal antibodies to CD1a, CD83, CD86 or their isotype-matched controls (BD biosciences, San Diego, USA) for 30 min on ice, washed and analyzed by flow cytometry (FACSCanto II, BD). CD83 is only expressed on matured DCs so it was used as a marker to assess the immature status of the DCs generated from monocytes. CD86 expression was measured to check the activation status of the cells during the differentiation protocol and after cryopreservation.
Live, apoptotic and necrotic cells were discriminated by staining with Annexin V and propidium iodide (PI) on days 3, 6 and 8 according to the manufacturer's protocol. The cells were analyzed on a flow cytometer. Cells that were negative for both Annexin V and PI were not apoptotic or necrotic as translocation of the membrane phospholipid phosphatidylserine had not occurred and the plasma membrane was still intact. Therefore, Annexin V and PI double negative cells were considered to be viable cells; whereas both single and double positive cells were regarded as non-viable [15]. The flow cytometry data were analyzed using the BD FACSDiva software. On days 3 to 8 the viability of the cells was between 60- 80%.

Cytokine analysis
Supernatants from the DC stimulation assays were collected after 48 hours of stimulation and analyzed for the presence of cytokines (IL-12p70, TNF-α, IL-1β and IL-10) using a cytometric bead-based immunoassay that enables multiplex measurements of soluble cytokines in the same sample [16], according to the manufacturer's protocol (BD biosciences). The limits of sensitivity for detection were as follows: IL-12p70 0.6 pg/mL, TNF-α 0.7 pg/mL; IL-1β 1.1 pg/mL and IL-10 0.13 pg/mL. The flow cytometry data were analysed using the BD FCAP software.

Statistical analysis
Mixed general linear model using restricted maximum likelihood was used to determine the statistical differences between cytokines produced by DCs differentiated from fresh monocytes and DCs differentiated from frozen monocytes and frozen iDCs stimulated with different stimuli within 3 donors. Each assay was performed in duplicate. A two-

sided p-value of 0.05 or lower was considered to be significant. The statistical analysis was performed by using SAS software (version 9.1, SAS Institute Inc., Cary, NC, USA).

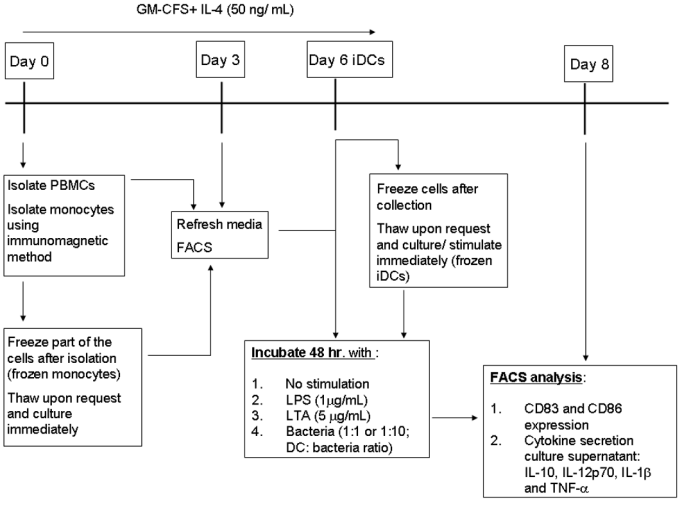

Figure 1 Schematic overview of the differentiation and stimulation protocol.

Results and Discussion

It was not clear from the current literature whether or not cryopreserved monocytes or iDCs could be used to simplify and standardize a method for measuring bacteria-DC immune responses, including for example probiotic screening. To investigate the effects of cryopreservation on the functional properties of DCs stimulated with bacteria or the bacterial TLR agonists we describe a standardized method for generating DCs from peripheral blood monocytes. In the absence of DC maturation factors the cultured monocytes differentiate into immature DCs in the presence of GM-CSF and IL-4. Several monocyte isolation procedures have been described in the literature but in our hands the immunomagnetic enrichment method described here reproducibly yielded iDCs from different donors as revealed by the expression of a range of DC markers using flow cytometry. In this study differences between cryopreserved cells and fresh cells were compared within multiple donors. The freshly generated iDCs were stimulated on day 6 of the protocol whereas the cryopreserved iDCs (frozen on day 6) were thawed the following day and then stimulated immediately. In the case of cryopreserved monocytes these cells were thawed, differentiated into iDCs and stimulated on day 6 (Fig. 1).

To determine the maturation status of cryopreserved iDCs or those generated from fresh monocytes or cryopreserved monocytes, CD1a, CD83 and CD86 staining was performed after day 3 and day 6 as shown in the differentiation protocol (Fig. 1). The maturation markers were similarly expressed at low levels on unstimulated iDCs that were freshly prepared, cryopreserved or generated from cryopreserved monocytes (Fig. 1). Immature DCs expressed high levels of CD1a, a specific DC marker, and low levels of CD83 and CD86 compared to DCs that were treated with maturation factors, such as LPS or TNF-α and IL-1β. Taken together, these findings demonstrate that cryopreservation of monocytes or iDCs does not affect expression of maturation markers compared to freshly prepared iDCs.

The maturation marker expression on iDCs differentiated from freshly isolated monocytes or cryopreserved monocytes and cryopreserved iDCs generated from the same batch of blood was compared by stimulation with LPS, LTA or different bacterial strains. A similar activation status was observed for iDCs from fresh and cryopreserved monocytes, in agreement with previous publications [11, 13]. In contrast to other studies we found that iDCs frozen and thawed on day 6 of the differentiation protocol (Fig. 2) had increased expression levels of CD83 and CD86 compared to fresh iDCs (Fig. 2).

IL-12p70 was measured in the supernatant of stimulated DCs as it is known to be a potent promoter of Th1 type immune responses. IL-12p70 could be measured in the supernatants of LTA stimulated DCs generated from freshly isolated monocytes but not from cryopreserved monocytes (Fig. 3). Similarly, the IL-12p70 response to bacterial stimulation (but not to LPS) was also significantly decreased. Additionally, IL-12p70 secretion was up to 10 fold lower for cryopreserved iDCs after stimulation with bacteria (*L. plantarum* and *L. fermentum*, at two different ratios, $p<0.05$). IL-12p70 secretion was significantly lower for cryopreserved iDCs than for freshly generated iDCs, especially

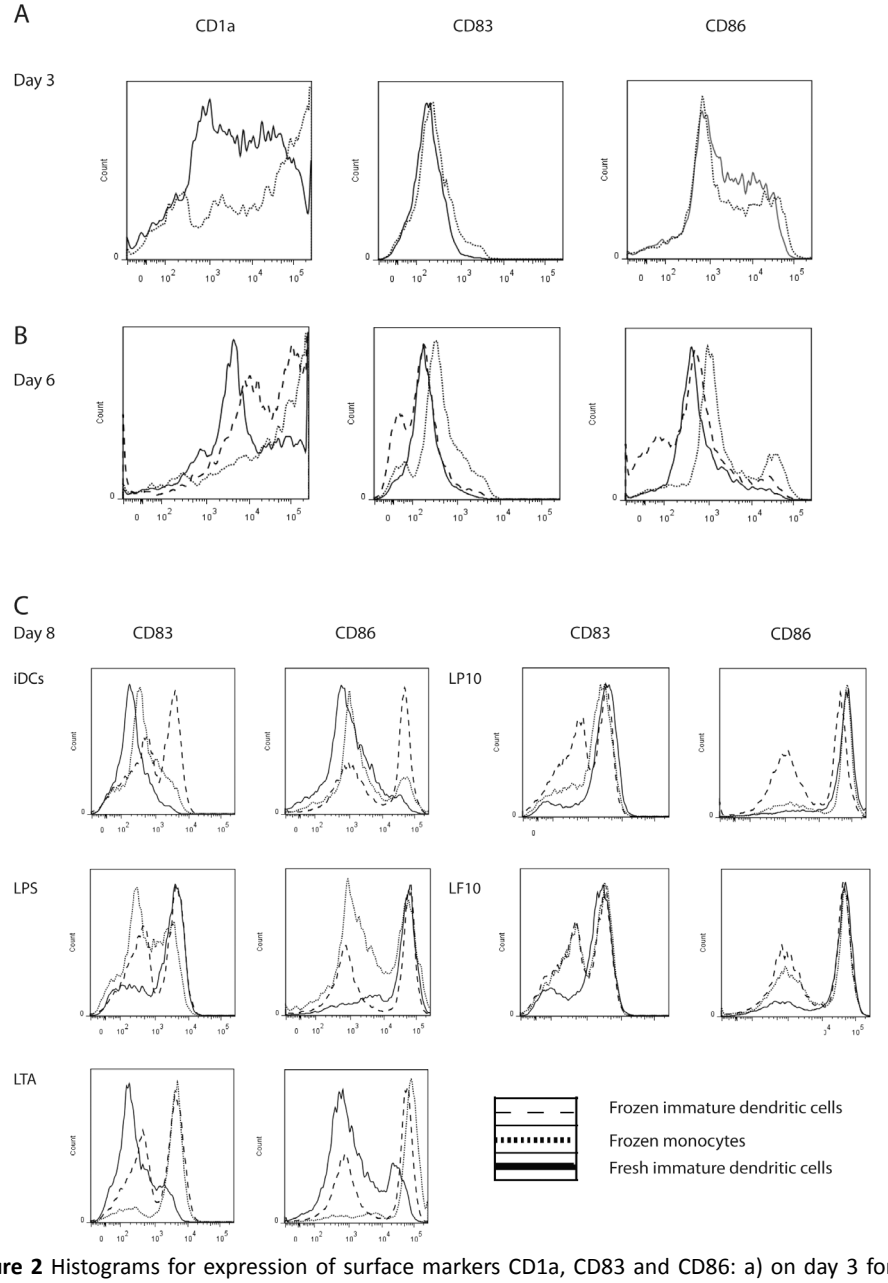

Figure 2 Histograms for expression of surface markers CD1a, CD83 and CD86: a) on day 3 for iDCs derived from fresh and frozen monocytes. b) on day 6 for iDCs derived from fresh monocytes, frozen monocytes and day 6 frozen iDCs. c) on day 8 iDCs unstimulated or after stimulation with LPS, LTA or bacteria (*L. plantarum* or *L. fermentum*) bacteria: DC ratio 10:1. One of three representative experiments is shown.

after stimulation with LPS or LTA ($p<0.05$) (Fig. 3). Thus, cryopreservation of monocytes and iDCs affects IL-12p70 secretion upon stimulation with bacteria, LTA or LPS. The only exception was with cryopreserved monocytes stimulated with the TLR-4 agonist LPS. As LPS is often used as a control for iDC activation, it is important to be aware of the differences observed with other stimuli.

Measurements of TNF-α secretion gave similar results to that for IL-12p70. Indeed, DCs derived from cryopreserved monocytes produced less TNF-α after stimulation with LTA ($p<0.05$) compared to freshly generated DCs. Also, after stimulation with bacteria (*L. plantarum* and *L. fermentum*, both ratios, $p<0.01$), there was a striking difference in the amounts of secreted TNF-α. For cryopreserved iDCs a significant decrease in TNF-α was only seen following stimulation with *L. fermentum* stimulation at two different ratios ($p< 0.01$). No significant effects of cell cryopreservation on TNF-α production were observed using LPS as a stimulus (Fig. 3).

Cryopreservation of monocytes did not induce of IL-1β secretion, except when stimulated with highest ratio of *L. plantarum*. The cryopreserved DCs follow the same patterns as DCs generated from freshly isolated monocytes, but in most cases the amount of IL-1β produced was lower (Fig. 3).

Interestingly, the IL-10 secretion by stimulated cryopreserved iDCs was slightly increased compared to freshly prepared DCs. In contrast, DCs generated from cryopreserved monocytes produced significantly less IL-10 than DCs derived from freshly prepared monocytes or cryopreserved DCs ($p<0.01$). IL-10 is a critical regulatory T-cell cytokine that suppresses IL-12 production and consequently other Th1 cytokines such as IFN-γ and TNF-α. The suppression of IL-12 in the gut by IL-10 prevents an inflammatory cascade of Th1 cytokines and cellular migration. IL-10 and IL-12 are often used to screen for the immunomodulatory properties of probiotic bacteria and other nutritional compounds as the ability of different lactobacilli to induce a high ratio of IL-10/IL-12 production in immune cells is associated with protection from inflammatory diseases [6, 7].

Our results demonstrate that cryopreservation of monocytes or iDCs leads to an impaired cytokine response, particularly for IL-12p70. This is in contrast with the results of Lewalle et al. [13] but in agreement with those of Hayden et al. [11]. In the study by Hayden et al. IL-12p70 was the only cytokine measured, and LPS or pro-inflammatory cytokines were used as maturation factors [11] whereas Lewalle et al. used CD40L and IFN-γ as maturation stimuli. In our study the effect of cryopreservation on IL-12p70 secretion was much greater (reduced up to 250-fold or no secretion) than that shown by Hayden et al. (reduced up to 6 fold). These differences in the IL-12p70 response could be due to the use of different maturation stimuli.

Immature DCs are often used to investigate the immunomodulatory properties of probiotics and here we show for the first time that cryopreserved monocytes and cryopreserved iDCs have an altered cytokine response to microbial stimulation, which is associated with the skewing of T helper cells responses towards Th2. These findings have implications for the methods used in bacterial DC immune assays and highlight the importance of comparing different cytokines and stimuli in immune cell cryopreserva-

tion protocols.

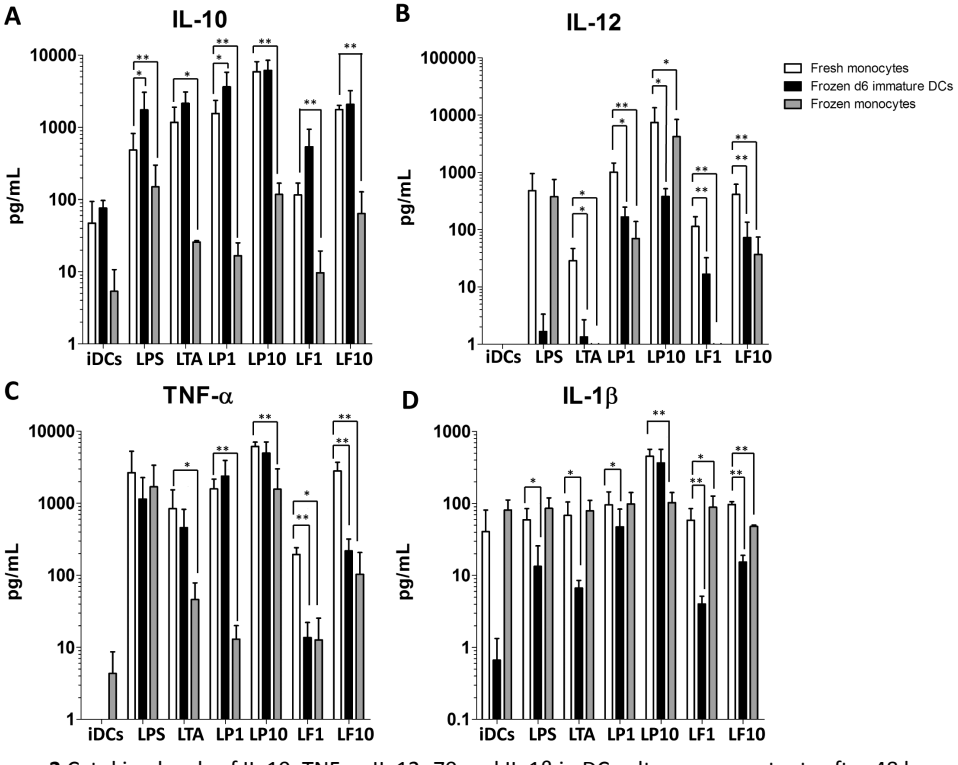

Figure 3 Cytokine levels of IL-10, TNF-α, IL-12p70 and IL-1β in DC culture supernatants after 48 hours: iDCs were unstimulated (negative control) or stimulated with LPS, LTA or bacteria (*L. plantarum* or *L. fermentum*) at ratios of 1:1 or 10:1 bacteria per DC. Amounts of secreted cytokines are plotted on a logarithmic scale. White bars represent DCs derived from fresh monocytes, grey bars represent frozen day 6 iDCs and black bars represent DCs derived from frozen monocytes. A two-sided p-value of 0.05 or lower was considered to be significant indicated *p <0.05 or ** p<0.01. The average data is shown from three experiments. Error bars indicate SD.

References

1. Macpherson, A.J. and T. Uhr, Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. Science, 2004. 303(5664): p. 1662-5.
2. Rescigno, M., et al., Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat Immunol, 2001. 2(4): p. 361-7.
3. Miller, H., et al., Intestinal M cells: the fallible sentinels? World J Gastroenterol, 2007. 13(10): p. 1477-86.
4. Banchereau, J., et al., Immunobiology of dendritic cells. Annu Rev Immunol, 2000. 18: p. 767-811.
5. Wells, J., et al., Epithelial Crosstalk at the Microbiota-Mucosal Interface. Proc Natl Acad Sci U S A, In press.
6. Foligne, B., et al., Correlation between *in vitro* and *in vivo* immunomodulatory properties of lactic acid bacteria. World J Gastroenterol, 2007. 13(2): p. 236-43.
7. Kwon, H.K., et al., Generation of regulatory dendritic cells and CD4+Foxp3+ T cells by probiotics administration suppresses immune disorders. Proc Natl Acad Sci U S A, 2010. 107(5): p. 2159-64.
8. Christensen, H.R., H. Frokiaer, and J.J. Pestka, Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. J Immunol, 2002. 168(1): p. 171-8.
9. Meijerink, M., et al., Identification of genetic loci in *Lactobacillus plantarum* that modulate the immune response of dendritic cells using comparative genome hybridization. PLoS One, 2010. 5(5): p. e10632.
10. Buchler, T., et al., Dendritic cell-based immunotherapy for the treatment of hematological malignancies. Hematology, 2003. 8(2): p. 97-104.
11. Hayden, H., et al., Cryopreservation of monocytes is superior to cryopreservation of immature or semi-mature dendritic cells for dendritic cell-based immunotherapy. J Immunother, 2009. 32(6): p. 638-54.
12. John, J., et al., Cryopreservation of immature monocyte-derived dendritic cells results in enhanced cell maturation but reduced endocytic activity and efficiency of adenoviral transduction. J Immunol Methods, 2003. 272(1-2): p. 35-48.
13. Lewalle, P., et al., Freezing of dendritic cells, generated from cryopreserved leukaphereses, does not influence their ability to induce antigen-specific immune responses or functionally react to maturation stimuli. J Immunol Methods, 2000. 240(1-2): p. 69-78.
14. Hori, S., et al., Freeze-thawing procedures have no influence on the phenotypic and functional development of dendritic cells generated from peripheral blood CD14+ monocytes. J Immunother, 2004. 27(1): p. 27-35.
15. Vermes, I., et al., A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods, 1995. 184(1): p. 39-51.
16. Morgan, E., et al., Cytometric bead array: a multiplexed assay platform with applications in various areas of biology. Clin Immunol, 2004. 110(3): p. 252-66.

Chapter 6

A comparative study of the immunomodulatory properties of potential probiotics *in vitro* and *in vivo* using a mouse model of peanut allergy

M. Meijerink, J.M. Wells, N. Taverne, M. de Zeeuw Brouwer, B. Rappard, K. Venema and J. van Bilsen

Submitted

Abstract

Background
Peanut allergy accounts for the majority of severe food-related allergic reactions and requires the development of new prevention and treatment methods. Probiotics may be considered for treatment of allergic individuals on the basis of their immune modulating properties.

Objective
To screen the immunomodulatory properties of 28 commercially available bacterial strains *in vitro* using human peripheral blood mononuclear cells (PBMCs) and investigate selected strains for their *in vivo* immunomodulatory potential in an established mouse peanut allergy model.

Methods
Cytokine profiles of the probiotic strains were determined by *in vitro* co-culturing with human PBMCs from four different donors. Three strains were selected to investigate their immunomodulatory role in the peanut allergic response by analyzing peanut-specific antibodies, mast cell degranulation and *ex vivo* cytokine production by splenocytes.

Results
The probiotic strains induced highly variable cytokine profiles in PBMCs. *L. salivarius* HMI001 (HMI001), *L. casei* Shirota (LCS) and *L. plantarum* WCFS1 (WCFS1) were selected for further investigation due to their distinct patterns of IL-10, IL-12 and IFN-γ induction. Prophylactic treatment with both HMI001 and LCS attenuated the Th2 phenotype in the mouse model (reduced mast cell responses and *ex vivo* IL-4 and/or IL-5 production). In contrast, WCFS1 augmented the Th2 phenotype (increased mast cell and antibody responses and *ex vivo* IL-4 production).

Conclusion and Clinical relevance
In vitro PBMC screening was useful in selecting strains with anti-inflammatory and Th1 skewing properties. In case of HMI001 (inducing a high IL-10/IL-12 ratio) and LCS (inducing high amounts of IFN-γ and IL-12) partial protection was seen in a mouse peanut allergy model. Selection of certain strains may worsen the allergic reaction as shown in the case of WCFS1. Although further research is needed to validate the immunomodulatory mechanisms involved *in vivo,* this approach is likely to be useful for selecting strains for translational research in humans.

Introduction

Several species of the genus *Lactobacillus* are naturally present in the human intestinal tract and have been evaluated for their probiotic activity. Oral administration of certain probiotic strains has shown promising results in human clinical trials and experimental animal models of inflammatory bowel disease, irritable bowel syndrome and allergy [1-9]. Results obtained from *in vitro* and *in vivo* studies with probiotics indicate a strong role for immunomodulation and enhancement of the epithelial barrier function [10, 11]. Proposed immunomodulatory mechanisms include down-regulation of inflammatory responses through the modulation of dendritic cell (DC) function and subsequent expansion or induction of regulatory T cells (Tregs) producing anti-inflammatory cytokines such as TGF-β and IL-10, which suppresses inflammatory reactions in allergy and colitis [12, 13]. Additionally certain strains of *Lactobacillus* have been demonstrated to modulate T-cell responses to co-administered antigen towards a Th1-type immune response leading to an inhibition in IgE production and an increase in Th1 cytokines [9, 14-17]. Probiotics can decrease the IL-6 production by lamina propria mononuclear cells and PBMCs and increase their IL-10 and IL-12 production [18-21]. As IL-6 can skew Tregs and effector T cells into IL-17-producing T cells (Th17), its decrease in mucosal tissues might support Treg functions in the mucosa [22]. IL-12 promotes the differentiation of naïve T cells into Th1 cells and augments natural killer (NK) cell activity [23] leading to increased IFN-γ and further skewing the immune response from a Th2 to a Th1 response. Exposure to probiotics in the gut can also stimulate the intestinal epithelial cells (IEC) to secrete anti-inflammatory cytokines, such as TSLP and TGF-β, which can promote the differentiation of immature DCs into tolerogenic DCs which play a key role in regulating oral tolerance through the induction of regulatory Foxp3+ T cells expressing gut-homing receptors [13, 24, 25]. Several studies have shown that the immunomodulatory properties of lactic acid bacteria differ among species [26-28] and even between strains of the same species [29-33].

Given the potential for probiotics to modulate host immune responses there is ongoing interest in their application in the treatment and prevention of food allergy. Food allergy represents a failure to attain oral tolerance leading to elevated levels of Th2 cytokines such as IL-4, IL-5, higher levels of allergen specific IgE and increased mast cell degranulation. There is evidence that differences in Treg activity may play a role in the development and resolution of food allergies. Karlsson et al. showed that children who outgrew cow's milk allergy had higher numbers of Tregs with more potent function compared to children who had remained allergic [34]. Smith et al. demonstrated a link between lower numbers of Tregs and the development of food allergy to egg in infants up to the age of 12 months [35]. This is in line with the study by Shreffler et al. who showed that infants with higher numbers of allergen-specific Tregs had milder disease and a more favorable outcome than those with lower numbers of Tregs [36].

Of all food allergies, peanut allergy deserves extra attention as it is mostly frequently associated with anaphylaxis and fatal outcome [37]. Furthermore, peanut allergy usu-

ally does not resolve itself and therefore has a high impact on the quality of life of the individual [38]. The prevalence of peanut allergic sensitisation varies between 1-2% (US and Canada) to 10% in the UK, of whom 2% have a clinical peanut allergy [39]. Currently allergen avoidance is the only way to prevent allergic responses [40] but in the case of peanut allergy, accidental ingestion is common due to the trace amount of peanut in many food products and cross-contamination during food processing [41]. Most human studies on probiotics and allergy have focused on the role of specific *Lactobacillus* strains in the management and prevention of eczema, allergic eczema and allergic rhinitis [7]. Recent reviews on the large number of clinical trials performed so far provide stronger evidence for prevention of atopic disease rather than the management of atopic eczema and support the idea that probiotics deserve to be further explored. To date, there are only a few strains that have been reasonably well documented in clinical studies and they mostly show a positive but temporary effect on atopic dermatitis and eczema, in regard to allergic diseases [42-50].

As there are only a few studies comparing potential probiotic strains *in vitro* and *in vivo* our aim was to investigate the effects of three well characterized probiotic strains, which differ in their immunomodulatory properties, on the prevention of peanut allergy. To select strains for this study strains of *Lactobacillus* and *Bifidobacterium* isolated from different commercially available products were screened for their immunomodulatory properties in a co-culture assay with human PBMCs. Based on these results *L. plantarum* WCFS1, *L. salivarius* HMI001 and *L. casei* Shirota were tested for their prophylactic effect in a mouse model of allergic sensitization to peanut allergen.

Materials and Methods

Bacterial strains

Twenty-eight different strains comprising 12 species of probiotics or potential probiotic strains from commercially available products were tested in PBMC co-culture assays (Table 1). The species of the strains isolated from a product containing a mixture of bacteria were checked by 16S rDNA sequencing (data not shown). The bacteria were grown overnight to stationary phase in anaerobic conditions at 37°C in the recommended medium. The bacteria were then recovered by centrifugation, washed twice in phosphate buffered saline (PBS, pH=7.4), resuspended at approximately $1*10^{10}$ colony forming units (CFU)/mL in PBS containing 20% glycerol, and stored in aliquots at – 80 °C prior to use. The exact number of bacterial CFU in a thawed aliquot was determined by plating serial dilutions of the cultures on MRS agar or MRS plus cysteine for the *Bifidobacterium* sp.

Human PBMC co-culture assays

The study was approved by the Wageningen University Ethical Committee and was performed according to the principles of the Declaration of Helsinki. Buffy coats from four blood donors were obtained from the Sanquin Blood bank Nijmegen, Netherlands. A written informed consent was obtained before the sample collection. PBMCs were isolated by density gradient centrifugation (Ficoll-Plaque PLUS, Amersham, Roosendaal, Netherlands). PBMCs were cultivated in Iscove's Modified Dulbecco's Medium (IMDM) containing 1% heat-inactivated human AB serum, 1% penicillin, and 1% streptomycin (v/v) (Invitrogen, Breda, Netherlands). The cells were left unstimulated or were stimulated with lipopolysaccharide (LPS, 1 µg/mL), Concanavalin A (ConA, 5 µg/mL) or with bacteria in a 1:1 ratio with mononuclear cells. After 24hr incubation, the concentrations of IL-10, IL-12p70, and IFN-γ present in the co-culture supernatants were determined using the Becton and Dickinson (BD, San Diego, USA) cytometric bead array and flow cytometry (FACSCantoII, BD, San Diego, USA). The limits of sensitivity for detection were as follows: IL-10 (13 pg/mL); IL-12p70 (0.6 pg/mL); and IFN-γ (1.8 pg/mL).

Mice

Female C3H/HeOuJ mice, specific pathogen-free, were purchased from Charles River Laboratories (Lyon, France) and were maintained under barrier conditions in macrolon type III cages with wood chips bedding, at mean temperature of 22 ± 2°C, a relative humidity of at least 40% and not exceeding 70% and a 12-h light/dark cycle. Drinking water and standard laboratory food pellets were provided ad libitum. Mice were 6 weeks old at the start of probiotic dosing. The welfare of the animals was maintained in accordance with the general principles governing the use of animals in experiments of the European Communities (Directive 86/609/EEC) and Dutch legislation (The Experiments on Animals Act, 1997).

Chemicals, reagents and monoclonal antibodies
Cholera toxin (CT) was purchased from List Biological Laboratories, Inc (Campbell, CA, USA). All other chemicals were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands) unless otherwise stated.

Table 1: Strains used in study

Species	Strain	Origin/ source
B. animalis	DN 173 010	Danone
B. lactis	Bb12	Chr Hansen
B. lactis	Bi-07	Danisco
B. longum	Sanostol	Altana
L. acidophilus	La-5	Chr Hansen
L. acidophilus	NCFM	Danisco
L. acidophilus	R0052	Rossell
L. casei	Shirota	Yakult
L. casei	Immunitas	Danone
L. casei	TNO 15532	TNO collection
L. casei	R0215	Rossell
L. fermentum	HMI002	HMI collection
L. gasseri	PA 16/8	Merck Selbstmedikation
L. johnsonii	LC-1	Nestle
L. plantarum	WCFS-1	TIFN
L. plantarum	299v	Probi
L. plantarum	256	TNO collection
L. plantarum	R1012	Rossell
L. reuteri	ATCC55730	BioGaia
L. reuteri	DSM20016	DSM
L. reuteri	RC-14	Urex Biotec Inc
L. rhamnosus	LGG	Valio
L. rhamnosus	HOWARU Lr-32	Danisco
L. rhamnosus	Sanostol	Altana
L. rhamnosus	R0011	Rossell
L. rhamnosus	GR-1	Urex Biotec Inc
L. salivarius	HMI001	HMI collection
L. salivarius	FortaFit Ls-33	Danisco

Preparation of peanut extract (PE)
Peanut was kindly donated by Imco Nut Products, the Nut Company (Doetinchem, Netherlands) and was supplied by Golden Peanut, plant at Alpharetta (GA, USA). Peanut protein extract was made by blending 500 g peanut with 500 mL 20 mM Tris buffer (pH 7.2). After 2 h blending at intervals of 20 min for 1 min at room temperature, the aqueous fraction (peanut extract) was collected by subsequent centrifugation (10,000 g). The peanut extract was stored at -70 °C in aliquots prior to use.

Sensitization protocol
Groups of 8 mice were orally exposed to PBS (control) or PE plus cholera toxin (CT) (allergic sensitization) by intragastric dosing of PBS or 6 mg PE plus 10 µg CT in 200 µL. Oral exposure was performed on days 0, 1, 2, 10, 17 and 24. Mice were intragastrically administered $1x10^9$ CFU of the different lactobacilli strains, diluted in 300 µL 0.2 M $NaHCO_3$. for three times per week, starting 14 days prior to the sensitization phase (day -14) until scheduled necropsy (day 31). The effect of LCS was tested in a separate animal experiment to HMI001 and WCFS1. To compare the results of the different experiments, the measured parameters (i.e. mMCP-1 level, antibody levels and cytokines levels) were normalized to the vehicle-treated PE sensitized mice.

Measurement of serum mouse mast cell protease-1 (mMCP-I)
At day 30, mice were orally challenged with PE by intra-gastric gavage (0.4 mL of 30 mg/mL). Blood samples were collected 30 minutes after oral challenge. Serum levels of mMCP-I were determined by ELISA according to the manufacturer's instructions (Moredun Scientific Ltd, Midlothian, Scotland).

Measurement of serum IgG1, IgG2a, and IgE antibodies
Blood samples were collected on day 31 and stored at -20°C until analysis. Levels of PE-specific IgG1and IgG2a in serum were determined by ELISA and levels of PE-specific IgE by sandwich ELISA. Plates (NUNC Immuno Maxisorp plate, Roskilde, Denmark) were coated overnight with 10 µg/mL PE (for IgG1 and IgG2a detection) or with 1 µg/mL purified rat anti-mouse IgE (BD Pharmingen) in PBS, followed by 1.5 h blocking with ELISA buffer. Each test serum was incubated for 2-2.5 h. For PE-specific antibody levels, a sera pool of mice, that were sensitized for three weeks with PE plus alum by intraperitoneal injection each week, was used as reference serum and a standard curve of the reference serum was included to determine antibody levels (in arbitrary units (AU)). For detection of PE-specific IgG1 and IgG2a, alkaline phosphatase-conjugated anti-IgG1 or anti-IgG2a, were added (1 h at RT). Subsequently, alkaline phosphatase buffer (Sigma) was used for the color reaction, which was stopped with a 3M NaOH and absorbance was measured at 405 nm using a Benchmark Plus Microplate Spectrophotometer (Bio-Rad Laboratories, Richmond, CA, USA).
To measure PE-specific IgE antibodies, a PE-digoxigenin (DIG) conjugate solution (diluted in High Performance ELISA Buffer, from Sanquin, San Diego, CA, USA) was added

(1 h at RT). The coupling of DIG to PE was performed according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). After incubation (1.5 h at RT) with peroxidase-conjugated anti-DIG fragments (Roche Diagnostics, Mannheim, Germany), tetramethylbenzidine substrate solution was added and the color reaction was stopped with 1M H_2SO_4. Absorbance was measured at 450 nm.

Cell culture and cytokine measurement

On the day of necropsy (day 31), mouse spleens were processed to obtain single cell suspensions for *ex vivo* cytokine analysis. A suspension with a viable cell concentration of 5 x 10^6 cells/mL was prepared in enriched RPMI-1640 medium (RMPI 1640 with Glutamax™ and 25 mM HEPES buffer supplemented with 10% heat inactivated FCS and 1% penicillin/ streptomycin). The splenocytes were stimulated in the presence or absence of PE (200 µg/mL) for 96 hours at 37°C and 5% CO^2. Culture supernatants were stored in -80°C for cytokine measurements by multiplex analyses (BenderMed Systems, Germany; mouse Th1/Th2 plex).

Statistics

Data were analyzed using GraphPad Prism 4 software. The significance of differences between group means were determined by using one-way ANOVA with the Bonferroni *post hoc* test. Statistical analyses were performed following logarithmic transformation (to achieve normal distribution). $P<0.05$ was considered to be statistically significant.

Results

Co-culture of hPBMCs with lactobacilli and bifodobacteria modulates production of IL-10, IL-12, and IFN-γ

PBMCs from four different healthy donors were cultured in the absence or presence of 28 different bacterial strains comprising 12 species as well as LPS or ConA. Even though the levels of cytokines induced by the probiotic strains differed between donors, the ranking of the strains was highly consistent for each cytokine showing that the strain immune profiles were reproducible between donors. The different bacterial strains differed considerably in their ability to modulate PBMC immune responses (Fig. 1) as shown by the large range of cytokine responses: IL-10 (6 – 3789 pg/mL: 632-fold), IL-12p70 (2 – 927 pg/mL: 464-fold), IFN-γ (1.8 pg/mL – 7.3 ng/mL: 4000-fold).

For some species the level of induced cytokines were similar for the different isolates (strains) tested within that species (e.g *L. salivarius*) whereas for other species the strain immune profile varied considerably, especially for the *L. casei* strains where IL-10 and IL-12p70 differed by 3-fold and 35-fold, respectively. Interestingly the *B. animalis* strain was not capable of inducing IL-10, IL-12p70 and IFN-γ. The *L. salivarius* HMI001 (HMI001) induced high amounts of IL-10 and low amounts of IL-12p70 and IFN-γ, while the *L. plantarum* WCFS1 strain (WCFS1) induced less IL-10, and moderate amounts IL-12p70 and IFN-γ (Table 2). Also *L. casei* Shirota (LCS) a probiotic previously shown to attenuate allergic responses [9] showed an interesting profile by inducing high amounts of Th1 cytokines, IFN-γ and IL-12p70, but low amounts of IL-10. Based on these distinct immunoprofiles the three strains were tested for their prophylactic effect in a mouse model of allergic sensitization to peanut allergen. Strain HMI001 has a relatively high IL-10 /IL-12 ratio compared to other strains and was predicted to reduce the development of allergy by enhancing regulatory mechanisms to counteract allergic sensitization. The LCS strain was predicted to attenuate exaggerated Th2 responses due to the relatively high induction of Th1 cytokines such as IFN-γ which inhibits proliferation of Th2 cells. Strain WCFS1 was selected because it produces moderate amounts of pro- and anti-inflammatory cytokines and had an IL-10/IL-12 ratio which was lower than that of HMI001 and higher than the LCS strain.

PE-specific antibody responses are modulated by treatment with lactobacilli

In order to investigate if prophylactic treatment with probiotics modulates the PE-specific immune responses, mice were treated three times a week with $1x10^9$ CFU of HMI001, WCFS1 or LCS starting 2 weeks before the sensitization phase with PE and CT and ending on day 31.

Compared to non-sensitized mice, all PE sensitized mice developed PE-specific IgG1, IgG2a, and IgE antibody responses, indicating that all mice became sensitized (Fig. 2). While administration of WCFS1 caused a significant increase in the PE-specific IgG1, IgG2a and IgE antibody levels, administration of HMI001 lead to a significant decrease in IgE levels (Fig. 2). In contrast, administration of the LCS strain had no significant effect

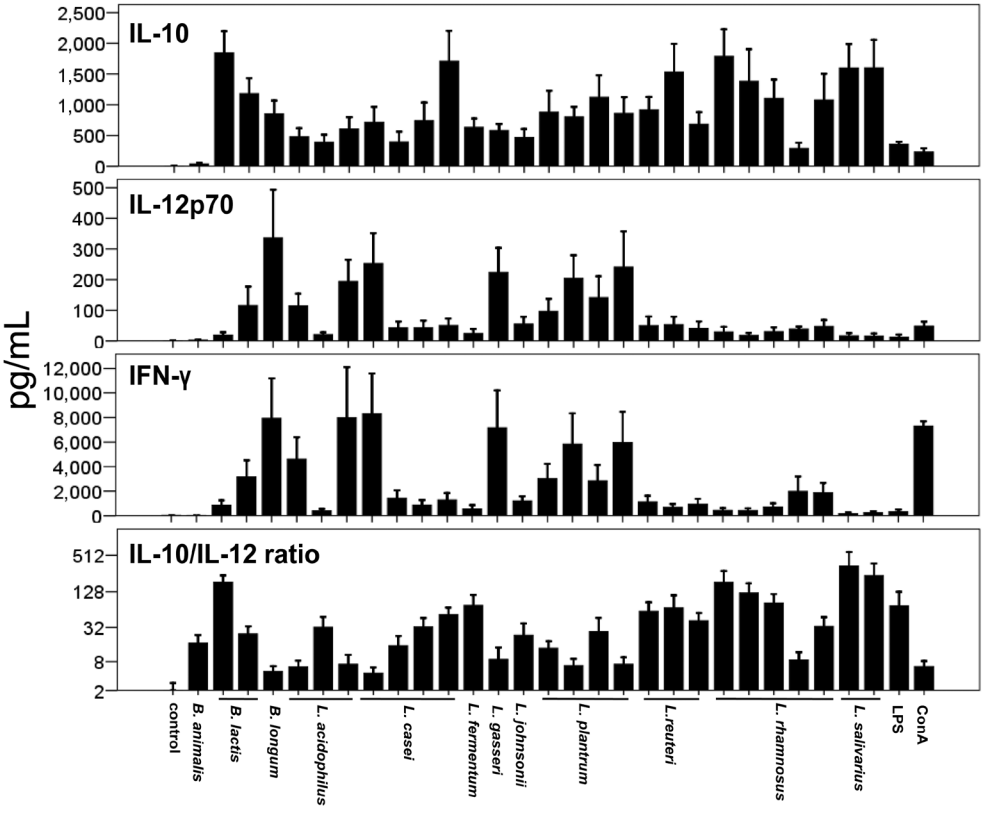

Figure 1: Effect of 28 probiotic strains on the cytokine production of human PBMCs (n=4 donors).

on the PE-specific antibody titers.

In PE sensitized mice mast cell degranulation is modulated by treatment with lactobacilli
After a six week probiotic treatment and a four week oral exposure regime to PBS or PE with CT, all mice received an oral challenge with PE. Thirty minutes after PE challenge the serum concentration of mMCP-I was measured as a read-out for the type I hypersensitivity response leading to mast cell degranulation. Control mice sensitized to PE had significantly higher levels of mMCP-1 in the serum after peanut allergen challenge compared to non-sensitized mice (Fig 2). Mice administered WCFS1 had significantly higher levels of serum mMCP-1 compared to the control, whereas serum mMCP-1 was lower in the mice given LCS or HMI001 (Fig. 2).

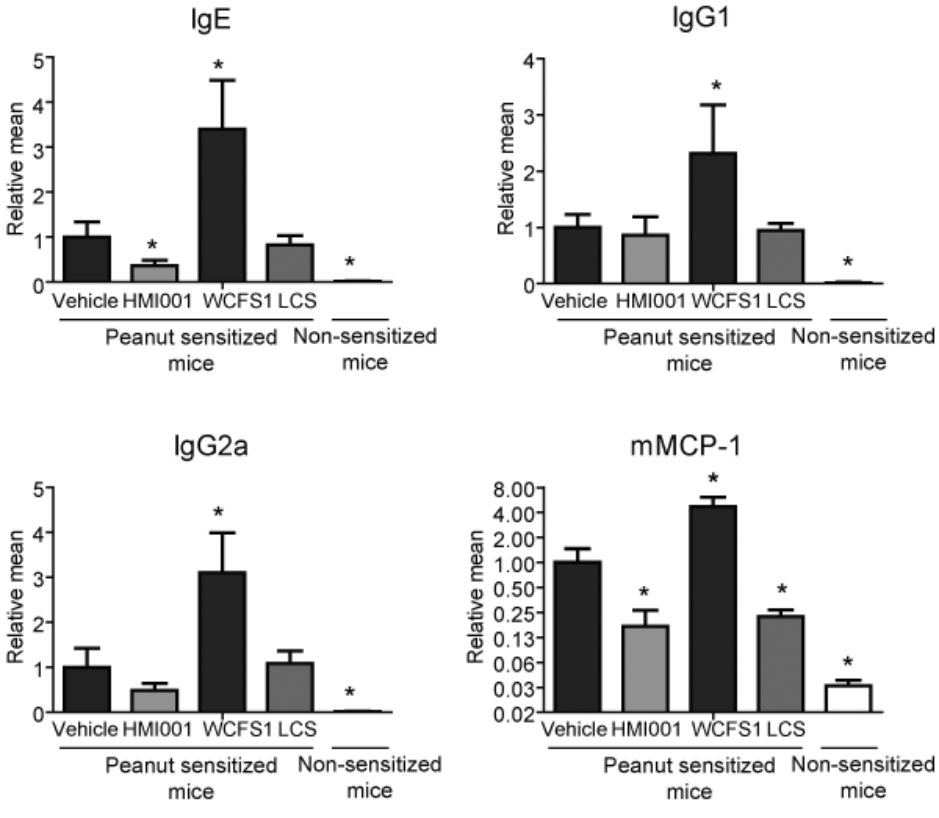

Figure 2: Effect of probiotic treatment *in vivo* on mast cell degranulation and PE-specific antibody response in a mouse model ofpeanut allergy. Data was normalized to sensitized vehicle treated mice. Values for sensitized vehicle treated mice were for HMI001 and WCFS1: mMCP-1 200 AU, IgG1 1229 AU, IgG2a 12588 AU and IgE 2332 AU and for LCS: mMCP-1 6 , IgG1 7 , IgG2a 4 and IgE 3 .
Note that the values for LCS are 2log antibody titers. *P <0.05 compared to the vehicle treated PE sensitized mice.

Treatment with lactobacilli modulated the ex vivo *cytokine response to peanut allergen*
Splenocytes from PE sensitized and non-sensitized mice were stimulated *ex vivo* in the presence or absence of PE. Unstimulated splenocytes secreted low amounts of cytokines (data not shown). PE stimulated splenocytes from the vehicle control group of peanut sensitized mice produced increased amounts of IL-4, IL-5, and IL-10 compared to splenocytes from non-sensitized mice (Fig. 3). Prophylactic treatment with HMI001 reduced IL-5 production compared to PE sensitized control mice, but this was not significant. A reduction in levels of induced Th2 cytokines IL-4 and IL-5 was also observed by treatment of PE-sensitized mice with LCS. In contrast, WCFS1 augmented the Th2 phenotype as shown by an increase in IL-4 production compared to the vehicle control (not significant) (Fig. 3). IL-10 and IFN-γ levels were not affected by any of the lactobacilli.

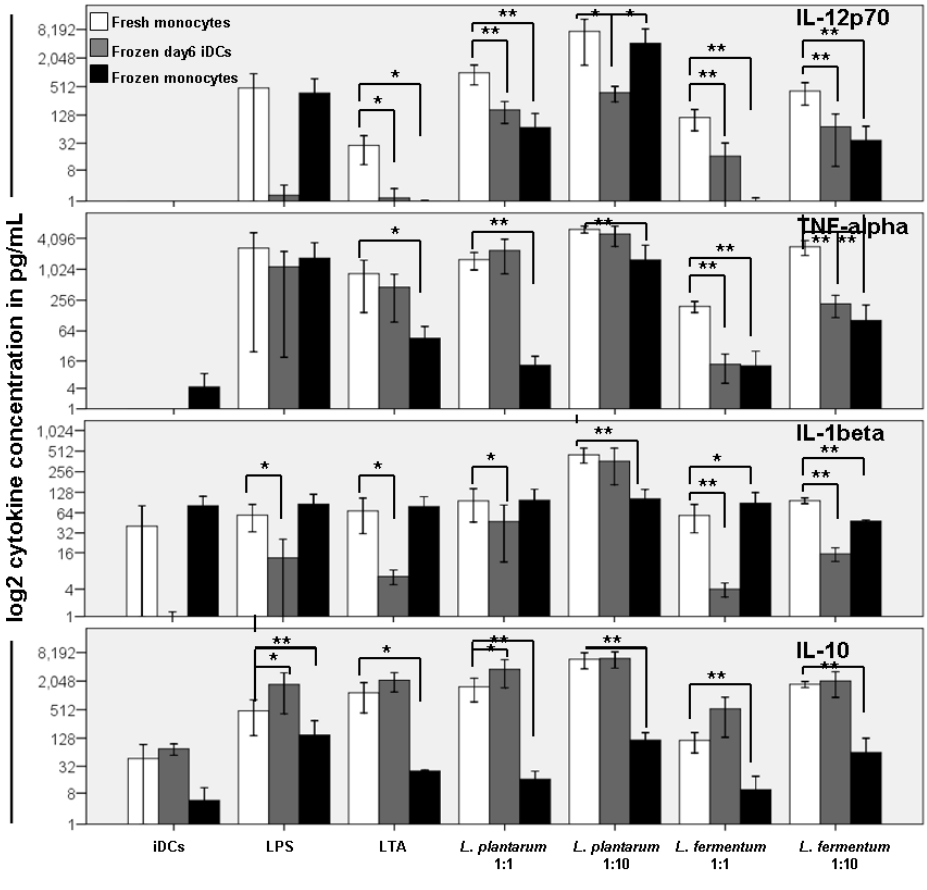

Figure 3: Effect of probiotic treatment on *ex vivo* cytokine production of PE-stimulated splenocytes from peanut sensitized mice. Data was normalized to sensitized vehicle treated mice. Values for sensitized vehicle treated mice were for HMI001 and WCFS1: IL-4 213 pg/mL, IL-5 328 pg/mL, IL-10 5585 pg/mL and IFN-γ 25599 pg/mL. and for LCS: IL-4 42 pg/mL, IL-5 156 pg/mL, IL-10 1622 pg/mL and IFN-γ 4647 pg/mL *P <0.05 compared to the vehicle treated PE sensitized mice.

Discussion

In this study we determined the immunomodulatory properties of 28 different bacterial strains, isolated from commercially available products, in a co-culture assay with human PBMCs. The different bacterial strains varied considerably in their ability to modulate PBMC immune responses. The strain differences in IL-10 (632-fold) and IL-12 (464-fold) induction, are much higher than that reported for different *Bifidobacterium longum* strains (8-fold) [29] and for multiple *Lactobacillus* and *Bifidobacterium species* in PBMC co-culture assays (10-15-fold) [15, 29, 51-53]. For some species, i.e. *L. salivarius*, the levels of induced cytokines were similar for the different strains within the species, whereas large variations in cytokine levels were measured using different strains of *L. casei*. These results are in line with other studies describing very different and even opposing effects of different species of *Lactobacillus* on DC activation [26]. Similarly, co-culture of DCs with different strains of *B. longum* led to variable cytokine responses and co-culture with PBMCs led to the secretion of either pro-inflammatory or regulatory cytokines [29]. Large variation in the ability of different *Lactobacillus* and *Bifdobacterium* strains to induce IL-12p70, IL-10, tumor necrosis factor alpha (TNF-α) and IFN-γ release in co-culture with PBMCs was also reported by Foligne et al. [51]. The ability of the strains to confer protection against TNBS-induced colitis *in vivo* correlated with their potential to induce high amounts of IL-10 and low amounts of IL-12. Furthermore strains inducing high IL-10/IL-12 ratios in MLNs have recently been shown to induce tolerogenic DCs and expand the CD4+ Treg population in mice [13].

Based on the PBMC immune profiles of the strains tested in this study three different potential probiotic strains were selected to investigate their immunomodulatory properties in an established mouse model of peanut allergy. The *L. salivarius* strains (including the HMI001 strain) were capable to produce high amount of IL-10 and low amounts of IL-12p70 (i.e. a high IL-10/ IL-12 ratio; Fig. 1). Prophylactic treatment with HMI001 treatment prior to sensitization to PE led to a slight, but significant, decrease in IgE titres compared to the vehicle-treated mice. In addition, HMI001 treatment also led to a significant diminution in mast cell degranulation (7-fold lower) upon oral challenge with peanut allergen. A reduction in mMCP-I is considered to be clinically relevant, as mediators released from mast cells cause clinical symptoms in allergic patients. Furthermore, prophylactic treatment with HMI001 reduced the *ex vivo* Th2-associated IL-5 production, although this was not significant (p=0.15). A recent study showed that probiotics selected on the ability to induce a high IL-10/ IL-12 ratio can suppress experimental immune disorders in mice, such as inflammatory bowel disease, atopic dermatitis, and rheumatoid arthritis, by inducing Tregs [13]. This suggests a possible mechanism for the prophylactic effects of HMI001 in the peanut allergy model but flow cytometric analysis did not reveal any changes in the percentage of CD4+CD25+Foxp3+ T cells or CD4+CD25-Foxp3+ T cells in lactobacilli treated mice (data not shown). One possibility is that the Tregs in the HMI001 treated group have a greater suppressive activity on activated T cells as described in a recent immunodulation study using mixtures of

probiotics [13].
LCS induced high amounts of IFN-γ and IL-12p70, but low amounts of IL-10 in co-culture with PBMCs (Fig. 1), and was selected on the basis that it might counter balance the Th2 cell induced hyper responsiveness, by promoting Th1 cell development via IFN-γ and IL-12. Although prophylactic treatment with LCS did not significantly modulate the PE-specific antibody responses compared to vehicle-treated PE sensitized mice, Th2 cytokine production (IL-4 and IL-5) in *ex vivo* stimulated splenocytes was significantly reduced. This is compatible with a skewing of the immune response towards Th1 and the immunomodulatory properties of the strain *in vitro*. Furthermore, LCS treatment led to a significant decrease in mast cell degranulation (7-fold lower) upon oral challenge with peanut allergen. LCS treatment was previously shown to reduce ovalbumin-specific IgE and IgG1 responses and diminished systemic anaphylaxis by promoting a dominant Th1-type response mediated by IL-12 induction in a mouse model for food allergy [54, 55]. Furthermore LCS administration has been shown to attenuate seasonal allergic rhinitis in adults [9]. However, LCS did not decrease allergic responses to peanut extract in Brown Norway rats [56].
The *L. plantarum* strain WCFS1 was selected because it produced moderate amounts of IL-10, IL-12 and IFN-γ compared to other strains and had an IL-10/IL-12 ratio which was lower than that of HMI001 and higher than the LCS strain. In the PE sensitization model, treatment with *L. plantarum* strain WCFS1 increased PE-specific antibody levels 3-fold compared to vehicle-treated peanut sensitized mice, which was accompanied by a marked increase in mast cell degranulation after challenge. Additionally, WCFS1 treatment augmented the *ex vivo* production of the Th2 cytokine IL-4 in PE- stimulated splenocytes. Interestingly, a study on Peyer's Patches (PP)-DCs showed that antigenic challenge of PP-DCs from sensitized C3H/HeJ but not Balb/c mice in the presence of IL-4 failed to inhibit the production of IL-10 and produced less IL-12p70, by that increasing the susceptibility to food allergy [57]. IL-4 promotes IL-12p70 production by DCs by suppressing the production of IL-10 by DCs themselves [58]. Taken together these data indicate that treatment with WCFS1 increased PE-specific humoral response and the development of type I hypersensitivity to peanut allergen. Based on these results we suggest that WCFS1 may be a useful strain to enhance immunity during infection or immune responses to vaccination, but not in conditions of allergy.
The mouse peanut allergy model used in this study mimics the clinical and immunologic characteristics of peanut allergy in human subjects [59, 60]. In this mouse model the anaphylaxis induced by intragastric sensitization and challenge with peanut extract closely reflects the clinical characteristics in human subjects. This is based on the model described by Li et al., showing that peanut-induced anaphylaxis was IgE mediated, and mast cell degranulation and histamine release were associated with the anaphylactic symptoms [59]. Therefore we think that results obtained in our model are relevant to the human population.
Enhanced levels of PE-specific IgE and mast cell degranulation have been correlated as a worsening of food allergy [59, 61]. In contrast, helminth infections are known to in-

crease a Th2 response, also characterized by an increase in IgE and IL-10, which protects against allergies. In the latter case, the stimulation of the Th2 response is accompanied by induction of local regulatory mechanisms, such as TGF-β production in the intestine [62-64]. An increase in TGF-β can lead to expansion of Tregs which may modulate allergic responses [65]. However in this study the increase in PE specific IgE was coupled to increased mast cell degranulation and heightened sensitization to PE.

Several *in vitro* experiments have evaluated the effects of lactobacilli strains on the modulation of the immune response. Different strains have been shown to induce maturation of DCs [26] stimulate the production of IFN-γ, IL-12, IL-10, and IL-18 [15], activate human monocytes [66], inhibit the production of Th2 classified cytokines, and induce IL-10-producing Tregs [16]. One or more of these immunomodulatory properties may play a role in the prevention of allergic sensitization. Different studies showed that differences in Treg activity may play a role in the development and resolution of food allergies, but for probiotic treatment this might not be the only protective mechanism. Using an animal model of food allergy, Zhang et al. showed that oral feeding of bifidobacteria favored the generation of IL-10+ DCs in the intestine, enhanced the number of Tregs and suppressed the Th2 pattern of inflammation [67]. In the current study flow cytometric analysis did not reveal any changes in the percentage of CD4+CD25+ and Foxp3+T cells in any of the lactobacilli treated mice compared to vehicle-treated mice (data not shown). Although it has been shown that naturally occurring CD4+CD25+ Tregs are important for the modulation of the PE allergic response [60, 68], it could be that other regulatory cells, such as Tr1 (IL-10 secreting) or Th3 (TGF-β secreting) cells play a more imperative role in probiotic modulation [69]. In food allergy multiple mechanisms might play simultaneous roles, making it difficult to predict the applicability of probiotics to treat food allergy. If more detailed knowledge of the mechanisms underlying their effect becomes available, it might well be possible to improve therapy by using cocktails of different probiotics, combining specialized regulatory features of the various probiotics.

Screening potential probiotics using human PBMCs was useful in selecting strains with predicted anti-inflammatory and Th1 skewing properties. In the case of HMI001 (inducing a high IL-10/IL-12 ratio) and LCS (inducing high amounts of IFN-γ and IL-12) partial protection was seen in a mouse peanut allergy model. Selection of strains with different profiles may worsen the allergic reaction as shown in the case of WCFS1. Although further research is needed to validate the immunomodulatory mechanisms involved *in vivo*, this approach is likely to be useful for selecting strains for translational research in humans.

Acknowledgement

This work was carried out within the research program of the Netherlands Consortium for Systems Biology (NCSB), which is part of the Netherlands Genomics Initiative / Netherlands Organization for Scientific Research. Reference serum for IgG1, IgG2a and IgE ELISA and PE-digoxigenin were a kindly gift to TNO from IRAS.

References

1. Fabia, R., et al., The effect of exogenous administration of *Lactobacillus reuteri* R2LC and oat fiber on acetic acid-induced colitis in the rat. Scand J Gastroenterol, 1993. 28(2): p. 155-62.
2. Gionchetti, P., et al., Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: a double-blind, placebo-controlled trial. Gastroenterology, 2000. 119(2): p. 305-9.
3. Madsen, K.L., et al., *Lactobacillus* species prevents colitis in interleukin 10 gene-deficient mice. Gastroenterology, 1999. 116(5): p. 1107-14.
4. Mao, Y., et al., The effects of *Lactobacillus* strains and oat fiber on methotrexate-induced enterocolitis in rats. Gastroenterology, 1996. 111(2): p. 334-44.
5. Mimura, T., et al., Once daily high dose probiotic therapy (VSL#3) for maintaining r emission in recurrent or refractory pouchitis. Gut, 2004. 53(1): p. 108-14.
6. Rembacken, B.J., et al., Non-pathogenic Escherichia coli versus mesalazine for the treatment of ulcerative colitis: a randomised trial. Lancet, 1999. 354(9179): p. 635-9.
7. Kalliomaki, M., et al., Guidance for Substantiating the Evidence for Beneficial Effects of Probiotics: Prevention and Management of Allergic Diseases by Probiotics. J Nutr, 2010.
8. Rijkers, G.T., et al., Guidance for Substantiating the Evidence for Beneficial Effects of Probiotics: Current Status and Recommendations for Future Research. J Nutr, 2010.
9. Ivory, K., et al., Oral delivery of *Lactobacillus casei* Shirota modifies allergen-induced immune responses in allergic rhinitis. Clin Exp Allergy, 2008. 38(8): p. 1282-9.
10. Resta-Lenert, S.C. and K.E. Barrett, Modulation of intestinal barrier properties by probiotics: role in reversing colitis. Ann N Y Acad Sci, 2009. 1165: p. 175-82.
11. Wells, J.M., L.M. Loonen, and J.M. Karczewski, The role of innate signaling in the homeostasis of tolerance and immunity in the intestine. Int J Med Microbiol, 2009.
12. Delcenserie, V., et al., Immunomodulatory effects of probiotics in the intestinal tract. Curr Issues Mol Biol, 2008. 10(1-2): p. 37-54.
13. Kwon, H.K., et al., Generation of regulatory dendritic cells and CD4+Foxp3+ T cells by probiotics administration suppresses immune disorders. Proc Natl Acad Sci U S A, 2010. 107(5): p. 2159-64.
14. Matsuzaki, T., et al., The effect of oral feeding of *Lactobacillus casei* strain Shirota on immunoglobulin E production in mice. J Dairy Sci, 1998. 81(1): p. 48-53.
15. Miettinen, 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(12): p. 6058-62.
16. Pochard, P., et al., Lactic acid bacteria inhibit TH2 cytokine production by mononuclear cells from allergic patients. J Allergy Clin Immunol, 2002. 110(4): p. 617-23.
17. Daniel, C., et al., Selecting lactic acid bacteria for their safety and functionality by use of a mouse colitis model. Appl Environ Microbiol, 2006. 72(9): p. 5799-805.
18. Hessle, C., L.A. Hanson, and A.E. Wold, Lactobacilli from human gastrointestinal mucosa are strong stimulators of IL-12 production. Clin Exp Immunol, 1999. 116(2): p. 276-82.
19. Fujiwara, D., et al., The anti-allergic effects of lactic acid bacteria are strain dependent and mediated by effects on both Th1/Th2 cytokine expression and balance. Int Arch Allergy Immunol, 2004. 135(3): p. 205-15.
20. Matsumoto, S., et al., Probiotic *Lactobacillus*-induced improvement in murine chronic inflammatory bowel disease is associated with the down-regulation of pro-inflammatory cytokines in lamina propria mononuclear cells. Clin Exp Immunol, 2005. 140(3): p. 417-26.

21. Shida, K., et al., Induction of interleukin-12 by *Lactobacillus* strains having a rigid cell wall resistant to intracellular digestion. J Dairy Sci, 2006. 89(9): p. 3306-17.
22. Dominitzki, S., et al., Cutting edge: trans-signaling via the soluble IL-6R abrogates the induction of FoxP3 in naive CD4+CD25 T cells. J Immunol, 2007. 179(4): p. 2041-5.
23. Papamichail, M., et al., Natural killer lymphocytes: biology, development, and function. Cancer Immunol Immunother, 2004. 53(3): p. 176-86.
24. Smits, H.H., 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(6): p. 1260-7.
25. Wells, J.M., et al., Microbes and Health Sackler Colloquium: Epithelial crosstalk at the microbiota-mucosal interface. Proc Natl Acad Sci U S A, 2010.
26. Christensen, H.R., H. Frokiaer, and J.J. Pestka, Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. J Immunol, 2002. 168(1): p. 171-8.
27. Maassen, C.B., et al., Strain-dependent induction of cytokine profiles in the gut by orally administered *Lactobacillus* strains. Vaccine, 2000. 18(23): p. 2613-23.
28. Niers, L.E., et al., Selection of probiotic bacteria for prevention of allergic diseases: immunomodulation of neonatal dendritic cells. Clin Exp Immunol, 2007. 149(2): p. 344-52.
29. Medina, M., et al., Differential immunomodulatory properties of *Bifidobacterium longum* strains: relevance to probiotic selection and clinical applications. Clin Exp Immunol, 2007. 150(3): p. 531-8.
30. Meijerink, M., et al., Identification of genetic loci in *Lactobacillus plantarum* that modulate the immune response of dendritic cells using comparative genome hybridization. PLoS One, 2010. 5(5): p. e10632.
31. van Hemert, S., et al., Identification of *Lactobacillus plantarum* genes modulating the cytokine response of human peripheral blood mononuclear cells Submitted, 2010.
32. Vissers, Y.M., et al., Differential effects of *Lactobacillus acidophilus* and *Lactobacillus plantarum* strains on cytokine induction in human peripheral blood mononuclear cells. FEMS Immunol Med Microbiol, 2010.
33. Hougee, S., et al., Oral treatment with probiotics reduces allergic symptoms in ovalbumin-sensitized mice: a bacterial strain comparative study. Int Arch Allergy Immunol, 2010. 151(2): p. 107-17.
34. Karlsson, M.R., J. Rugtveit, and P. Brandtzaeg, Allergen-responsive CD4+CD25+ regulatory T cells in children who have outgrown cow's milk allergy. J Exp Med, 2004. 199(12): p. 1679-88.
35. Smith, M., et al., Children with egg allergy have evidence of reduced neonatal CD4(+) CD25(+)CD127(lo/-) regulatory T cell function. J Allergy Clin Immunol, 2008. 121(6): p. 1460-6, 1466 e1-7.
36. Shreffler, W.G., et al., Association of allergen-specific regulatory T cells with the onset of clinical tolerance to milk protein. J Allergy Clin Immunol, 2009. 123(1): p. 43-52 e7.
37. Sampson, H.A., L. Mendelson, and J.P. Rosen, Fatal and near-fatal anaphylactic reactions to food in children and adolescents. N Engl J Med, 1992. 327(6): p. 380-4.
38. Le, T.M., et al., Reported food allergy to peanut, tree nuts and fruit: comparison of clinical manifestations, prescription of medication and impact on daily life. Allergy, 2008. 63(7): p. 910-6.
39. Sicherer, S.H., et al., US prevalence of self-reported peanut, tree nut, and sesame allergy: 11-year follow-up. J Allergy Clin Immunol, 2010. 125(6): p. 1322-6.
40. Lee, L.A. and A.W. Burks, New insights into diagnosis and treatment of peanut food allergy. Front Biosci, 2009. 14: p. 3361-71.

41. Clark, A.T. and P.W. Ewan, Good prognosis, clinical features, and circumstances of peanut and tree nut reactions in children treated by a specialist allergy center. J Allergy Clin Immunol, 2008. 122(2): p. 286-9.
42. Abrahamsson, T.R., et al., Probiotics in prevention of IgE-associated eczema: a double-blind, randomized, placebo-controlled trial. J Allergy Clin Immunol, 2007. 119(5): p. 1174-80.
43. Folster-Holst, R., et al., Prospective, randomized controlled trial on *Lactobacillus rhamnosus* in infants with moderate to severe atopic dermatitis. Br J Dermatol, 2006. 155(6): p. 1256-61.
44. Gruber, C., et al., Randomized, placebo-controlled trial of *Lactobacillus rhamnosus* GG as treatment of atopic dermatitis in infancy. Allergy, 2007. 62(11): p. 1270-6.
45. Kalliomaki, M., et al., Probiotics during the first 7 years of life: a cumulative risk reduction of eczema in a randomized, placebo-controlled trial. J Allergy Clin Immunol, 2007. 119(4): p. 1019-21.
46. Kukkonen, K., 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(1): p. 192-8.
47. Moro, G., et al., A mixture of prebiotic oligosaccharides reduces the incidence of atopic dermatitis during the first six months of age. Arch Dis Child, 2006. 91(10): p. 814-9.
48. Passeron, T., et al., Prebiotics and synbiotics: two promising approaches for the treatment of atopic dermatitis in children above 2 years. Allergy, 2006. 61(4): p. 431-7.
49. Taylor, A.L., J.A. Dunstan, and S.L. Prescott, Probiotic supplementation for the first 6 months of life fails to reduce the risk of atopic dermatitis and increases the risk of allergen sensitization in high-risk children: a randomized controlled trial. J Allergy Clin Immunol, 2007. 119(1): p. 184-91.
50. Viljanen, M., et al., Probiotics in the treatment of atopic eczema/dermatitis syndrome in infants: a double-blind placebo-controlled trial. Allergy, 2005. 60(4): p. 494-500.
51. Foligne, B., et al., Correlation between *in vitro* and *in vivo* immunomodulatory properties of lactic acid bacteria. World J Gastroenterol, 2007. 13(2): p. 236-43.
52. Miettinen, M., J. Vuopio-Varkila, and K. Varkila, Production of human tumor necrosis factor alpha, interleukin-6, and interleukin-10 is induced by lactic acid bacteria. Infect Immun, 1996. 64(12): p. 5403-5.
53. Niers, L.E., et al., Identification of strong interleukin-10 inducing lactic acid bacteria which down-regulate T helper type 2 cytokines. Clin Exp Allergy, 2005. 35(11): p. 1481-9.
54. Shida, K., et al., *Lactobacillus casei* inhibits antigen-induced IgE secretion through regulation of cytokine production in murine splenocyte cultures. Int Arch Allergy Immunol, 1998. 115(4): p. 278-87.
55. Allakhverdi, Z., et al., Thymic stromal lymphopoietin is released by human epithelial cells in response to microbes, trauma, or inflammation and potently activates mast cells. J Exp Med, 2007. 204(2): p. 253-8.
56. de Jonge, J.D., et al., *Lactobacillus casei* Shirota does not decrease the food allergic response to peanut extract in Brown Norway rats. Toxicology, 2008. 249(2-3): p. 140-5.
57. Temblay, J.N., et al., Production of IL-12 by Peyer patch-dendritic cells is critical for the resistance to food allergy. J Allergy Clin Immunol, 2007. 120(3): p. 659-65.
58. Yao, Y., et al., Interleukin (IL)-4 inhibits IL-10 to promote IL-12 production by dendritic cells. J Exp Med, 2005. 201(12): p. 1899-903.
59. Li, X.M., et al., A murine model of peanut anaphylaxis: T- and B-cell responses to a major peanut allergen mimic human responses. J Allergy Clin Immunol, 2000. 106(1 Pt 1): p. 150-8.
60. van Wijk, F., et al., The CD28/CTLA-4-B7 signaling pathway is involved in both allergic

sensitization and tolerance induction to orally administered peanut proteins. J Immunol, 2007. 178(11): p. 6894-900.
61. Li, X.M., et al., Food Allergy Herbal Formula-1 (FAHF-1) blocks peanut-induced anaphylaxis in a murine model. J Allergy Clin Immunol, 2001. 108(4): p. 639-46.
62. Yazdanbakhsh, M. and P.M. Matricardi, Parasites and the hygiene hypothesis: regulating the immune system? Clin Rev Allergy Immunol, 2004. 26(1): p. 15-24.
63. Flohr, C., R.J. Quinnell, and J. Britton, Do helminth parasites protect against atopy and allergic disease? Clin Exp Allergy, 2009. 39(1): p. 20-32.
64. Flohr, C., et al., Reduced helminth burden increases allergen skin sensitization but not clinical allergy: a randomized, double-blind, placebo-controlled trial in Vietnam. Clin Exp Allergy, 2010. 40(1): p. 131-42.
65. Dittrich, A.M., et al., Helminth infection with Litomosoides sigmodontis induces regulatory T cells and inhibits allergic sensitization, airway inflammation, and hyperreactivity in a murine asthma model. J Immunol, 2008. 180(3): p. 1792-9.
66. Miettinen, M., et al., Lactobacilli and Streptococci activate NF-kappa B and STAT signaling pathways in human macrophages. J Immunol, 2000. 164(7): p. 3733-40.
67. Zhang, L.L., et al., Oral *Bifidobacterium* modulates intestinal immune inflammation in mice with food allergy. J Gastroenterol Hepatol, 2010. 25(5): p. 928-34.
68. van Wijk, F., et al., CD4+CD25+ T cells regulate the intensity of hypersensitivity responses to peanut, but are not decisive in the induction of oral sensitization. Clin Exp Allergy, 2007. 37(4): p. 572-81.
69. Allez, M. and L. Mayer, Regulatory T cells: peace keepers in the gut. Inflamm Bowel Dis, 2004. 10(5): p. 666-76.

Chapter 7

Immunomodulating effects of orally administered *L. plantarum* strains in a mouse vaccination model

M. Meijerink, L. Loonen, N. Taverne, E. Kranenbarg, B. Rappard, P. de Vos, R. Pels Rijcken, H.F.J. Savelkoul, J. van Bilsen[#] and J.M. Wells[#]

Submitted

[#] Authors equally contributed to the paper

Abstract

Background
Influenza is a globally important respiratory pathogen which causes a high degree of morbidity and mortality annually. Both mucosal and systemic immunity contributes to resistance to influenza infection and disease. Probiotics, including *L. plantarum* strains, may be considered as adjuvants to increase the efficacy of influenza vaccine.

Objective
To test whether intragastric (i.g) administration of selected *L. plantarum* strains, possessing distinct immunomodulatory properties, can modulate systemic immune responses to an influenza vaccine in mice.

Methods
We established an influenza vaccination model in young female Balb/c mice. Modulation of cellular immune responses to vaccination by six *L. plantarum* strains was studied by measurement of delayed type hypersensitivity (DTH) responses to the vaccine, cytokines, chemokines and innate receptors gene expression measurements in mesenteric lymph node cells and by cytokine secretion of mesenteric lymph node cells stimulated *ex vivo* with ConA and vaccine HA antigen. The vaccine-specific serum antibody titres for IgG1 and IgG2a subclasses were measured in serum by ELISA.

Results
Oral (i.g.) administration of *L. plantarum* strains LMG18021 and WCFS1 significantly modulated the immune responses to an intranasal influenza vaccine. LMG18021 augmented the Th1 phenotype in the mouse model (increased DTH, higher IgG2a/IgG1 ratio, higher IL-12 and TNF-α expression levels and higher antigen specific response). In contrast, WCFS1 augmented the Th2 response (higher IgG1 response, higher IL-13 and lower TNF-α expression levels).

Conclusion and Clinical relevance
L. plantarum strains LMG18021 and WCFS1 are promising candidates to test in an intranasal influenza vaccination and challenge model. *In vitro* immune assays with human dendritic cells are useful tools to select candidate strains for immune modulation studies in mice.

Introduction

Influenza is an important respiratory pathogen which causes a high degree of morbidity and an estimated 250,000 to 500,000 deaths each year worldwide (WHO). Vaccination is still the most effective method of preventing mortality and morbidity, despite the improvements in antiviral therapy with amantadine or rimantadine. The efficacy of antiviral therapy is about 70% in treating influenza type A infection [1]. In high-income countries influenza vaccination is used to reduce mortality in people aged over 65 and other risk groups. The current inactivated vaccines are aimed at eliciting high-levels of virus neutralizing antibody to influenza surface HA antigen thereby inhibiting binding to the host cells which provides efficient protection from influenza infection. However, due to the high mutation rate of the virus, caused by antigenic shift and antigenic drift, a particular vaccine formulation is effective for at most about one year. Therefore each year, three strains are chosen for development of the seasonal vaccine in the Northern and Southern Hemispheres based on the 'The WHO Global Influenza Surveillance Network of National Influenza Centres'. The efficacy of influenza vaccine varies each year depending on the match between vaccine subtypes and circulating viral strains, patient's age, and pre-existing immunity. Poor immunogenicity may also be a problem with certain vaccines as exemplified by the development of vaccines for the pandemic H5N1 avian influenza. In this case two 90 µg doses of the inactivated split virus were required to produce levels of seroconversion observed with only a single dose of the seasonal flu vaccine [2]. Despite the current vaccination policies influenza infection still remains a severe burden particularly in elderly individuals over 60 years [3-4].

In young healthy adults, the inactivated influenza vaccine provides protective clinical efficacy in 70-90% of cases, but this is reduced to only 17-53% in elderly individuals [5]. In addition to the inactivated vaccine a live attenuated influenza vaccine (LAIV) has been licensed by the Food and Drug Administration (FDA) since 2003. The LAIV contains live but attenuated influenza viruses, and is sprayed into the nostrils rather than injected intramuscularly. As the FDA have approved the use LAIV in healthy children and adults from 2-49 years of age, it could be used to control pandemic influenza outbreaks. To improve the efficacy of vaccines several approaches have been investigated such as increasing the antigen dose in the vaccine (Centers for Disease Control and Prevention, 2010), using alternative routes of administration such as intradermal injection [6] and using mucosal immune adjuvants. None of these strategies are fully efficacious or suffer from specific drawbacks. Therefore novel approaches are still needed to increase the efficacy of oral vaccines such as polio, rotavirus and influenza. One attractive and relatively easily implemented approach could be to utilize dietary supplementation with immune stimulating probiotics.

Probiotics have been shown to enhance specific immune responses to vaccination in young children and in adults. In a study on cholera vaccination several probiotic strains were tested for their effect on antibody responses to the commercial oral cholera vaccine [7]. The probiotic strains were given daily over a period of three weeks in which

the oral cholera vaccine was also administered. In the early phase of the response serum IgG responses tended to increase in most probiotic treated groups and significant increases were observed for *Bifidobacterium lactis* B1-04 and *Lactobacillus acidophilus* La-14 compared to controls [7]. However, no effects on the final end point immunoglobulin titres to the vaccine were found [7]. The effects of *L. rhamnosus* LGG on a rhesus-based rotavirus vaccine were shown to significantly increase humoral antibody IgM responses but not cell-mediated immunity (T cell responses to antigen stimulation). In a different study consumption of LGG or *L. acidophilus* CRL431 for five weeks doubled neutralization antibody titres to an oral polio vaccine given in the fifth week [8]. In the group consuming probiotics a marked increase in polio-virus specific IgA was observed after vaccination. The effects of LGG and *L. lactis* on a *Salmonella typhi* Ty21a vaccine were shown to not significantly increase numbers of IgA, IgG and IgM secreting cells [9]. There was however a trend towards a higher antigen-specific IgA levels in subjects treated with LGG treatment. Together these findings show that specific probiotic strains can enhance the immunogenicity of orally administered vaccines such as rotavirus [10], *Salmonella* [9], polio [8], and cholera [7].

Although the focus of much research on probiotics and immunity has centered on enteric infections and oral vaccination [7-10], there is also evidence that probiotics can stimulate immune responses to respiratory pathogens [11]. Furthermore a recent study on LGG as an immune adjuvant for LAIV in healthy adults showed significantly improved protective serum responses to one of the three viruses present in the vaccine [12]. This has important implications for the use of probiotics to stimulate immunity to respiratory pathogens and enhance responses to other intranasally delivered vaccines.
Little is known about the mechanisms underlying the positive effects of probiotics on mucosal vaccines but it is generally assumed that this involves stimulation of innate immunity via microbe associated molecular patterns (MAMPs). Despite the fact that different MAMPs are generally conserved in major groups of bacteria, strikingly different immune responses to different species and strains are observed *in vitro* [13-14]. This should be given more consideration in relation to *in vivo* studies as it may reveal the underlying mechanisms and explain the different effects of probiotic strains observed *in vivo*. Recently probiotic strains that induce high IL-10 to IL-12 cytokine ratios in mouse bone marrow derived dendritic cells (DCs) were shown to expand the CD4+ Foxp3+ regulatory T cell population after oral administration [15]. This was linked to a beneficial effect in different mouse models of inflammatory disease but it might have converse effects with respect to enhancement of immunity or vaccination. The *in vitro* immunomodulatory properties of probiotics have been largely ignored in previous studies on their use as adjuvants but it warrants further investigation.

As certain strains of probiotic lactobacilli have been shown to enhance immunity to respiratory pathogens and intranasally administered vaccines such as LAIV, we undertook a mechanistic study to compare the effects of different selected strains on an intrana-

sal influenza vaccine. In general the probiotic field suffers from a lack of comparative studies using the same models and assays with different strains *in vitro* and *in vivo*. In addition to LGG which was taken as a control, we selected 6 strains of *Lactobacillus plantarum* that have strikingly different immunomodulatory properties *in vitro* (Table 1). This selection was based on previous studies examining the *in vitro* immune responses of human monocyte derived DCs and peripheral mononuclear blood cells (PBMCs) to 42 different *L. plantarum* strains [13-14]. First we established an influenza vaccination model in Balb/c mice that would be sensitive to immunomodulation by lactobacilli. Cellular immune responses were assessed after vaccination by measurement of delayed type hypersensitivity (DTH) responses and cytokine responses of mesenteric lymph node (MLN) cells after polyclonal stimulation with concanavalin A (Con A) or the vaccine. In addition mRNA levels of different cytokines, chemokines and innate receptors were measured in naïve MLN cells after vaccination. Modulation of the humoral immune response to vaccination by different lactobacilli was assessed by measurement of the vaccine-specific serum antibody concentrations.

Table 1: Immunomodulating properties of selected strains in DCs [13] .

Strain	IL-10	IL-12	TNF	IL-10/IL-12 ratio
KOG18	335 (61)	5421 (337)	4167 (139)	2 (0.4)
CIP104448	67(10)	1006 (43)	582 (73)	8 (0.0)
LMG18021	135 (22)	1331 (76)	1285 (141)	12 (2.2)
CIP104450	595 (106)	3347 (248)	5441 (70)	15 (2.9)
WCFS1	173 (30)	3118 (231)	1895 (182)	3 (0.4)
B1839	37 (5)	53 (3)	42 (8)	2 (0.3)

Data presents average (SEM) of 5 donors.

Materials and Methods

Bacterial strains

Bacteria, stored at – 80 °C in de Man,Rogosa Sharpe (MRS) medium plus 10% glycerol, were streaked on MRS solidified with 1.4% agar and incubated overnight at 37 °C. Cultivation was carried out until an optical density (OD)600 1- 1.5 was obtained. Bacteria were harvested by centrifugation, washed twice in 0.2 M $NaHCO_3$, and resuspended in 0.2 M $NaHCO_3$ plus 10% glycerol to an estimated concentration of $2x10^9$ colony forming units (CFU) per mL. Bacterial suspensions were dispensed into aliquots and stored at –80 °C. Aliquots of these stock suspensions were used to determine the actual number of CFU per mL. On each day of dosing, an aliquot of each bacterial strain was thawed and diluted in 0.2 M $NaHCO_3$ buffer to a dose concentration of 10^9 CFU/mL.

Mice

Female Balb/c mice, specific pathogen-free, were purchased from Harlan (Correzzana, Italy) and were maintained under barrier conditions in macrolon type III cages with wood chips bedding, at mean temperature of 22 ± 2 °C, a relative humidity of between 40% and 70% and a 12-h light/dark cycle. Drinking water and standard laboratory food pellets were provided ad libitum. Mice were 6 weeks old at the start of probiotic dosing. The welfare of the animals was maintained in accordance with the general principles governing the use of animals in experiments of the European Communities (Directive 86/609/EEC) and Dutch legislation (The Experiments on Animals Act, 1997).

Dose responsiveness to the intranasally administered Influvac vaccine

The tri-valent influenza vaccine Influvac 2010/ 2011 (Solvay, Weesp, The Netherlands) contains an A/California/7/2009 (H1N1)-like strain (A/California/7/2009 NYMC X-179A reass., 15 µg haemagglutinin (HA)/ dose), an A/Perth/16/2009 (H3N2)-like strain (A/Wisconsin/15/2009 NYMC X-183 reass., 15 µg HA/ dose) and a B/Brisbane/60/2008-like strain (B/Brisbane/60/2008, 15 µg HA/ dose). In order to determine the appropriate vaccine dose to use in the immunization model with probiotics, five groups of mice (n=4) were intranasally administered 0.1, 0.3, 0.9, 1.8 or 3.6 µg of the Influvac 2010/2011 vaccine. One group (n=4) was not immunized with the influenza vaccine and served as a control group. To obtain a positive reference serum, 4 mice were intramuscularly immunized with 9 µg influenza HA vaccine on days 0 and 14. Serum from these mice was pooled and used as a standard serum in the antibody ELISAs.

Vaccination and probiotic dosing protocol

The study comprised 9 groups of 6 week old Balb/c mice. Group 1 (n=10) consisted of vaccine control mice that were immunised with the influenza vaccine and given a buffer instead of lactobacilli as indicated below. Groups 2-8 (n=10) were immunised with the influenza vaccine and received one of the lactobacilli strains. Group 9 (n=5) were administered a buffer instead of the vaccine and lactobacilli. The bacterial strains ($1x10^9$

CFU/ dose) were diluted to a volume of 250 µl and were administered three times a week by oral gavage (day 0-40). Groups 1-8 were intranasally immunized with a dose of 3.6 µg influenza HA (Influvac 2010/2011) on days 14 and 28.

Detection of HA-specific IgG1 and IgG2a in serum

Vaccine-specific antibody concentrations were measured in serum after days 0, 28 and at the end of the intervention period (day 44). To do this, flat bottom plates (NUNC Immuno Plate, Roskilde, Denmark) were coated (overnight, 2-10 °C) with 2 µg HA/mL, dissolved in 0.2 M $NaHCO_3$. After washing the plates three times in wash buffer (phosphate buffered saline (PBS) / 0.1% v/v Tween-20 solution; Sigma-Aldrich), the plates were blocked with 1% w/v Bovine Serum Albumin (BSA) in PBS (1 hr, RT). After removing the blocking buffer, serial dilutions of the serum (in PBS / 1% w/v BSA) were incubated in duplicate (1.5 hr, RT). Eight dilutions of serum were used for ELISAs, the starting dilution of the serum was 15 times for IgG1 and 50 times for IgG2a. The plates were washed again 3 times in wash buffer and then incubated for 1.5 hr at RT with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG1 (AbD Serotec, Dusseldorf, Germany) or goat anti-mouse IgG2a [AbD Serotec] diluted 10.000 times in PBS / 1% BSA. After washing the plates three times, the HRP detection substrate 3,3',5,5'-tetramethylbenzidine solution was added for approximately 15 minutes at RT before stopping the reaction with 2N H_2SO_4. Optical density was measured at 450 nm using a Benchmark Plus Microplate Spectrophotometer (Bio-Rad Laboratories, Richmond, CA). The standard curves were modeled using the four-parameter logistic regression and were used to calculate the antibody-concentrations.

Delayed type hypersensitivity response

The delayed type hypersensitivity (DTH) reaction was evaluated by assessing ear swelling reactions. On day 42, the animals were intradermally injected with Influvac HA vaccine in both ears (20 µL per ear, containing 1.8 µg HA). Ear thickness was measured in duplicate using a digital micrometer (Mitutoyo, Veenendaal, the Netherlands), shortly before challenge (reference ear thickness) and 24 and 48 hours after challenge. The ear swelling was calculated by subtracting the basal ear thickness from the value at 24h or 48h after challenge.

Gene transcript quantification in MLNs

Total RNA was isolated from the MLNs of each group using RNeasy kit (Qiagen) with a DNase digestion step. The quantity of RNA was determined using the nanodrop device (Thermoscientific, Wilmington, USA). Reverse transcription was performed on 0.5 µg of total RNA using qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, USA). The optimized blend of random and oligo(dT) primers provides robust, consistent, and unbiased first-strand synthesis over a broad range of RNA template concentrations. PRIMER EXPRESS (Applied Biosystems, Foster City, CA, USA) and primer3 software were used to design primers for use in real-time quantitative PCR (qPCR) (Table 2). Primers for

IL-10 (PPM03017B-200) and IL-13 (PPM03021A-200) were ordered from Qiagen (Venlo, The Netherlands). For qPCR 5 µl cDNA and forward and reverse primers (300 nM each) were added to 12.5 µl GoTaq qPCR Master Mix (Promega, Leiden, The Netherlands) and demineralized water was added to a final volume of 25 µl. qPCR (2 min 95 °C, 40 cycles of 15 s at 95 °C, 60 s at 60 °C followed by 2 min at 60 °C) was carried out on a Rotorgene 2000 real-time cycler (Corbett Research, Sydney, Australia). Raw data were analysed with comparative quantitation of the Rotor-gene Analysis Software V5.0. Changes in transcript levels were calculated relative to the vaccinated control mice according to the Pfaffl equation: Ratio = (E_{target}) Ct $_{target}$ (control-sample)/$(E_{reference})$Ct $_{reference}$ (control-sample) [16]. Two internal reference genes (L32 ribosomal protein and β-actin) were included in every q-PCR experiment and the results were similar using either gene for standardisation. To control for possible DNA contamination in the RNA samples 'non-RT'-controls were included in all experiments and no amplification above background levels was observed. No-template controls were also included for each gene in each run and no amplification above background levels was observed. Specificity of the amplification was ensured by checking the melting temperature and profile of each melting curve. The PCR product of each template was checked at least once by DNA sequencing (Baseclear, Leiden, The Netherlands).

Cell culture and cytokine measurement

On the day of necropsy (day 44), mouse MLNs were gently disrupted to obtain single cell suspensions for *ex vivo* cytokine analysis. The cell suspensions were adjusted to viable 5 x 10^6 cells/mL in enriched RPMI-1640 medium (RMPI 1640 with Glutamax™ and 25 mM HEPES buffer supplemented with 10% heat inactivated FCS and 1% penicillin/streptomycin). The MLN cells were stimulated in the presence or absence of HA vaccine antigen (5 µg/ mL) for 72 hours or the mitogenic T cell stimulant Con A (5 µg/ mL) (Sigma) for 24 hours at 37°C and 5% CO_2. Culture supernatants were stored in -80°C until cytokines were measured by multiplex analyses (Mouse Th1/Th2/ Th17 CBA kit, BD).

TLR signalling assays

TLR signalling assays were performed on stably transformed cell lines of human embryonic kidney cells (HEK293) expressing TLR-2 and TLR-6 and transfected with a reporter plasmid (pNiFTY) containing firefly luciferase under the control of the human NF-κB promoter. HEK293 cells do not produce TLRs, but when stably transformed with TLRs they can activate NF-κB upon addition of the appropriate TLR agonists. TLR transfected cells were seeded at 5 x 10^5 cells/cm^2. Cells were incubated with different probiotic strains (1:15 cell to bacteria ratio), or Pam2CSK4 (Invivogen) agonists as a control (5 µg/mL). After six hours incubation the medium was replaced with Bright-Glo luciferase assay buffer (Promega) and luminescence intensity was measured in a Spectramax M5 reader (Molecular devices) within 15 minutes. TLR-2 ligands signal through two types of TLR-2 receptors, one linked to TLR-1 and one to TLR-6. TLR-2/6 is known to be activated by MAMPs associated with Gram-positive bacteria, i.e. lipoteichoic acid and diacylated

lipoproteins and unmethylated CpG DNA.

Table 2: Primer sequences used for qPCR

Primer	Sequence
Fw L32	5'-GCC CAA GAT CGT CAA AAA GA-3'
Rv L32	5'-ATT GTG GAC CAG GAA CTT GC-3'
Fw IL-2	5'-CCT GAG CAG GAT GGA GAA TTA CA-3'
Rv IL-2	5'-TCC AGA ACA TGC CGC AGA G-3'
Fw IL-4	5'-ACA GGA GAA GGG ACG CCA T-3'
Rv IL-4	5'-GAA GCC CTA CAG ACG AGC TCA-3'
Fw IL-12p40	5'-GGA AGC ACG GCA GCA GAA TA-3'
Rv IL-12p40	5'-AAC TTG AGG GAG AAG TAG GAA TGG-3'
Fw IL-17A	5'-TTC ATC TGT GTC TCT GAT GCT-3'
Rv IL-17A	5'-TTG ACC TTC ACA TTC TGG AG-3'
Fw TNF-α	5'-CAT CTT CTC AAA ATT CGA GTG ACA A-3'
Rv TNF-α	5'-TGG GAG TAG ACA AGG TAC AAC CC-3'
Fw IFN-Y	5'-TCT TGG CTT TGC AGC TCT TC-3'
Rv IFN-Y	5'-TGT TGC TGA TGG CCT GAT TG-3'
Fw TGF-β	5'-GAA GGC AGA GTT CAG GGT CTT-3'
Rv TGF-β	5'-GGT TCC TGT CTT TGT GGT GAA-3'
Fw Foxp3	5'-CCC ATC CCC AGG AGT CTT G-3'
Rv Foxp3	5'-CCA TGA CTA GGG GCA CTG TA-3'
Fw IDO	5'-TGG CGT ATG TGT GGA ACC G-3'
Rv IDO	5'-CTG CAT AAG ACA GAA TAG GAG GC-3'
Fw PD1L	5'-ATG CTG CCC TTC AGA TCA CAG-3'
Rv PD1L	5'-TGG TTG ATT TTG CGG TAT GGG-3'
Fw CTLA-4	5'-AGA ACC ATG CCC GGA TTC TG-3'
Rv CTLA-4	5'-CAT CTT GCT CAA AGA AAC AGC AG-3'
Fw B7.1	5'-ACC CCC AAC ATA ACT GAG TCT-3'
Rv B7.1	5'-TTC CAA CCA AGA GAA GCG AGG-3'
Fw B7.2	5'-TGT TTC CGT GGA GAC GCA AG-3'
Rv B7.2	5'-CAG CTC ACT CAG GCT TAT GTT TT-3'
Fw ICOSL	5'-GAC TGA AGT CGG TGC AAT GGT-3'
Rv ICOSL	5'-TGG GTT TTC GAT TTG CCA ATA GA-3'
Fw β-Actin	5'-GGG ACC TGA CGG ACT AC-3'
Rv β-Actin	5'-TGC CAC AGG ATT CCA TAC-3'

Statistics

Data were analyzed using GraphPad Prism 4 software. The significance of differences between group means were determined by using one-way ANOVA with the Bonferroni *post hoc* test. Statistical analyses were performed following logarithmic transformation (to achieve normal distribution). $P<0.05$ was considered to be statistically significant.

Results

The intranasal vaccine-dose response

In order to determine a vaccine-dose that would be suitable for an immunomodulation study with lactobacilli, the mouse were intranasally administered 0.1, 0.3, 0.9, 1.8 or 3.6 µg of Influvac 2010/2011 vaccine on days 0 and 14. There was a clear dose–response relationship between the vaccine dose and vaccine specific antibody responses (Fig. 1A and B). Even at the lowest dose of 0.1 µg, vaccine-specific antibody responses were detected by d14, but no high levels of IgG2a were detectable at d28. The response to the lowest dose however, was significantly lower than those of the higher vaccine doses. In the vehicle group, no vaccine specific antibody titres were measured. In the group vaccinated with 3.6 µg of HA, all mice responded with high antibody titres to the vaccine but these responses were still 385-fold (1334 AU) and 22-fold (22624 AU) lower than the titres obtained for IgG1 and IgG2a respectively in mice immunized intramuscularly with 9 µg HA (500.000 AU). These results indicated that a dose of 3.6 µg of HA would enable us to study both the potentiation and attenuation of vaccine antibody responses and was therefore used in subsequent experiments to test the immunomodulatory effects of different *Lactobacillus* strains on intranasal immunization.

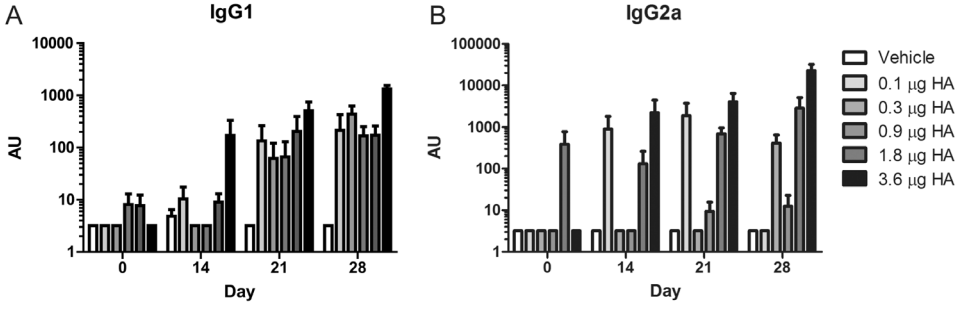

Figure 1 *Vaccine-dose responses in mice after intranasal immunization with different amounts of Influvac 2010/2011.* Vaccine-specific IgG1 (a) and IgG2a (b) concentration in arbitrary units (AU) in serum of mice. The concentrations were calculated relative to pooled serum (500 times diluted) from intramuscular vaccinated animals, with a defined concentration of 1000 AU. Error bars indicate SEM; n = 4.

Selection of Lactobacillus *strains for the immunization study*

Based on the distinct immune profiles seven *L. plantarum* strains were tested for their prophylactic effect in a mouse vaccination model (Table 1). Strains KOG18 and CIP104448 were both stimulating relatively high levels of Th1 promoting cytokines TNF-α and IL-12, and were predicted to enhance cell-mediated immunity. However the IL-10 and IL-12 ratio differed for KOG18 (low ratio) and CIP104448 (high ratio), which might influence their ability to enhance regulatory T cells. Strain WCFS1 was selected because it produces moderate amounts of both pro- and anti-inflammatory cytokines. Strains LMG18021

and CIP104450 were chosen for their intermediate stimulating capacities of all tested cytokines. Strain B1839 was selected because it induced barely detectable amounts of pro- and anti-inflammatory cytokines and was predicted not to enhance vaccination. *L. rhamnosus* LGG was selected as a control based on its previous use in influenza vaccination and challenge studies [8-10, 17-19].

Cytokine mRNA levels in unstimulated MLN cells
IL-12 mRNA levels were significantly higher in mice treated with CIP104450 or LMG18021 than that in control (vaccinated) mice (Fig. 2A). TNF-α mRNA levels were also significantly higher in the LMG18021 group but significantly lower in the mice administered WCFS1 or LGG. IL-13 mRNA levels tended to be higher in mice treated with WCFS1 than in the control group, but this difference was not statistically significant (p=0.062). IL-4 mRNA levels were lower in most groups treated with *L. plantarum* strains except for strain CIP104448. IL-2 mRNA levels were lower in KOG18, CIP104448 and LGG. There were no significant differences in IL-10, IL-17A, TGF, IFN-γ , Foxp3, IDO, PD1L, ICOSL, CTLA-4, B7.1 and B7.2 mRNA expression levels (Fig. 2A and B).

In these qPCR assays there were no significant differences between the control groups of vaccinated and non-vaccinated mice. These data suggest that strains LMG18021 and CIP104450 induce more Th1 skewing responses in unstimulated lymphoid cells draining the mucosal tissue. The increased levels of IL-13 and lower levels of TNF-α observed in the WCFS1 group are suggestive of increased Th2 responses in the mucosa-associated lymphoid tissue.

Ex vivo stimulation of MLN cells.
Cytokine production was measured in the culture supernatants after *ex vivo* stimulation of MLN cells with ConA or the vaccine antigen HA(Fig 3A). Strikingly, WCFS1 treated mice showed a significant decrease in the IL-10, TNF-α, IL-2 and IL-6 response to ConA compared to the vaccine control (Fig. 3A, data IL-2 and IL-6 not shown). Significantly lower IL-10 was also observed in the KOG18, CIP104450, CIP104448 treated mice (Fig. 3A). There were no significant effects of the *Lactobacillus* strains on the levels of IL-17A, IL-4 and IFN-γ compared to vaccinated mice administered a buffer control instead of lactobacilli (data not shown).
As expected the *ex vivo* cytokine response to HA was significantly lower in non-vaccinated mice compared to all the vaccinated groups. IL-2 and IFN-γ responses were not altered in the probiotic treated mice (data not shown).Interestingly MLN cells from CIP104450, LMG18021, and CIP104448 treated mice showed a significant increased IL-10, IL-17A, IL-6 and IL-4 in response to the vaccine (Fig. 3B). CIP104450 treated mice showed also an increased TNF-α vaccine induced cytokine response. As these differences were not apparent in ConA stimulated MLN cells, it can be concluded that the lactobacilli administration induced antigen-specific changes in the mucosal immune response.

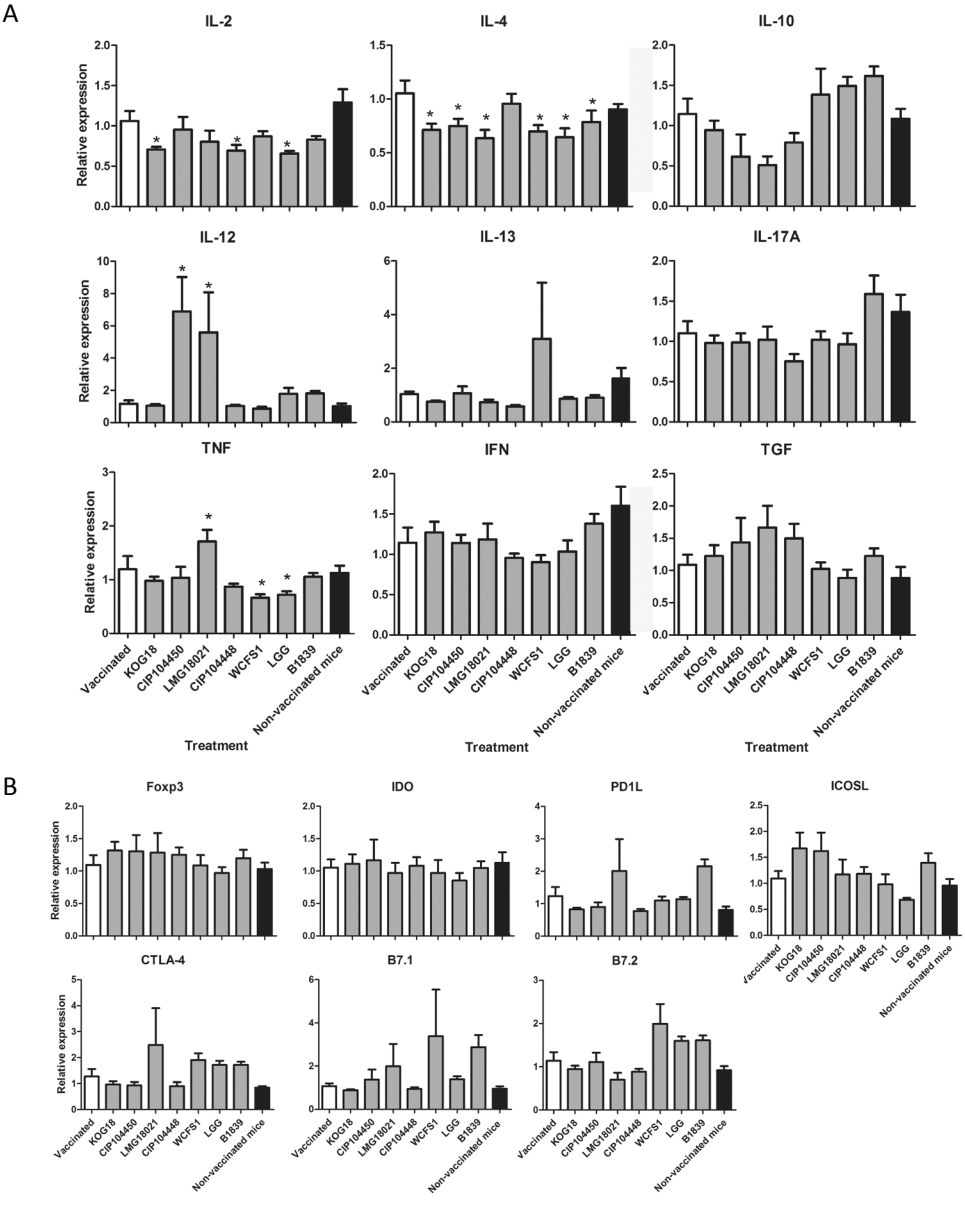

Figure 2 *Gene transcript quantification in unstimulated MLN cells*. qPCR was used to determine gene-specific mRNA levels relative to the housekeeping gene L32. * indicates $p < 0.05$ compared to vaccinated mice group, tested using ANOVA; error bars indicate SEM; n = 5 in sham group, n = 10 in all other groups.

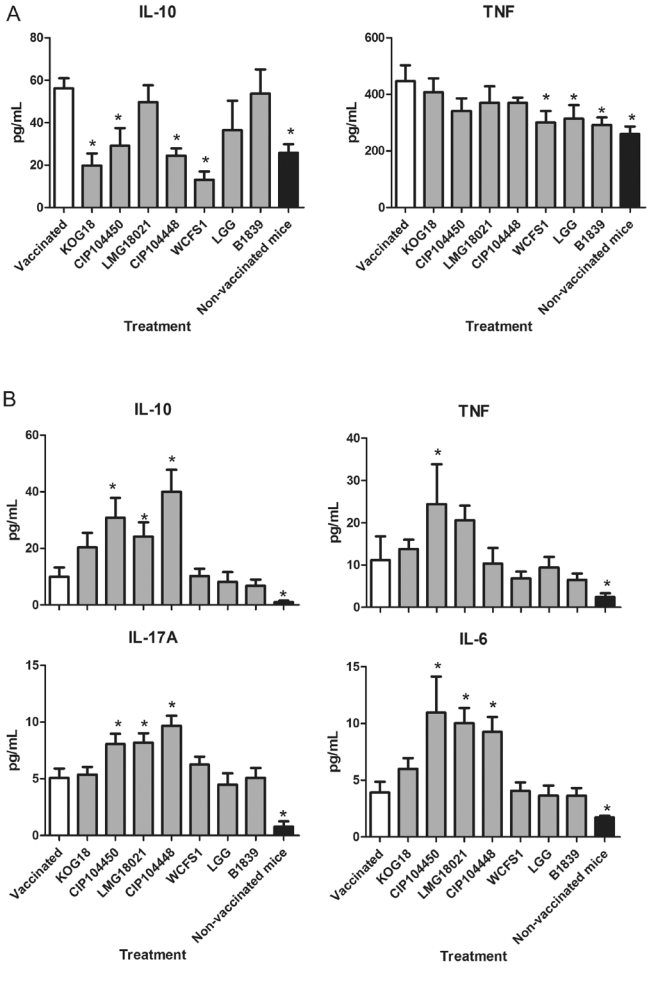

Figure 3 Ex vivo *cytokine responses in stimulated MLN cells*. MLN cells were stimulated with ConA 5 µg/mL (A) or HA antigen 5µg/mL (B) in pg/ mL. Probiotic treatment led to an increased vaccine-specific (HA) induced cytokine response *ex vivo*. *indicates $p < 0.05$ compared to vaccinated mice group, tested using ANOVA; error bars indicate SEM; n = 5 in sham group, n = 10 in all other groups.

Effect of lactobacilli on vaccine DTH response

The generation of a delayed-type hypersensitivity (DTH) response was used as a primary measurement of Th1-mediated immune response to HA although other cell types have been suggested to play a potential role in this hypersensitivity reaction [20]. On day 42, the DTH reaction was evaluated by assessing ear swelling reaction (Fig. 4). The DTH response in non-vaccinated mice was significantly lower than in all other groups of vaccinated mice. Administration of KOG18, CIP104450, LMG18021, LGG or B1839

resulted in a significant increase of the DTH response 24 hours after challenge, with LGG having the strongest effect. The WCFS1 strain gave a significant DTH response after 48 h but not 24 h after challenge. Strain CIP104448 (showing a high IL-10 to IL-12 ratio in DCs) did not induce a significant DTH response compared to the vehicle control mice. In conclusion some of the lactobacilli were able to increase the DTH response to the vaccine compared to the control vaccine group.

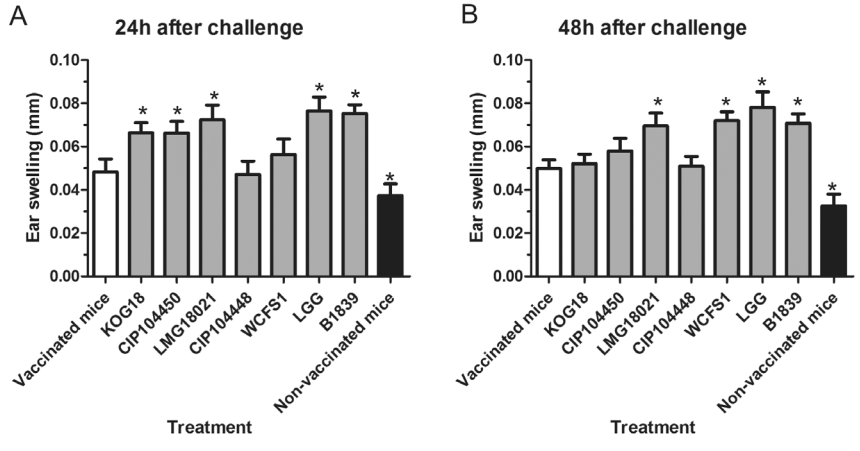

Figure 4 *Ear thickness after intradermal challenge*. Lactobacilli treatment leads to an increased DTH response. DTH responses to the vaccine measured by ear swelling after a) 24h and b) 48h. * indicates $p < 0.05$ compared to vaccinated mice group, tested using ANOVA; error bars indicate SEM; n = 5 in sham group, n = 10 in all other groups.

Modulation of vaccine-specific antibody titres by administration of lactobacilli

After the first immunization with Influvac 2010/2011, all groups had HA-specific antibody-titres at day 28, except the non-vaccinated mice (data not shown). The IgG1-titres increased > 20 fold after the second immunization, whereas IgG2a increased >35 fold. As expected, no HA-specific antibody titers were detected in the serum of non-vaccinated mice at day 44. The evaluation of the effects of lactobacilli-treatment on IgG1 titres, revealed that mice orally treated with WCFS1, had significantly higher HA-specific IgG1 titres (2-fold increase) (p=0.044) compared to the vaccine control group, administered a buffer instead of lactobacilli (Fig. 5A). This trend was also observed in KOG18-treated mice although it was not highly significant (p=0.055). Mice treated with LMG18021, CIP104448, LGG and B1839 showed statistically significantlower IgG2a titres as compared to the vaccine control group (Fig. 5B).

In mice, IgG2a antibodies are generally associated with Th1 activity, while IgG1 antibody responses are associated with Th2 activity, based on the ability of IFN-γ and IL-4 to stimulate and cross-regulate the production of these IgG subclasses [21]. Therefore, IgG2a/IgG1 ratios give an indication of relative Th1/Th2 balance and revealed that ad-

ministration of LMG18021 significantly increased the Th1 response to HA (p=0.008; Fig. 5C). CIP104450 administration also elevated the antigen-specific IgG2a/ IgG1 ratio by 3-fold compared to the vehicle control group, although this was not significant (p=0.07).

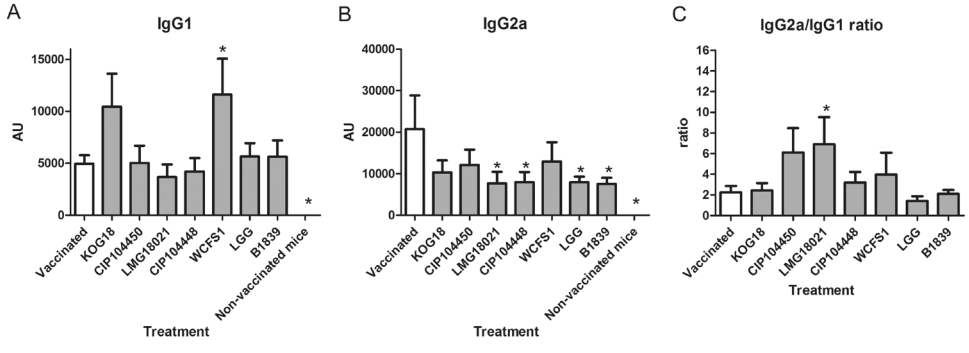

Figure 5 *HA-specific serum antibody titres for subclasses IgG1 (A) and IgG2a (B) in arbitrary units (AU) and the IgG2a/IgG1 ratio (C).* The concentrations were calculated relative to a pooled serum (500 times diluted) from intramuscular vaccinated animals, with a defined concentration of 1000 AU. * indicates $p < 0.05$ compared to vaccinated mice group, tested using ANOVA; error bars indicate SEM; n = 5 in sham group, n = 10 in all other groups. Note that some data points were left out in the calculation of the IgG2a/IgG1 ratio, due to the low value in IgG1, using Dixon's Q test.

L. plantarum strains differentially activate TLR-2

HEK-reporter cell lines that express TLR-2 and TLR-6 were used to compare the capacity of the *L. plantarum* strains to activate TLR-2/6 signaling (Fig. 6). Exposure of the TLR-2/6-expressing HEK-reporter cell line to LMG18021 strongly activated NF-κB compared to the other strains and the medium control.

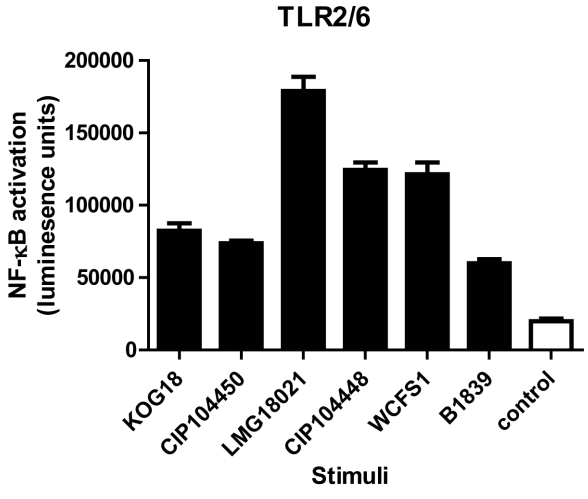

Figure 6 TLR activating properties selected *L. plantarum* strains
NF-κB pathway activation measured by a luminescence reporter in HEK cell lines expressing TLR-2/6 after exposure to different *L. plantarum* strains. The ratios of HEK reporter to bacterial cell were 1:15 and error bars indicate SEM; n=5.

Discussion

Here we showed that oral administration of certain strains of lactobacilli could enhance humoral antibody responses and modulate T cell responses to an intranasal influenza vaccine in mice. This may have important implications for the current public health strategy of reducing influenza-related mortality and morbidity by annual vaccination. One of the high-risk groups for complications due to influenza infection is elderly over 60 years for which the seasonal tri-valent inactivated vaccine (TIV) given by intramuscular injection is the current standard. Despite current vaccination policies, the elderly population has the highest infection rates and novel strategies are needed to improve the efficacy of the influenza vaccine [3]. Recent studies indicate that the intranasally administered LAIV is safe in elderly subjects and tended to have a higher efficacy than the inactivated vaccine in people over 70 years [22]. Thus dietary supplementation with specific strains of *L. plantarum* strains might further increase the efficacy of LAIV.

Serum antibody responses are believed to correlate with protection efficacy of TIV for influenza [23-24]. A specific-HA antibody response was only detected in groups of mice administered the vaccine. Of the six *L. plantarum* strains evaluated in this model, the WCFS1 strain significantly increased HA-specific antibody responses to the intranasal vaccine compared to the vaccine control group, a trend which was also seen with strain KOG18. Interestingly both these strains induce the lowest IL-10 to IL-12 cytokine ratio in human DC co-culture assays except for strain B1839 which induces hardly any cytokine responses in this assay (Table 1). In the group of mice administered WCFS1 we observed a significant decrease in IL-10 and TNF-α production after polyclonal stimulation of MLN cells with ConA. In unstimulated MLN cells, mRNA levels of the Th2 cytokine IL-13, also tended to be higher than in the vaccine control group but this did not reach significance ($p= 0.062$). In the WCFS1 group the TNF-α mRNA levels were lower than in the vaccine control group suggesting increased Th2 responses in the lymphoid tissue. Moreover a significantly higher IgG1 response ($p= 0.044$) was observed in the WCFS1 group compared to the vaccine control which in mice indicates a skewing of the vaccine T helper response towards Th2. Interestingly, WCFS1 was recently shown to increase sensitization to peanut extract in an allergic sensitization model (Meijerink et al 2011, submitted), further supporting the idea that this strain enhances Th2 responses. Taken together these results indicate that oral administration of WCFS1 strain can enhance humoral immune responses to the intranasal vaccine.
Several *Lactobacillus* strains appeared to increase DTH responses after vaccination compared to the vaccine control group suggesting Th1-mediated vaccine responses. For strain LMG18021 this was also reflected in the significantly higher HA-specific IgG2a to IgG1 antibody ratio. The Fc portion of IgG2a interacts with complement factors [25] and activating Fc receptors [26-28] with a high affinity which efficiently activates receptor-mediated effector function. This stimulates the antibody dependent cell-mediated cytotoxicity [29] and opsonisation and subsequent phagocytosis by macrophages [30], which

is important in the contribution to the clearance of influenza virus from the infected host [31]. The Fc portions of IgG1 antibodies are binding with a lower affinity to the Fc receptor than the Fc portion of IgG2a, [32-33] and increased IgG2a antibody titres to influenza have been associated with increased vaccination efficacy [31, 34-36]. *In vitro* LMG18201 induces relatively high amounts of Th1 promoting cytokines and relatively low amounts of IL-10 compared to the other strains. The induction of influenza-specific memory T cells producing IFN-γ (Th1 cytokine) is vital for a rapid response to influenza reinfection [37]. The importance of IFN-γ in protection against influenza has also been demonstrated in genetically altered mice [38]. The exact reasons why some strains like LMG18021 promote Th1 responses *in vitro* and *in vivo* are unknown but recent insights come from a study of a phosphoglycerol transferase gene mutant in *L. acidophilus* [39]. The mutant is unable to synthesize lipoteichoic acid (LTA) and in comparison to the wild type strain induction of IL-12 was substantially reduced. Moreover, IL-10 levels were increased in co-culture with human monocyte derived DCs [39]. As LTA is an agonist of TLR-2/6, the reduction in Th1 promoting cytokines observed in immune assays with the mutant may reflect a reduced ability to stimulate innate signalling via the TLR-2/6 pathway. Circumstantial evidence supporting this hypothesis comes from our finding that the Th1 promoting strain LMG18021 was the strongest inducer of NF-κB activation in a TLR-2/6 reporter cell assay, which is also reflected in the significantly increased expression of TNF-α in naive MLN cells. LMG18021, CIP104448 and CIP104450, which induce the highest ratio of IL-10 to IL-12 among the strains tested, significantly enhanced the *ex vivo* HA-specific induction of IL-10, IL-17A, IL-6 and IL-4 in MLN cells. Elevation of these cytokines was not observed in MLN cells polyclonally stimulated with ConA, a lectin which cross-links the T cell receptor, thereby mimicking the T cell receptor activation without the requirement of co-stimulatory signals. ConA polyclonal stimulation of T cells is often used to assess their maximal immune responsiveness *ex vivo*. These results show that these strains specifically enhance the antigen-specific HA response to vaccination.

The mucosal immune system contains a large network of immune cells that traffic from inductive sites to effector sites in the mucosal tissues. Immunization studies have previously shown that cells traffic from the gut to the lungs, to enhance local immunity to clear *Pseudomonas aeruginosa* or *Haemophilus influenza* [40-41]. Thus the HA-specific response measured in the MLN cells might be due to trafficking of vaccine-primed antigen presenting cells or T cells from the nasal mucosa to the MLNs. This might be a mechanism by which orally administered lactobacilli can enhance responses to an intranasal vaccine. HA-specific immune responses in the MLN cells could also result from some of the intranasal vaccines being swallowed but this seems less likely as the protocol has been optimized to specifically avoid this possibility. Furthermore, much higher doses of purified antigen are generally needed to elicit responses by the oral route of administration than via the intranasal route.

Evidence to support the influence of intestinal microbiota on respiratory immune responses comes from recent work on mice treated with antibiotics [42]. Commensal

bacteria are crucial in maintaining immune homeostasis of the intestine, but also in regulating immune defense against respiratory tract influenza A infection [42]. Oral antibiotic treatments resulted in defective CD4+ T, CD8+ T and B cell immunity following intranasal infection with influenza virus. Rectal inoculation of CpG (TLR-9 agonist), poly I:C (TLR-3 agonist) and to a lesser extent peptidoglycan/cell wall components (TLR-2 agonists) could restore immunity in the lung. This indicates that signals coming from distal commensal bacteria may be sufficient to support immune priming in the lung. Antibiotic treatment led to impaired respiratory distribution, steady state synthesis of pro–IL-1β, pro–IL-18, and NLRP3 and reduced DC migration from lung to the draining lymph nodes.

Several human and animal studies have shown the potential of probiotics to function as immune adjuvants. In one study healthy volunteers developed higher vaccine-specific IgA antibodies compared to the placebo group, consuming lyophilized LGG for seven days and receiving an attenuated oral *Salmonella typhi* vaccine capsule on days 1, 3 and 5 [9]. Additionally healthy volunteers consuming fermented milk containing *L. acidophilus* La1 and bifidobacteria had an increased *S. typhi* specific serum IgA, compared to the placebo group [45]. In a study with oral rotavirus vaccine infants supplemented with LGG had higher titres of IgA and higher numbers of rotavirus-specific IgM-secreting cells than the placebo group [10]. The administration of LGG before an oral polio booster, has also been associated with increased poliovirus-neutralizing antibody titres and poliovirus- specific IgA and IgG [8]. In a placebo-controlled trial using a supplement containing *L. paracasei*, elderly patients receiving the influenza and pneumococcal vaccines had increased NK cell activity and IL-2 secretion in PBMCs and a decreased number of infections when compared with placebo group [46]. In another randomized placebo-controlled trial, elderly patients who received Actimel containing *L. casei* DN-114 001 for six weeks had higher influenza-specific antibody titres after intramuscular vaccination [19]. In a trial of healthy adults, *L. fermentum* CECT5716 improved the immunogenicity of an intramuscular applied inactivated tri-valent influenza vaccine, showing an increased number of NK cells in blood, increased levels of serum Th1 cytokines and an increase in antigen specific IgA [47]. Additionally the incidence of influenza-like illness after vaccination was lower in the probiotic treated group. Collectively, these studies clearly indicate the potential for specific strains of lactobacilli to enhance immune responses to vaccines administered via the intranasal or oral route.

Besides the adjuvant effects of oral lactobacilli on vaccination, several studies in humans and animals that support the role of specific strains of *Lactobacillus* in the prevention of influenza infection. In an animal study, where mice pups were fed *L. casei* Shirota supplement for 3 weeks before inoculation with influenza virus, the probiotic treated pups had better survival (40% versus 14%). Moreover the *L. casei* Shirota treated mice had lower viral titres in the nasal lavages, higher pulmonary NK cell activity, and higher IL-12 production by MLN cells compared with control mice [48]. In a separate study intranasal administration of *L. casei* Shirota, was reported to enhance cellular immunity in the respiratory tract and to protect against influenza virus infection in mice [49].

Furthermore, oral administration of *L. casei* Shirota activated not only the systemic immune system but also the local immune system and ameliorated influenza virus infection in aged mice [50]. In a mouse model of influenza infection with an H1N1 strain, 3 days of intranasal exposure to LGG was significantly associated with a lower frequency of accumulated symptoms and a higher survival rate than in control mice [17]. Additionally using the same mouse model of influenza infection, oral administration of LGG or *L. gasseri* TMC0356 for 19 days was associated with lower clinical symptom scores and pulmonary virus titres as compared with control mice [18]. In studies of children in day-care centers, administration of LGG was associated with a decrease in upper respiratory infections and with the duration of infections [51-52].

In our study the probiotic LGG was included as it has been shown to confer protective effects in challenge studies with influenza [17-18] and to enhance the efficacy of intramuscular influenza vaccination [19]. In contrast in our study there was only an enhanced DTH response observed in the mice treated with LGG, but no effect on HA-specific antibodies in serum and on *ex vivo* cell response. The challenge studies did not show any data on antibody titres in serum [17-18], so the serum levels might also be unaffected, indicating that LGG might be more beneficial for NK cell function, which was not measured in our study.

Although the most common vaccination route of influenza A is intramuscular, we hypothesized that intragastric probiotic treatment could lead to an enhanced respiratory immunity. It has been shown that mucosal immune responses can be more efficiently achieved by the mucosal administration of vaccines, i.e. oral, rectal, vaginal or intranasal. Additionally, intranasal vaccines are easier to administer and avoid the use of needles and syringes leading to decreased risk of infection from contaminated blood, better public compliance and reduced costs. Nevertheless many vaccines could benefit from strategies to enhance the efficacy and the required type of immune response in different target populations. Our results suggest that *L. plantarum* strains LMG18021 and WCFS1 could be used as probiotics to enhance immunity to intranasally administered vaccines such as LAIV. Whether these increased vaccine-specific antibody titres and enhanced *ex vivo* vaccine induced T cell responses we have observed with certain lactobacilli will finally lead to increased protection has to be investigated in a future mouse study. In this study HA-specific IgA responses in the nasal lavages were too low to quantify in our assay. As intranasal vaccination has the potential to enhance protection by eliciting vaccine-specific secretory IgA in the respiratory tract which could block virus entry and neutralize intracellular virus assembly, more sensitive methods should be used to qualify the IgA in the nasal lavage or other time points should be taken to investigate the effects of probiotics. Additionally NK cell activity could also be altered by probiotic administration, as this seems to be another important route of the beneficial effects of for instance probiotic LGG [17-18, 48-49], therefore NK cell activity assays could be also included in future studies with these strains.

In conclusion, this study shows that oral administration of specific strains of lactobacilli can enhance humoral or Th1-mediated immune responses to an intranasal influenza

vaccine. Thus oral consumption of selected immunomodulatory probiotics may be one strategy to enhance vaccine responses to intranasal vaccination against influenza.

References

1. Hayden, F.G., Antivirals for pandemic influenza. J Infect Dis, 1997. 176 Suppl 1: p. S56-61.
2. Treanor, J.J., et al., Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. N Engl J Med, 2006. 354(13): p. 1343-51.
3. Simonsen, L., et al., Mortality benefits of influenza vaccination in elderly people: an ongoing controversy. Lancet Infect Dis, 2007. 7(10): p. 658-66.
4. Lang, P.O. and J.P. Michel, [Vaccination: a programme for the whole life]. Rev Med Suisse, 2010. 6(270): p. 2118-24.
5. Goodwin, K., C. Viboud, and L. Simonsen, Antibody response to influenza vaccination in the elderly: a quantitative review. Vaccine, 2006. 24(8): p. 1159-69.
6. Holland, D., et al., Intradermal influenza vaccine administered using a new microinjection system produces superior immunogenicity in elderly adults: a randomized controlled trial. J Infect Dis, 2008. 198(5): p. 650-8.
7. Paineau, D., et al., Effects of seven potential probiotic strains on specific immune responses in healthy adults: a double-blind, randomized, controlled trial. FEMS Immunol Med Microbiol, 2008. 53(1): p. 107-13.
8. de Vrese, M., et al., Probiotic bacteria stimulate virus-specific neutralizing antibodies following a booster polio vaccination. Eur J Nutr, 2005. 44(7): p. 406-13.
9. Fang, H., et al., Modulation of humoral immune response through probiotic intake. FEMS Immunol Med Microbiol, 2000. 29(1): p. 47-52.
10. Isolauri, E., et al., Improved immunogenicity of oral D x RRV reassortant rotavirus vaccine by *Lactobacillus casei* GG. Vaccine, 1995. 13(3): p. 310-2.
11. Lykova, E.A., et al., [Disruption of microbiocenosis of the large intestine and the immune and interferon status in children with bacterial complications of acute viral infections of the respiratory tract and their correction by high doses of bifidumbacterin forte]. Antibiot Khimioter, 2000. 45(10): p. 22-7.
12. Davidson, L.E., et al., *Lactobacillus* GG as an immune adjuvant for live-attenuated influenza vaccine in healthy adults: a randomized double-blind placebo-controlled trial. Eur J Clin Nutr, 2011. 65(4): p. 501-7.
13. Meijerink, M., et al., Identification of genetic loci in *Lactobacillus plantarum* that modulate the immune response of dendritic cells using comparative genome hybridization. PLoS One, 2010. 5(5): p. e10632.
14. van Hemert, S., et al., Identification of *Lactobacillus plantarum* genes modulating the cytokine response of human peripheral blood mononuclear cells. BMC Microbiology, 2010. 10(1): p. 293.
15. Kwon, H.K., et al., Generation of regulatory dendritic cells and CD4+Foxp3+ T cells by probiotics administration suppresses immune disorders. Proc Natl Acad Sci U S A, 2010. 107(5): p. 2159-64.
16. Pfaffl, M.W., A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res, 2001. 29(9): p. e45.
17. Harata, G., et al., Intranasal administration of *Lactobacillus rhamnosus* GG protects mice from H1N1 influenza virus infection by regulating espiratory immune responses. Lett Appl Microbiol, 2010. 50(6): p. 597-602.
18. Kawase, M., et al., Oral administration of lactobacilli from human intestinal tract protects mice against influenza virus infection. Lett Appl Microbiol, 2010. 51(1): p. 6-10.
19. Boge, T., et al., A probiotic fermented dairy drink improves antibody response to influenza vaccination in the elderly in two randomised controlled trials. Vaccine, 2009. 27(41):

p. 5677-84.
20. Black, C.A., Delayed type hypersensitivity: current theories with an historic perspective. Dermatol Online J, 1999. 5(1): p. 7.
21. Finkelman, F.D., et al., Lymphokine control of *in vivo* immunoglobulin isotype selection. Annu Rev Immunol, 1990. 8: p. 303-33.
22. De Villiers, P.J., et al., Efficacy and safety of a live attenuated influenza vaccine in adults 60 years of age and older. Vaccine, 2009. 28(1): p. 228-34.
23. Murphy, B.R., et al., Temperature-sensitive mutants of influenza virus. 3. Further characterization of the ts-1(E) influenza A recombinant (H3N2) virus in man. J Infect Dis, 1973. 128(4): p. 479-87.
24. Clements, M.L., et al., Serum and nasal wash antibodies associated with resistance to experimental challenge with influenza A wild-type virus.JClinMicrobiol,1986.24(1):p.157-60.
25. Neuberger, M.S. and K. Rajewsky, Activation of mouse complement by monoclonal mouse antibodies. Eur J Immunol, 1981. 11(12): p. 1012-6.
26. Gessner, J.E., et al., The IgG Fc receptor family. Ann Hematol, 1998. 76(6): p. 231-48.
27. Heusser, C.H., C.L. Anderson, and H.M. Grey, Receptors for IgG: subclass specificity of receptors on different mouse cell types and the definition of two distinct receptors on a macrophage cell line. J Exp Med, 1977. 145(5): p. 1316-27.
28. Unkeless, J.C. and H.N. Eisen, Binding of monomeric immunoglobulins to Fc receptors of mouse macrophages. J Exp Med, 1975. 142(6): p. 1520-33.
29. Kipps, T.J., et al., Importance of immunoglobulin isotype in human antibody-dependent, cell-mediated cytotoxicity directed by murine monoclonal antibodies. J Exp Med, 1985. 161(1): p. 1-17.
30. Takai, T., et al., FcR gamma chain deletion results in pleiotrophic effector cell defects. Cell, 1994. 76(3): p. 519-29.
31. Huber, V.C., et al., Fc receptor-mediated phagocytosis makes a significant contribution to clearance of influenza virus infections. J Immunol, 2001. 166(12): p. 7381-8.
32. Nimmerjahn, F., et al., FcgammaRIV: a novel FcR with distinct IgG subclass specificity. Immunity, 2005. 23(1): p. 41-51.
33. Nimmerjahn, F. and J.V. Ravetch, Divergent immunoglobulin g subclass activity through selective Fc receptor binding. Science, 2005. 310(5753): p. 1510-2.
34. Arulanandam, B.P., M. O'Toole, and D.W. Metzger, Intranasal interleukin-12 is a powerful adjuvant for protective mucosal immunity. J Infect Dis, 1999. 180(4): p. 940-9.
35. Hovden, A.O., R.J. Cox, and L.R. Haaheim, Whole influenza virus vaccine is more immunogenic than split influenza virus vaccine and induces primarily an IgG2a response in BALB/c mice. Scand J Immunol, 2005. 62(1): p. 36-44.
36. Moran, T.M., et al., Th2 responses to inactivated influenza virus can be converted to Th1 responses and facilitate recovery from heterosubtypic virus infection. J Infect Dis, 1999. 180(3): p. 579-85.
37. Deng, Y., et al., Age-related impaired type 1 T cell responses to influenza: reduced activation *ex vivo*, decreased expansion in CTL culture *in vitro*, and blunted response to influenza vaccination *in vivo* in the elderly. J Immunol, 2004. 172(6): p. 3437-46.
38. Bot, A., S. Bot, and C.A. Bona, Protective role of gamma interferon during the recall response to influenza virus. J Virol, 1998. 72(8): p. 6637-45.
39. Mohamadzadeh, M., et al., Regulation of induced colonic inflammation by *Lactobacillus acidophilus* deficient in lipoteichoic acid. Proc Natl Acad Sci U S A, 2011. 108 Suppl 1: p. 4623-30.
40. Dunkley, M.L., R.L. Clancy, and A.W. Cripps, A role for CD4+ T cells from orally immunized rats in enhanced clearance of Pseudomonas aeruginosa from the lung.

Immunology, 1994. 83(3): p. 362-9.
41. Kyd, J.M., M.L. Dunkley, and A.W. Cripps, Enhanced respiratory clearance of nontypeable Haemophilus influenzae following mucosal immunization with P6 in a rat model. Infect Immun, 1995. 63(8): p. 2931-40.
42. Ichinohe, T., et al., Microbiota regulates immune defense against respiratory tract influenza A virus infection. Proc Natl Acad Sci U S A, 2011. 108(13): p. 5354-9.
43. Fitzgerald, K.A., NLR-containing inflammasomes: central mediators of host defense and inflammation. Eur J Immunol, 2010. 40(3): p. 595-8.
44. Duggan, J.M., et al., Synergistic Interactions of TLR2/6 and TLR9 Induce a High Level of Resistance to Lung Infection in Mice. J Immunol, 2011.
45. Link-Amster, H., et al., Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intake. FEMS Immunol Med Microbiol, 1994. 10(1): p. 55-63.
46. Bunout, D., et al., Effects of a nutritional supplement on the immune response and cytokine production in free-living Chilean elderly. JPEN J Parenter Enteral Nutr, 2004. 28(5): p. 348-54.
47. Olivares, M., et al., Oral intake of *Lactobacillus fermentum* CECT5716 enhances the effects of influenza vaccination. Nutrition, 2007. 23(3): p. 254-60.
48. Yasui, H., J. Kiyoshima, and T. Hori, Reduction of influenza virus titer and protection against influenza virus infection in infant mice fed *Lactobacillus casei* Shirota. Clin Diagn Lab Immunol, 2004. 11(4): p. 675-9.
49. Hori, T., et al., Effect of intranasal administration of *Lactobacillus casei* Shirota on influenza virus infection of upper respiratory tract in mice. Clin Diagn Lab Immunol, 2001.8(3):p.593-7.
50. Hori, T., et al., Augmentation of cellular immunity and reduction of influenza virus titer in aged mice fed *Lactobacillus casei* strain Shirota. Clin Diagn Lab Immunol, 2002. 9(1): p.105-8.
51. Hatakka, K., 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(7298): p. 1327.
52. Hojsak, I., et al., *Lactobacillus* GG in the prevention of nosocomial gastrointestinal and respiratory tract infections. Pediatrics, 2010. 125(5): p. e1171-7.

Chapter 8

General discussion

Marjolein Meijerink

The aim of this thesis was to get a better understanding of the molecular mechanism of host responses to probiotics. Beneficial effects of strains of probiotics have been established in the treatment and prevention of various intestinal disorders, including allergic diseases and diarrhea. However the precise molecular mechanisms and the strain dependent factors involved are poorly understood. This knowledge would facilitate the selection of the most effective strains or strain combinations and give new insights into how to measure the health effects of probiotic in animal models and eventually in humans.

Two key questions were central to the research described in this thesis:

1. Which bacterial factors determine the immunomodulatory properties of specific probiotic strains?
2. Can the *in vivo* activity of a probiotic be predicted from its *in vitro* immunomodulatory properties?

Initially immune responses were studied to different probiotic strains and different strains of the same species, namely *L. plantarum*, in order to determine the natural variation in the response (*Chapter 3* and *4*). In *Chapters 3* and *4* this was combined with genomics data in a statistical approach to identify genetic loci in *L. plantarum* that influenced the specific cytokine responses. Specific strains were then selected on the basis of their immune responses *in vitro* for studies in mouse models of peanut sensitization (*Chapter 6*) and influenza vaccination (*Chapter 7*). The aim of the *in vivo* work was to test the predicted immune effects of selected strains on allergic sensitization and vaccination and gain further mechanistic insights into the immunomodulatory properties of these strains.
In the last part of this general discussion relevance of this thesis to industry and society is discussed and concluding remarks are given for future research.

Overview of probiotic mechanisms

Over the past ten years, the role of the intestinal bacteria in shaping human physiology and immunity has become increasingly evident [1-7]. This has stimulated the interest in using probiotics to benefit human health and prevent disease, such as allergy and colitis. Probiotics are defined as living organisms that, when administered in sufficient numbers, are beneficial to the host [8]. The most common genera used as probiotics are *Lactobacillus* and *Bifidobacterium*. Probiotic effects seen in human or animal studies are species specific and even strains of the same species may differ in their ability to elicit beneficial effects [9]. In addition, the effects of probiotics are dependent on dose, formulation, route and frequency of delivery as well as the animal model or human study population. Probiotic effects may result from modulation of the composition and/ or activity of the commensal microbiota, and/ or their stimulation of the immune system including innate signaling pathway in epithelial cells (ECs). Probiotics can influ-

ence the production of the short chain fatty acids (SCFAs) butyrate, propionate and acetate that have potent anti-inflammatory activities [10-12]. Probiotics can make the gut environment more favorable to the growth of bacteria considered to be beneficial (e.g. of the genera *Lactobacillus* and *Bifidobacterium*) and less favorable for pathogens by lowering the intestinal pH, through the production of SCFAs and lactic acid and improving colonization resistance (Fig. 1, General introduction). Some probiotics are suggested to act in the lumen of the gut by producing antibacterial molecules such as bacteriocins [13]. Others enhance the mucosal barrier by increasing the production of innate defense molecules, including goblet cell–derived mucins [14] and trefoil factors and Paneth cell defensins via innate signaling pathways [15, 16]. Probiotics can also mediate their beneficial effects by promoting adaptive immune responses (secretory immune globulin A, regulatory T cells (Tregs), interleukin-(IL)10, NK cell activity) [17-19] in a strain-dependent fashion. Although probiotics are proposed to act via different mechanisms, this thesis is specifically focused on the immunomodulatory capacities of probiotics.

The fate of orally consumed probiotics and implications for understanding mechanisms of action

Different species of lactic acid bacteria (LAB) vary in their capacity to survive passage through the stomach, and passage only transiently through the gastrointestinal tract [20, 21]. Studies on colonization with orally consumed lactobacilli have shown that they persist only during the periods of dosing and/ or for relatively short periods of time after administration [22-24]. The mucus present in the intestine presents a substantial barrier to microbial contact with ECs, although some commensal species are known to degrade and colonize the mucus (reviewed in [25]).
Mucus is a viscous gel-like matrix formed by large heavily glycosylated proteins called mucins. The mucus layer contains several antimicrobial factors and secreted immunoglobulins that are directed against specific microbial antigens. Specialized secretory cells called goblet cells found throughout the entire intestinal tract secrete and produce mucins. The composition of the mucus barrier varies throughout the gastrointestinal tract and is rapidly turned over, and is regulated by innate signaling pathways in goblet cells [26]. In mice, the colonic mucus consists of two layers extending 150 µm above the epithelial surface which have a similar protein composition [27]. The inner firmly adherent layer is thinner (50 µm) densely packed and devoid of bacteria, whereas the outer layer is less dense and colonized by bacteria [28]. In the small intestine the mucus layer is thinner than in the colon or even absent in places [25, 29]. The firmly adherent mucus is approximately 15 µm thick whereas the thickness of the outer mucus layer varies throughout the small intestine but is the thickest in the ileum, where the bacterial content of the small intestine is highest [25, 29]. The barrier function of the mucus is evident from studies on human biopsy material showing that most commensal bacteria are present either in suspension in the lumen or trapped in the mucus [30]. Other

challenges to the persistence of bacteria in the small intestine are the fast transit time (ranging from 15 minutes to 5 hours with a mean of about 84 min) [31] and the presence of bile acids, which limit the bacterial growth and even kill most probiotic species, although many LAB can possess bile salt hydrolases [32].

The dome epithelium otherwise known as follicle-associated epithelium (FAE) covering the large aggregates of lymphoid follicles in the Peyer's patches (PP) of the small intestine lacks or has limited numbers of goblet cells [33-35] and may therefore be more accessible to consumed probiotics (Fig. 1). Microfold (M) cells present in the FAE are specialized in the uptake of particulate antigens, including bacteria and viruses which they transport to the basal membrane in endosomes [36]. M cells differ in structure compared to their surrounding enterocytes with fewer lysosomes, more mitochondria, a lack of mucous glycocalyx covering their surface, and a basolateral extracellular space that surrounds interdigitated lymphoid cells. In addition, the pattern and dominance of M cell surface receptors is different to that of enterocytes [37-39]. It has been shown that human M cells utilize receptor dependent transport mechanisms for bacterial uptake [40]. Orally administered nanoparticles and GFP-labeled commensals are taken up by M cells in the PP and the same may be true for probiotics [36, 41, 42]. M cell mediated uptake of bacteria is also likely to occur in the isolated lymphoid follicles (ILFs) associated with the colonic epithelium. Several studies have shown that commensal bacteria can translocate by an intracellular route through the ECs lining the intestine and subsequently travelling via the lymph to the mesenteric lymph nodes (MLN) [36, 43-46]. This could also be important in the activation of the intestinal immune system.

In the sub-epithelial dome of the PP, dendritic cells (DCs) present bacteria sampled by M cells and subsequently activate the adaptive immune system by migration to the follicular area and presentation of antigen to naïve lymphocytes, or migrate to the MLNs through the efferent lymphatic ducts. In the MLNs or in the PP, mature DCs drive the differentiation of naïve CD4+ T lymphocytes into effector T cells, helper T (Th) cells (Th1, Th2, Th17) or Tregs. (Fig. 1).

DC subtypes that can open the epithelial tight junctions and send protrusions to the luminal side [48] play an important role in the sampling of luminal bacteria [49, 50]. The ability of DCs to produce IL-12 and prime naïve T cells for Th1 polarization to microbial stimuli can be abolished by *in vitro* conditioning monocyte-derived DCs with supernatant from intestinal eptihelial cells (ECs) [51]. Treatment of DCs with EC conditioned growth medium conditions them to produce high amounts of IL-10 and promote regulatory T cell and Th2 cell responses, a phenotype that was shown *in vitro* to be dependent on epithelial production of thymic stromal lymphopoietin (TSLP) [51]. Another important immunoregulatory cytokine produced abundantly by intestinal ECs and stromal cells in the intestine is transforming growth factor beta (TGF-β), that inhibits NF-κB dependent gene expression and the production of pro-inflammatory cytokines by macrophages and DCs [52, 53]. Additionally, TGF-β acts in concert with TSLP to induce a tolerogenic phenotype in monocyte derived DCs *in vitro* [54].

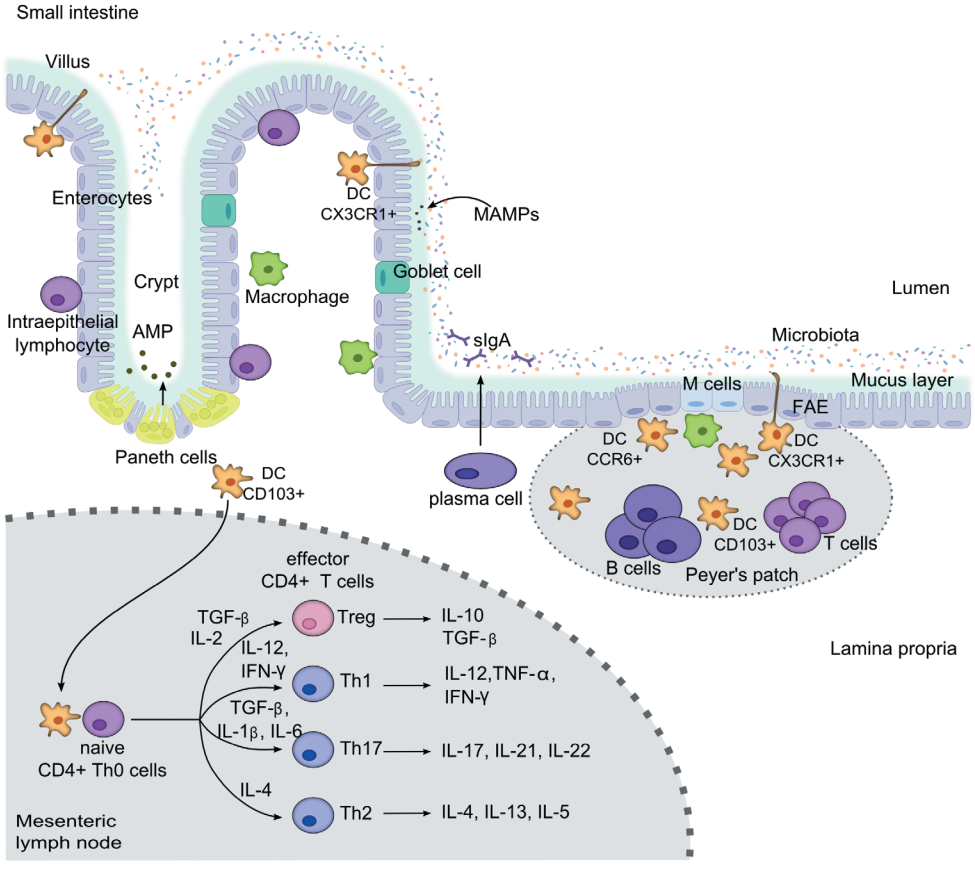

Figure 1 (modified From [47]): *The mucosal epithelium, lamina propria cells and gut-associated lymphoid tissue in the small intestine*. A single layer of intestinal epithelial cells (ECs) separates the luminal contents from the lamina propria (LP). In the basolateral space between ECs, are positioned intraepithelial lymphocytes. The epithelium is covered by a thick layer of mucus produced by goblet cells. Antimicrobial peptides (AMPs) and secretory antibodies provide additional protection mechanisms. Enterocytes and specialized Paneth cells in the crypts are able to secrete AMPs preventing bacterial growth in proximity to the epithelium. Plasma cells in the LP produce secretory immunoglobulin A (sIgA) that mediate bacterial exclusion. The luminal content is continuously sampled by specialized ECs and immune cells. In the follicle associated epithelium (FAE) of the Peyer's patches (PP) or isolated lymphoid follicles, microfold (M) cells transcytose bacteria and antigens. In the LP and PPs, CX3CR1+ macrophages and dendritic cells (DCs) sample microbes and antigens through the epithelium to support inflammatory responses. DCs, macrophages and M cells deliver antigens and bacteria to antigen presenting cells (APCs), such as CCR6+ DCs. Activated APCs then move to the follicular area of the PP or they migrate to the mesenteric lymph nodes (MLN) through the lymphatic system and present the antigen to naïve lymphocytes. In the MLN or in the PP, mature DCs drive the differentiation of naïve CD4+ T lymphocytes into effector T cells, helper T cells (Th1, Th2, Th17) or regulatory T cells (Tregs). The CD103+ population of DCs in the LP play a key role in generating inducible Tregs.

In summary, the small intestine seems to be the most likely place for interaction of probiotics with the immune system, due to a thinner or absent mucus layer, less competition of commensal bacteria and presence of M cells. Clearly DCs play a cardinal role in regulating tolerance and immunity in the mucosal tissues. Therefore this immune cell type is highly relevant and often used to study intestinal bacterial interactions *in vitro*.

In vitro co-culture assays to assess the immunomodulating properties of probiotics

In vitro assays are generally considered as useful approaches to select promising strains to test in *in vivo* animal models or in human trials although there is presently little data in the literature to strongly supports this view [55-57]. Candidate strains are often tested for their immunomodulatory potential in *in vitro* co-culture assays with different types of immune cells, such as human monocyte derived DCs, human peripheral mononuclear cells (PBMCs) and mouse bone marrow derived DCs [58]. Immune parameters such as IL-10, IL-12, tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) are often measured to characterize the strains tested. IL-10 production is typically measured, because it is an anti-inflammatory cytokine that suppresses IL-12 production and consequently IFN-γ production, thereby favouring a T-helper 2 (Th2) or a Treg response. In addition, IL-10 down-regulates antigen presentation and inhibits the activation of macrophages and thus the production of pro-inflammatory molecules and chemokines. IL-12 and TNF-α are commonly measured as production of these cytokines by DCs is associated with the induction of T-helper 1 (Th1) responses. Furthermore, IL-12 elicits IFN-γ production by T cells and by NK cells.

There are some important considerations and potential limitations to the use of *in vitro* co-culture assays. As mentioned before epithelial conditioning can also play an important role in the priming of DCs. DCs isolated from the PP behave differently compared to other subsets in the intestine. However, conditioned DCs probably may not reflect the response of all subtypes, e.g. in the PP or the epithelial-attached DCs. In addition, most immune co-culture assays with bacteria make use of antibiotics to prevent overgrowth and acidification of the culture, and therefore the probiotic strains will be killed, or arrested in growth during the incubation period [55, 59-61]. Mileti et al. used a method by which live bacteria are in co-culture with DCs and after 1h incubation, the cells are washed and the medium is replaced by an antibiotic containing medium [9]. In future experiments, this method might be a better alternative than co-culture in only antibiotic containing medium as it would allow interaction of DCs with live bacteria and the production of secreted bacterial factors including metabolites to occur.

In vitro assays for pre-screening of probiotics would benefit from standardized methods. Therefore in *Chapter 5* we used a cryopreservation method, which was previously investigated in a small number of studies [62-65] concerning the application of

DCs in clinical immunotherapy. These prior studies were limited to DC stimulations with synthetic ligands, cytokines or antibodies including lipopolysaccharide (LPS), TNF-α, IFN-γ and CD40L but not bacteria. However, we found that cryopreservation of both monocytes and immature DCs led to a skewed Th2 cytokine response to microbial stimulation. Therefore we consider that standardization of probiotic screening assays by the use of cryopreservation methods is currently not applicable. The detailed method for generating human monocyte derived DC described in *Chapter 5* may however be useful for developing standardized immune assays.

Variation in the immune response to different species and strains of lactobacilli
The *in vitro* cytokine responses of human PBMCs and DCs to lactobacilli and bifidobacteria can be strikingly different depending on both the species and the strain [59, 66-68]. In *Chapter 6* we determined the immunomodulatory properties of 12 different bacterial species encompassing 28 strains, isolated from commercially available products. The different bacterial strains varied considerably in their ability to modulate PBMC immune responses. The strain differences in IL-10 (632-fold) and IL-12 (464-fold) induction, was much higher than previously reported for *Bifidobacterium longum* strains (8-fold) [67] and for multiple *Lactobacillus* and *Bifidobacterium* species in PBMC co-culture assays (10-15-fold) [55, 67, 69-71].

In *Chapter 4* PBMC co-culture with 42 *L. plantarum* strains induced mean levels of IL-10 and IL-12 over a 14- and 16-fold range, respectively, and IL-10/IL-12 ratios over a 13.5-fold range. In *Chapter 3* we studied DC responses to these 42 *L. plantarum* strains and the amounts of IL-10 induced cytokines ranged from 28 pg/mL to 1095 pg/mL (39 fold) for IL-10 and from 20 to 11996 pg/mL (600 fold) for IL-12. From a comparison of IL-12 to IL-10 ratios it was clear that these cytokines can vary independently of each other resulting in strains with distinct pro-inflammatory and anti-inflammatory profiles (Fig. 2). This remarkable diversity in the immunomodulatory properties of different strains of the same species suggests that multiple genetic factors can influence the phenotype in immune assays. This was investigated further in *Chapter 3* and *4* and is discussed in the section “Identification of genetic loci in *L. plantarum* that influence cytokine responses of human immune cells”.

Immunomodulatory factors of bacteria

Using genetic approaches, several cell-wall associated molecules of lactobacilli have been shown to affect and/ or modulate the response of immune cells [72]. Innate signalling pathways are the most well characterised mechanisms by which lactobacilli can activate and induce effector responses in immune cells. The innate receptors involved in microbe detection are known as pattern recognition receptors (PRRs) and are expressed on immune cells and many other tissues including the intestinal epithelium. The Toll-like receptor (TLR) family of PRRs have been characterised in many animals

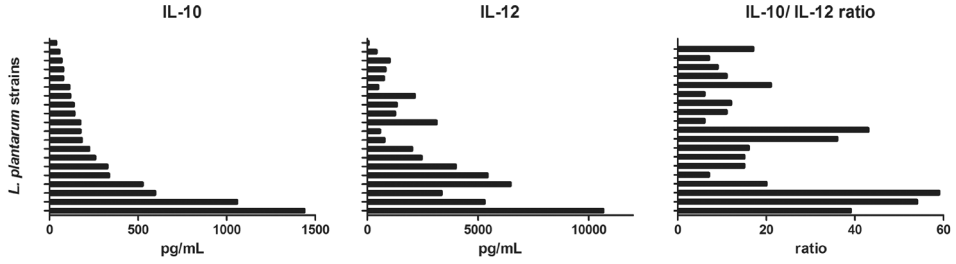

Fig. 2: IL-10, IL-12p70 production and the IL-10/IL-12p70 ratio by monocyte-derived DCs stimulated with 20 different *L. plantarum* strains. The graphs were produced using data from *Chapter 3*, [59] based on one of the 5 representative donors. The different strains induce strikingly different amounts of IL-10 and IL-12 and have distinct pro-inflammatory and anti-inflammatory profiles based on their IL-10/IL-12 ratios.

and their specificity for conserved microbe-associated molecular patterns (MAMPs) is well characterised for humans and rodents (see table 1 General introduction).
A different set of receptors known as the nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs), of which there are more than 20 family members, recognize intracellular ligands. NOD1 and NOD2 have been shown to detect the bacterial peptidoglycan (PGN) fragments meso-DAP and muramyl dipeptide, whereas the specificities of most of the other NLRs remains uncharacterized. Recently, a study showed that the anti-inflammatory capacity of *Lactobacillus salivarius* Ls33 in a trinitrobenzene sulfonic acid (TNBS) induced colitis model was driven by NOD2-mediated recognition of a specific PGN-derived muropeptide. This anti-inflammatory activity was mediated through the induction of regulatory CD103+ DCs via NOD2 signalling and the expansion CD4+ regulatory T cells [73].
Recently, several lectin and C-type lectin receptors (CLRs) have been characterized that are involved in the recognition and capture of antigens by antigen presenting cells (APCs). One such CLR known as DC-specific intracellular adhesion molecule-3 grabbing non-integrin (DC-SIGN) recognizes high-mannose-containing structures.

The di-acylated membrane anchors of lipoproteins and lipoteichoic acids bind to TLR-2 and TLR-6 promoting dimerization and MyD88-mediated activation of the canonical pathway of NF-κB. The capacity of different species to stimulate TLR-2 signalling varies considerably, even at the strain level for those species which have been extensively tested (Fig. 3). A D-alanyl transfer protein B (*dltB*)-mutant of *Lactobacillus plantarum* strain NCIMB8826 (WCFS1) was found to incorporate much less D-alanine in its teichoic acid (TA) than the wild-type strain [74]. This defect significantly impacted on the immunomodulatory properties of the bacterium, as shown by a reduction in the secretion of pro-inflammatory cytokines by PBMCs and monocytes in co-culture with the *dltB* mutant and wild type strain [74, 75]. Additionally in a recent study by Bron et al. the impact of wall TA (WTA) backbone in *L. plantarum* WCFS1 mutants was determined (Bron and

Meijerink, unpublished). Derivatives of *L. plantarum* WCFS1 were constructed that synthesize alternative WTA variants and were used for WTA isolation and biochemical analysis. The mutants were shown to either completely lack WTA, or produce WTA and LTA that lack D-alanine substitution, or contain ribitol-backbone WTA instead of the wild-type glycerol-containing backbone. A transcriptome analysis revealed which genetic determinants were involved in this alternative WTA backbone, and pinpointed ribose and arabinose as the possible biosynthetic precursors. Co-culture of these mutants with human DCs resulted in drastically decreased levels of pro-inflammatory cytokines as compared to the wild type (Bron and Meijerink, unpublished). Moreover, human embryonic kidney NF-κB reporter cell line experiments indicated that WTA dampens TLR-2 and TLR-1/2 signalling to different degrees, strongly depending on the backbone alditol, and to some extend on D-alanyl substitutions, whereas LTA signalling occurs via a TLR-2/6 dependent mechanism completely dependent on D-alanyl substitutions. This study shows that the WTA structure-function can also contribute to the high variability in the host immune response to different strains of lactobacilli. Therefore, strain-dependent differences in the peptidoglycan structure could explain why the *in vitro* cytokine responses of human PBMCs and DCs to lactobacilli can be strikingly different depending on both the species and the strain. Furthermore, sugars present in the cell wall and the post-translational modification of proteins with carbohydrates can also modulate the immune response by binding to C-type lectin receptors.

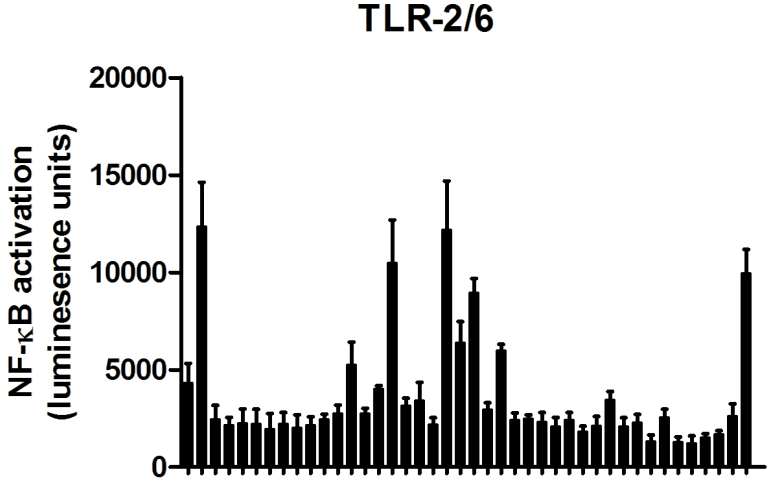

Fig. 3: *TLR-2/6 activating properties 42 different* L. plantarum *strains.* TLR signalling assays were performed on stably transformed cell lines of human embryonic kidney cells (HEK293) expressing TLR-2 and TLR-6 and transfected with a reporter plasmid containing the firefly luciferase under the control of the human NF-κB promoter. HEK293 cells do not produce TLRs, but when stably transformed with TLRs they can activate NF-κB upon addition of the appropriate TLR agonists. Cells were incubated with different *L. plantarum* strains (1:15 cell to bacteria ratio). for six hours incubation after which the luminescence intensity was measured

TLR-9 recognizes bacterial genomic DNA which unlike eukaryotic DNA contains a high frequency of unmethylated CpG motifs [76]. Different species of lactic acid bacteria might differ in their capacity to elicit TLR-9 signalling due to differences in C+G ccontent and the frequency of stimulatory motifs in the DNA. The expression of TLR-9 by immune cells is intracellular and endosomal, and in polarized ECs it is expressed on both the apical and basolateral membrane. In polarized ECs TLR-9 has been shown to have tolerogenic effects to chronic TLR challenges depending on the location of the stimulus [77]. Human epithelial HT-29 monolayers treated *in vitro* with bacterial DNA, including DNA from lactobacilli, delayed NF-κB activation, stabilized levels of IκB and attenuated IL-8 secretion in response to pro-inflammatory stimuli [78]. Additionally, a recent study reported similar effects for HT29 and T84 cell monolayers using DNA from *L. rhamnosus* GG (LGG) [79]. In addition, IL-10 deficient mice orally treated with purified DNA from VSL#3 showed reduced mucosal secretion of TNF-α and IFN-γ and improved histologic disease scores [78]. Applying neutralizing antibodies against type I IFN abolished the anti-inflammatory effects induced by TLR-9 agonists, whereas the administration of recombinant IFN-β mimicked the anti-inflammatory effects induced by TLR-9 agonists [80]. In contrast to anti-inflammatory effects, pro-inflammatory effects have been observed using bacterial DNA and synthetic oligonucleotides (ODN) containing CpG sequences *in vitro* and *in vivo* in combination with LPS challenge [81]. Interestingly, RAW 264.7 cells pre-exposed for 1-3h to CpG-ODN, augmented a pro-inflammatory cytokine response to LPS, whereas prolonged exposure of the cells for 6-9 h induced the suppression of inflammatory cytokines via the induction of IL-10 expression [81]. *In vivo* studies with experimental colitis showed conflicting effects of CpG-ODN, showing that CpG-ODN in active disease worsened inflammation [82] and prophylactic administration diminished colitis [80].
The large variation in strain immune profiles described in *Chapter 3* and *4* suggested that there could be some underlying strain-dependent genetic differences influencing the innate response to *L. plantarum*. As TLRs recognize highly conserved MAMPs, we hypothesized that the differences were due to modification or differential expression of MAMPs or other novel factors interacting with host immune cells. In *chapter 3* and *4* (discussed below) genetic loci in *L. plantarum* that affect the immune response to PBMCs and DCs were investigated using a gene-trait matching approach.

Identification of genetic loci in L. plantarum *that influence cytokine responses of human immune cells.*

To identify novel strain-specific genes and factors that modulate innate recognition by the host, the natural diversity in the immune response to different strains of *L. plantarum* was used in *Chapter 3* and *4*. Microarray based comparative genome hybridization of WCFS1 was used to provide a one-directional comparison of gene-content profiles per strain. In *Chapter 3* and *4* the secreted cytokine amounts or cytokine ratios for the *L. plantarum* strains were correlated with the presence or absence of specific genes by

regression using the Random Forest algorithm. Several genes or gene loci were identified to be potentially involved in the immunomodulation. These loci included genes encoding an N-acetyl-glucosamine/galactosamine phosphotransferase system, the LamBDCA quorum sensing system, a predicted transcriptional regulator gene (lp_2991) and components of the plantaricin (bacteriocin) biosynthesis and transport pathway. Deletion mutants of the candidate genes were constructed in the WCFS1 strain in order to validate their anticipated effect on cytokine induction.

One of the confirmed genes with immunomodulatory capacities in PBMCs was the *pts19ADCBR* locus coding for a cell membrane-associated N-acetyl-galactosamine/glucosamine phosphotransferase system, that might be important for utilization of different carbohydrates in the intestine. Another confirmed gene which correlated with immunomodulation of PBMCs was the *L. plantarum* WCFS1 *lamB*, encoding a processing/export protein of the autoinducer peptide quorum sensing two-component system (AIP-based QS-TCS) LamBDCA. The observed PBMC IL-10/IL-12 ratios for *L. plantarum* might either be mediated directly through the LamBDCA system and the cognate secreted peptide, or indirectly through cell products (e.g., polysaccharides) under the control of this regulatory system.

An unknown transcriptional regulator Lp_2991 was also identified, as influencing the immune response. A deletion mutant of lp_2991 led to a significantly higher secretion of IL-10, IL-12p70 and TNF-α in DCs compared to the wild-type control. This gene lies upstream of gtcA3, a gene encoding a putative teichoic acid glycosylation protein. Transcriptome analysis and qPCR data supported the hypothesis that Lp_2991 is a repressor of *gtcA3* transcription and points to this enzyme as being a prime candidate for the altered immune response. Interestingly GtcA3 is predicted to glycosylate TAs including LTA which is a known TLR-2 agonist capable of modulating immune cell responses. LTA mutants and mutants affecting D-alanine substitution in LTA have already been found to have effects on the immune response [74, 75]. All of the mutations introduced into the plantaracin locus affected the immune response and were predicted to inhibit plantaracin production. Thus it is possible that the plantaracins directly affect the immune response of DCs. Preliminary data suggest that specific plantaricins are indeed able to enhance cytokine expression of ECs after stimulation with LTA (Meijerink, unpublished work in progress).

Our results showed that *in silico* analysis led to the identification of novel factors that are involved in the immunomodulating capacities of *L. plantarum*. For the selection of new candidate probiotics, the presence or absence of these genes can be used. Several other factors are found to be involved in immunomodulation of the host in other genera and species. For example, AvrA is an avirulent bacterial factor found in *Salmonella*, that is found to stabilize the expression and distribution of tight junction proteins such as zonula occludens-1 (ZO-1) and the function of tight junctions *in vitro* and *in vivo* [83]. In addition, a study showed that AvrA has deubiquitinase activity which removes ubiquitins from ub-IκBα and inhibits activation of the pro-inflammatory NF-κB transcription

factor in cultured human ECs [84, 85]. Additionally, peptidoglycan N-deacetylation was shown to be a virulence factor in *Listeria* [86] and *Streptococcus* [87] species, playing an important role in evasion of the host innate immune response, which leads to survival of the pathogen *in vivo*. In addition, polysaccharide A (PSA) produced by *Bacteroides fragilis* was shown to be capable to activate T cell-dependent immune responses and homeostasis of the host immune system. PSA can provide protection in a *in vivo* colitis models through the IL-10 dependent repression of pro-inflammatory cytokines (reviewed in [88]).
The differences found between the WCFS1 and the deletion mutants of genes/loci identified in *Chapters 3* and *4* were much smaller than found among the 42 different *L. plantarum* strains, indicating that other factors play an important role in immunomodulation, and/ or that there are synergistic effects between one or more factors. Thus it might be insightful to sequence the other strains used in the study described above and apply the gene-trait matching approach to identify other genetic loci. A different approach would be to compare levels of different gene transcripts under different fermentation conditions with the cytokine responses to the bacteria (in progress, unpublished).

Use of probiotics to treat or prevent immune-related disorders

Most therapeutic or preventative applications of probiotics have been concerned with immune-related disorders such as atopy, colitis or have been aimed at enhancing aspects of innate immunity.
In mice, the Th1/Th2 hypothesis has proven to be a fruitful paradigm in describing the overreaction of either humoral immunity or cell immunity in different disease states such as allergy (Th2 dominance) and inflammatory bowel disease (IBD) and auto-immunity (Th1 dominance). Central to this hypothesis is that the T helper cells directing the different arms of the immune system express different cytokine patterns and that either pathway can down-regulate the other [89, 90]. In humans the Th1/Th2 paradigm has major inconsistencies, but remains a useful tool to measure imbalances in the immune system and evaluate immunomodulatory treatments especially in rodent models. T helper cell cytokine expression in humans rarely falls into exclusively pro-Th1 or -Th2 patterns. In addition to Th1 and Th2 cells as effector cells, there are also Th17 cells, which produce the pro-inflammatory cytokine IL-17 and are involved in the induction of inflammation and play an important role in IBD, multiple sclerosis, psoriasis and rheumatoid arthritis [91]. Moreover, the Tregs and APCs are equally important in regulating immune homeostasis. In the past few decades the incidence of allergies, autoimmunity and IBD (in industrialized countries) has risen and the simultaneous occurrence of Th1- and Th2-mediated disorders suggest that the simple Th1/Th2 balance assumption is unable to explain the underlying mechanisms [92]. Recently patients with type 1 diabetes and multiple sclerosis, and individuals with predisposition to allergy were shown to have a defective regulatory T cell activity [92]. Tregs are known to suppress

both Th1-and Th2-type immune responses through production of IL-10 and TGF-β. Recent evidence from *in vitro* and *in vivo* studies suggests that probiotics may mediate their beneficial effects through induction of Tregs, in addition to skewing of Th1 or Th2 responses.

The use of probiotics to prevent and treat Th2-mediated food allergy by skewing the immune response towards a more Th1/ Treg type has been described in several studies [93-98]. For example, a study by Shida et al. showed that in ovalbumin (OVA)-specific T cell receptor transgenic mice, intraperitoneal injection of *L. casei* Shirota led to an induced IL-12 response in serum. In this model of food allergy the applied probiotic strain skewed the splenocyte cytokine response towards Th1 and suppressed IgE and IgG1 response, which could be blocked by anti-IL-12 antibodies [93]. In a study by Kim et al. using a different food allergy model, mice were orally sensitized to OVA and cholera toxin (CT). In this model orally administered probiotic strains *B. bifidum* BGN4 and *L. casei* 911, both displaying *in vitro* Th1 skewing properties, were shown to decrease levels of OVA-specific IgE, total IgE and IgG1, decreased levels of mast cell degranulation and tail scabs [94]. Frossard et al. showed that oral application of a genetically modified *L. lactis*, engineered to secrete IL-10 could prevent allergic sensitization to β-lactoglobulin and attenuate the levels of antigen-specific serum IgE and total IgE and increase the antigen-specific IgA in the gut compared to the wildtype *L. lactis* [95]. This study shows the importance of IL-10 in food allergy. Numerous studies in animals and humans have shown protective or therapeutic effects of probiotics in other models or diseases including antibiotic induced diarrhoea, irritable bowel syndrome and colitis. Interestingly specific strains of probiotics have also shown encouraging results in mucosal vaccination studies and in protection from illness suggesting they can enhance immunity. Nevertheless demonstrating the beneficial effects of probiotics in healthy persons remains one of the major future challenges (discussed in section "The future potential of probiotics").

Linking in vitro and in vivo data on the immune effects of lactobacilli

Most *in vivo* studies are performed with one probiotic strain, which makes comparisons difficult. Indeed only a few studies have compared the *in vitro* immunomodulatory properties of different lactic acid bacteria with their *in vivo* properties. For instance, the ability of different lactobacilli to induce a high ratio of IL-10/ IL-12 or IL-10/ TNF-α production in immune cells correlates with their capacity to provide significant protection in TNBS induced colitis in mice and rats [55, 99, 100]. However, Mileti et al, demonstrated that *L. paracasei* B21060 and LGG induced a similar cytokine ratio in DCs, but did not show comparable protective effects *in vivo* in a mouse DSS model of colitis [9]. However, the strains used in this study were not associated with high IL-10/ IL-12 ratios (*L. plantarum*: 0,877; LGG: 1,222; *L. paracasei*: 1,176) as shown in the study by Foligne et al. (IL-10/IL-12 ratios >10), which might account for the apparent contrasting findings. Moreover, *L. paracasei* was a poor inducer of both pro- and anti-inflammatory cytokines *in vitro*.

Multiple strain comparison *in vivo* is the most preferred approach for comparing their potential probiotic functionality, to validate the immunomodulatory capacities *in vitro*. Foligne et al. showed that the ability of different lactobacilli to induce a high ratio of IL-10/IL-12 production in PBMCs was shown to correlate with their capacity to provide significant protection from TNBS induced colitis in mice [55]. Similarly a study by Kwon et al. identified bacterial strains that could specifically up-regulate CD4+Foxp3+ Tregs by co-culturing MLNs *ex vivo* with bacteria. These strains were selected on the basis of a high IL-10/IL-12 ratio (>4) and enhanced Foxp3 expression (>1.5-fold). This study underlines the important role of DCs in the induction of Tregs and how *in vitro* assays (pre-selection on IL-10 / IL-12 ratio and induction of Foxp3 levels in MLNs) can be used for the selection of probiotic strains and species that have therapeutic effects in models of IBD, atopic dermatitis and rheumatoid arthritis. To really prove this, it would have been better to show also results of strains with a IL-10/IL-12 ratio <4. A different study by Snel et al. showed that a methodology to compare candidate probiotic bacteria for their immunomodulatory properties in PBMCs from patients with a proven pollen allergy was appropriate to select strains with proven effect on the immune system *in vivo* [57]. This method using PBMCs from blood from birch pollen allergic patients can be an intermediate step between *in vitro* screening and testing for clinical efficacy.

In this thesis several strains with distinct *in vitro* immunomodulatory activity were tested in two different animal models. (i) a mouse model of sensitization to peanut extract (*Chapter 6*) and (ii) an intranasal mouse model of influenza vaccination (*Chapter 7*).
A model of peanut allergy was selected as it is one of the most common and severe food allergies. In *Chapter 6* human PBMC co-cultures with (potential) probiotics were used to determine the immunostimulating properties of different commercially available strains. Three strains were selected on basis of their distinct properties. One strain, *L. salivarius* HMI001, had a relatively high IL-10/IL-12 ratio compared to the other strains tested, and was predicted to enhance regulatory mechanisms to suppress the allergic sensitization to peanut, based on a study of Kwon et al., which showed that strains with a high IL-10/IL-12 ratio enhanced regulatory responses [56]. The second selected strain, *L. casei* Shirota (LCS), a probiotic previously shown to attenuate allergic responses [101] and inducing high secretion of IFN-γ and IL-12, was predicted to skew the antigen response towards Th1 as IFN-γ is known to inhibit proliferation of Th2 cells. The third strain, WCFS1, was selected as it induced moderate levels of IL-10 and IL-12 with a IL-10/IL-12 ratio that was lower than strain HMI001 but higher than LCS. The study in *Chapter 6* showed that screening these probiotics using human PBMCs was useful in selecting strains with predicted anti-inflammatory and Th1 skewing properties. In the case of HMI001 (inducing a high IL-10/IL-12 ratio) and LCS (inducing high amounts of IFN-γ and IL-12) attenuation of the allergic response was seen in the mouse peanut allergy model. Strikingly, we observed that certain strains may worsen the allergic reaction, as shown in the case of WCFS1, inducing moderate levels of IL-10 and IL-12. Although further research is needed to validate the immunomodulatory mechanisms involved *in*

vivo, this approach is likely to be useful for selecting strains for translational research in humans.

Most studies on probiotics and food allergy, evaluated the potential of prophylactic treatment of probiotics orally administered before or during allergen sensitization [93-96]. However, a recent study using mice intragastrically immunized with shrimp tropomyosin showed that a probiotic mixture (VSL#3) was able to inhibit established Th2 driven inflammation and the induced symptoms in this model. This study shows that probiotics might also be used in a therapeutical setting in food allergy [98]. This approach might be more relevant for allergic patients, as they already suffer from allergies and their symptoms need to be treated. However, for people with a genetic predisposition, it might be still relevant to look for preventive strategies to avoid the development of atopic disease/ allergy, particularly in infants.

As we observed that strain WCFS1 had a deteriorating effect in an allergic sensitization model we hypothesized that it might enhance humoral (Th2) mediated responses to a vaccine. This was tested in a mouse intranasal vaccination model using Influvac influenza vaccine (*Chapter 7*). Although the most common vaccination route of influenza A is intramuscular, we hypothesized that intragastric probiotic treatment could lead to an enhanced respiratory immunity. The other *L. plantarum* strains evaluated in this model were selected on basis of their cytokine inducing properties in co-culture with DCs and their TLR-2/6 activating properties and included strains inducing Th1 promoting cytokines or relatively high IL-10 /IL-12 cytokine ratios. As expected, strain WCFS1, inducing the lowest IL-10 to IL-12 cytokine ratio in DC co-culture, significantly increased HA-specific antibody responses to the intranasal vaccine compared to the vaccine control group. Several of the *Lactobacillus* strains appeared to increase delayed-type hypersensitivity (DTH) responses after vaccination compared to the vaccine control group indicating increased Th1-mediated vaccine responses. For strain LMG18021 this was also reflected in the significantly higher HA-specific IgG2a to IgG1 antibody ratio. LMG18021, CIP104448 and CIP104450 which have the highest IL-10 to IL-12 ratios of the strains tested, significantly enhanced the *ex vivo* HA-specific induction of IL-10, IL-17A, IL-6 and IL-4 in MLN cells. B1839 (a very low cytokine inducer in co-culture assays) was included as negative control. This strain did not enhance the HA-specific antibody response and the HA-specific immune response indicating that the immune-stimulatory properties of a strain are indeed important in mediating their effects on the host vaccine response.

Further research is needed to demonstrate that these effects on the vaccine response impact on protection to a challenge with influenza and to validate the immunomodulatory mechanisms involved. Nevertheless, the *in vivo* studies described in this thesis support other publications proposing that *in vitro* immune assays can be useful for predicting which candidate probiotic strains will be most effective *in vivo*. Positive results in animal models are encouraging but they raise a number of questions such as the suitability of mouse models for predicting applications in humans, variability of responses to probiotics in humans and the reasons for conflicting results reported in the literature even for the same probiotic strain. These topics are discussed in the following sections.

Considerations for further translational research

The assessment of probiotic functionality should preferably be tested directly in the target group, representing either the general population or a subgroup of subjects with a given condition, for example elderly, physically active subjects, allergic individuals or pregnant women. In addition, data from infants and young children cannot be extrapolated to the adult population or *vice versa*, as the immune system in early childhood is still developing, and is different from adults [102].

Human intervention studies focussing on for instance the risk reduction for a certain infection, should take into account that there is a lot of variation in immune status within the healthy population. Genetic variability strongly influences the immune response of the host [103]. Specific polymorphisms in genes encoding human leukocyte antigens [104] or proteins involved in the immune response can influence quantities of cytokines [105] in a healthy population or disease state. In addition, individuals could also respond differently to bacterial signals (TLR ligands) due to single nucleotide polymorphisms within TLR genes [106]. NOD2 mutation has been shown to be linked to increased risk for IBD, supporting the importance of SNPs and inter-individual differences in TLR signalling [107, 108]. Remarkably, a study where the virulence of H1N1 Neth/09 isolate was evaluated showed a considerably higher virulence in C57B/6 mice, compared to Balb/c mice indicating that the genetic background of mice can influence the virus susceptibility [109]. Additionally, host genetic variation has been shown to significantly impact on survival after infection with a highly pathogenic H5N1 influenza A virus [110].

Although it is clear that intestinal bacteria can shape human physiology and immunity [1-7], little is known about how differences in microbiota composition and/ or function can influence the results of clinical trials. Recent studies showed that intestinal microbes influence host immune development, immune responses and susceptibility to human diseases such as IBD, diabetes mellitus, and obesity (recently reviewed in [111-114]). Germ-free mice have been shown to have extensive defects in humoral antibody responses and lack the maturation of gut associated lymphoid tissues. In addition, specific commensal microbes can have a great impact on the mucosal T-cell response. For example segmented filamentous bacteria (SFB) have been shown to be potent stimulators of small intestinal Th17 cells, evidenced by the lack of Th17 cells in germ-free mice and their restoration after colonization with SFB [115, 116]. Interestingly, another commensal bacteria, *Bacteroides fragilis*, producing PSA, or purified PSA can treat or protect germ-free mice against the development of experimental colitis by inducing Foxp3+ Tregs, producing IL-10, via a TLR-2 and PSA dependent mechanism [6, 117]. A recent study showed the identification of three microbiota enterotypes, by combining 22 newly sequenced faecal metagenomes of individuals from four countries [118], highlighting the importance of a functional analysis to understand microbial communities.

The strain WCFS1 was included in an allergic sensitization (*Chapter 6*) and in an influenza mice model (*Chapter 7*) and interestingly in both models it enhanced the Th2 re-

sponses in the mice, by enhancing the allergic sensitization and the HA-specific IgG1 responses. This indicates that this strain is behaving similarly in the different mouse models in Balb/c and C3H/HeOuJ mice. Interestingly, WCFS1 (NCIM8826) has previously been shown to attenuate TNBS induced colitis [55], however its efficacy was not as good as some other strains used in the same study. Both Balb/c and C3H/HeOuJ are known to exhibit a predominant Th2 immunity [119, 120], and therefore it might be easier for the strain WCFS1 to further enhance the Th2 response. However we found in *Chapter 7* in the vaccination model, that the strain LMG18021 was able to skew the immune response towards Th1, although this strain of mouse is a genetically predisposed to a Th2 bias [120]. This suggests that probiotics can potentially have strong immunomodulatory effects in some models.

Many models used to assess the immune effects of probiotics have compromised epithelial integrity and altered gut microbiota, such as colitis, food allergy or infectious diarrhea models. This is also the case with the peanut sensitization model described in *Chapter 6* due to the use of cholera toxin which has been shown to increase paracellular permeability of the epithelium [121]. As an impaired barrier and altered microbiota may impact on immune sampling of orally consumed probiotics such models cannot be used to predict their impact in a healthy host (e.g. in the case of vaccination). However, the results obtained in our vaccination model (*Chapter 7*) showed that lactobacilli can indeed modulate the immune response of a healthy host.

Another interesting observation was that WCFS1 can be beneficial in the vaccination model by promoting a Th2 response, however the same strain is harmful in peanut sensitization model putting more emphasis on the fact that probiotics should be carefully selected on a strain by strain basis depending on what disease model is used or on the basis of the desired effect. It was interesting to see that WCFS1 modulated the immune response similarly in both the allergic sensitization and vaccination model. Therefore, it might be useful to test same strains in multiple models to have a better knowledge how the strains will immunomodulate in the target population.

Modulation of intestinal bacteria by probiotics

The strain-dependent effects of probiotics were shown both *in vitro* (*Chapter 3* and *4*) and *in vivo* (*Chapters 6* and *7*). In *Chapter 7*, six different *L. plantarum* strains were tested for their capacity to enhance the efficacy of the influenza vaccination. As expected the effects shown in this model were strain dependent, showing that some specific strains can enhance the HA-specific antibody levels or the HA-specific T cell response. It is possible that these effects are linked to the effects of the probiotic strain on the resident microbiota [122]. Little is known about how the microbiota composition affects specific immunity, although a recent study showed that oral antibiotic treatment resulted in defective CD4+ T, CD8+ T and B cell immunity following intranasal infection with influenza virus. Rectal inoculation of CpG, poly I:C and to a lesser extent peptidoglycan could restore immunity in the lung [1].

As some (anaerobic) strains are difficult or unable to culture (yet), techniques such as

pyrosequencing or 16S hybridization have recently emerged as powerful techniques to determine the composition of the microbiota [123] and could be used to investigate the effects of probiotics on microbiota composition. An important limitation of pyrosequencing is its relative inability to sequence longer stretches of DNA, rarely exceeding 100-200 bases with the first- and second-generation high throughput pyrosequencing chemistries. Compared to Sanger sequencing, nexgen pyrosequencing of individual genomes and assembly of many overlapping reads appear to yield comparable sequencing accuracy with error rates between 0.03%-0.07% depending on the study [124-127]. Genera and species are typically distinguished at levels of 95% and 97% pairwise sequence identities, respectively using 16S rRNA gene sequence data [128]. Metagenomics studies highlighted differences between colonic mucosa-associated and fecal populations in man [129-134]. To date the majority of studies have been performed on fecal material but probiotics may also affect the microbiota in the small intestine where most of the immune cells associated with the mucosal lymphoid tissue reside. However sampling the small intestine is relatively difficult in humans limiting the number of studies performed to date. Studies have shown that probiotics can inhibit the colonization of certain pathogenic groups such as *H. pylori* [135]. Several species of *Bifidobacterium* and *Lactobacillus* are generally associated with beneficial effects. However these species include strains that have both pro- as well as anti-inflammatory effects on the host (cells). Given the results described in this thesis and emerging evidence from the literature on the strain dependency of probiotic effects it now seems inappropriate to consider all strains of certain *Bifidobacterium* or *Lactobacillus* strains as being beneficial even though they may not be detrimental. Clearly any health benefit will depend on the strain (including its dose, frequency of dosis and formulation) and on the health status of the host.

Conflicting results in probiotic research

Some published animal studies and trials on probiotics have produced contradictory results even using the same strain. For example, LGG was shown to prevent atopic eczema or dermatitis [136, 137]. Subsequent studies on the use of LGG in the treatment of atopic eczema suggested a therapeutic effect [138-140], whereas more recent studies show no therapeutic or preventative benefits in the development of sensitization and atopic disease, particularly in infants with atopic dermatitis (AD) [141-143]. This could be partly explained by the inherent complexity of the allergic syndrome and the differences in study design, e.g. applied probiotic type (e.g. killed/ live or species), period of probiotic supplementation, different target population and additional treatment such as topical treatment or feeding hydrolysed infant formula [97], which is limiting the comparability of the results. Moreover, a recent study showed that the timing of the application of probiotics is crucial for their efficacy using a mouse model of allergic sensitization to birch and grass pollen [144]. Mucosal delivery of *L. paracasei* NCC2461 or *B. longum* NCC3001 at the time of sensitization and challenge led to a significant suppression of airway inflammation and down-regulated allergen-specific immune re-

sponse. However, when mice were treated with the individual bacterial strains before sensitization and challenge, only *B. longum* NCC3001 was protective [144]. Both strains induced similar amounts of IL-10 *in vitro*, however they exhibited different abilities to induce inflammatory cytokine production, with *B. longum* NCC3001 being the less pro-inflammatory strain. The results might be explained by the fact that a strain (like *B. longum* NCC3001) enhancing regulatory mechanisms could be efficient given before sensitization as well as during challenge, while *L. paracasei* NCC2461 having different immunomodulatory effects, could only skew the immune response during sensitization. Also the duration of probiotic feeding can impact on the probiotic effect. The results of different studies can also be influenced by mixing of probiotics with prebiotics or a hydrolysed whey formula or using probiotic combinations.

In *Chapter 7* the probiotic LGG was included as it has been shown to confer protective effects in challenge studies with influenza [145, 146] and enhanced the efficacy of intramuscular influenza vaccination [147]. Despite the fact that LGG increased DTH responses in our vaccination study (*Chapter 7*) the HA-specific antibodies in serum and *ex vivo* cell response were unaffected. The challenge studies did not show any data differences in antibody titers in serum [145, 146], so in that study the serum levels might also be unaffected, indicating that it might be that LGG can be more beneficial for NK cell function [145, 146], which was not a parameter measured in our study. This highlights the importance of standardizing biomarkers and immune assays to evaluate probiotics and their potential mechanisms of action.

The results of *in vivo* studies in this thesis (*Chapters 6* and *7*) showed that the tested strains all behaved differently in these models and that one cannot expect different species or strains of the same species to have the same effects *in vivo*. Additionally, differences in formulation may impact on the results obtained with the same strain, as shown in *Chapter 4*: differences in immune profiles were seen *in vitro* using strains harvested in stationary and exponential phase of growth. Similarly, a study by van Baarlen et al., showed that exponential phase and stationary phase grown *L. plantarum* WCFS1 eli-cited distinct transcript profiles in human duodenal tissue samples [148]. As discussed above host factors such as genetic differences in microbial responses and allergic pre-disposition, and also other environmental factors such as individual microbiota, diet and treatment with antibiotics may affect the outcomes of the studies.

Suitability of mouse models in translational research on probiotics

Testing potential probiotic strains directly in humans to treat diseases e.g. allergy, is correctly restricted for ethical reasons. However, translational research based on mouse studies are inherently complex due to the differences between mice and humans and uncertainties about the reliability of the inbred mouse models as predictors of efficacy in a human population. The immune system of humans and mice differs with respect to structural and functional aspects of the IgG isotypes and their binding affinities for FcγRI, FcγRIII, and FcεRI receptors. Nevertheless there are also clear similarities between humans and mice with respect to IgE and IgG antibodies that bind to the homologous

FcεRI and FcεRIII receptors, and with respect to their expression on different cells types e.g. mast cells, macrophages and DCs. In addition, some of the inflammatory mediators (e.g. histamine) are expressed in both humans and mice. Because of these similarities and despite of differences mentioned earlier, the mouse peanut allergy model described in *Chapter 6* mimics the clinical and immunological characteristics of peanut allergy in human subjects [149, 150]. This is based on the model described by Li et al., showing that peanut-induced anaphylaxis was IgE mediated, and mast cell degranulation and histamine release were associated with the anaphylactic symptoms. Thus the model seems likely to be relevant to the human population.

The future potential of probiotics

In the EU, the functional food industry is selling an increasing number of foods that bear nutrition and health claims. Health claims are statements given on labels, in advertising or other marketing products that health benefits can results from consuming a given product. In the case of probiotics, the main benefits attributed to probiotics are based on strengthen the gut barrier, modulation of the intestinal tract microbiota, enhancement of the mucosal integrity, and competitive exclusion of pathogens. Specific probiotics may also be associated with other health benefits, including prevention and reduction of symptoms of lactose intolerance, diarrhea and gut comfort (transit time, stool consistency), prevention of allergy response, mineral absorption, and inhibition of procarcinogen-activating enzymes (reviewed in [151]). Over the last decade industry has shown a growing interest in probiotics, but it is only recently that their health benefits have begun to be demonstrated in clinical trials [136, 152]. Health benefits should be demonstrated in humans by providing a scientific basis for establishing health claims related to food products. Health claim regulations are currently under intensive discussion in public as well as by experts, and new legislative developments have been implemented in the European Union, as well as in the United States, Australia/ New Zealand, India, China and Japan. In December 2006 the EU approved new European regulations on the requirement for nutrition and health claims food. This was considered necessary to harmonize the national rules for the use of health or nutritional claims on foods based on nutritional composition for the European market.
The European Food Safety Authority (EFSA) is responsible for verifying the scientific substantiation of the submitted claims. The advice given by the EFSA serves as a basis for the European Commission and Member States, to decide whether or not a claim will be accepted. To date probiotics represent 8% of the food ingredients for which applications for health claims have been made under this new legislation. However, up to now all health claims for probiotics related to gut and immune function have been rejected by EFSA. It would be incorrect to conclude that the regulatory hurdles imposed by the initial negative comments on probiotic health claims are only obstacles for researchers and industry. In fact this legislation could induce a boost in basic science as well as in clinical studies, supporting the launch of new products. The research should be

established in healthy people or at least in people without a defined disease. This is challenging due to the lack of accepted biomarkers to measure health in the absence of disease.

The EFSA guidance on the scientific requirements for health claims related to immune function states: "for the substantiation of claims on immune function many markers of the function of the immune system have been proposed as outcomes, including the numbers of various lymphoid subpopulations in the circulation, proliferative responses of lymphocytes, phagocytic activity of phagocytes, lytic activity of natural killer cells and cytolytic T cells, production of cellular mediators, serum and secretory immunoglobulin levels, delayed-type hypersensitivity responses, etc." EFSA also indicated that in the case of health claims concerning probiotics and vaccination, it is generally accepted that higher vaccination responses (as measured by increased numbers of individuals attaining protective levels, as well as by increments in antigen-specific antibody titers in groups of individuals) are beneficial. Therefore the EFSA stated that the stimulation of protective titers, as measured by increased numbers of individuals attaining protective levels, could be used to substantiate a health claim on the function of the immune system related to defense against pathogens. In *Chapter 7 L. plantarum* WCSF1 was shown to enhance the antibody titer levels compared to the vaccinated control group. Implying that the administered probiotic was increasing the defense against influenza. A follow up study should be performed to check the protective effect of these increased antibody titers in an influenza challenge model, to serve as basis for a health claim. These data can form a good starting point to explore the possibility of obtaining better protection, however additional data obtained from human trials with the vaccine are needed for a claim approved by the EFSA.

Concerning the prevention of allergy, the EFSA stated that "if it can be shown that if an alteration of a (immunological) marker(s) is accompanied by an improvement of clinical outcomes related to allergy (e.g. a reduced incidence, severity or frequency of allergic manifestations) then such alteration in the (immunological) marker might be considered beneficial in the context of a reduction of disease risk claim for allergy for that specific dietary intervention". In *Chapter 6* it was clear that prophylactic treatment with both HMI001 and LCS before sensitization of the mice with peanut extract, attenuated production of allergen-specific cytokine responses associated with allergic sensitization and mast cell activation marker mast cell protease-1 (MCP-1) upon challenge with the allergen. To obtain a health claim for humans these probiotics would need to be shown to prevent the development of allergy or reduce the severity of allergy (e.g. allergic eczema etc) in human individuals. EFSA stated that human studies are central for substantiation of health claims. Therefore a clinical trial(s) would need to be performed in humans to confirm the beneficial effect of the bacterial strains. However prior to human intervention studies it is important to establish the rationale for the particular probiotic strain and to gain information on the pre-clinical properties of the strain. Additionally, most probiotic studies in humans have been conducted in subjects who have been ill or critically ill. However the research/ effects should be also established in healthy people.

Safety issues of probiotics in humans

Probiotics have been advocated for the prevention and treatment of a wide range of diseases, and there is evidence for their efficacy in some clinical scenarios [97]. Given the increasingly widespread use of probiotics, a good understanding of their risks and benefits is imperative. Most probiotics are strains of bifidobacteria or lactobacilli, but species from other bacterial genera such as *Streptococcus*, *Bacillus* and *Enterococcus* have also been used as probiotics. There are concerns about the safety especially of the latter groups, as these genera contain many pathogenic species, particularly *Enterococcus* and *Streptococcus* (FAO/WHO. Guidelines for the evaluation of probiotics in food. 2002. Internet: http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf (accessed 27 May 2011)). Probiotics are often regulated as dietary supplements rather than as pharmaceuticals or biological products. Therefore there is usually no requirement to demonstrate safety, purity, or potency before marketing probiotics. In Europe, specific compositional legal requirements are for dietary supplements intended to be used by infants and young children. Unique to probiotics is however that they are alive when administered, and unlike other food or drug ingredients, possess the potential for infectivity or *in situ* toxin production.

Humans are regularly exposed to lactobacilli and bifidobacteria via their own microbiota, as well by consumption of foods such as yoghurt, cheese, sauerkraut and olives. In many traditional foods, these bacteria play an important role in preventing spoilage and the growth of pathogenic microorganisms [153]. Lactobacilli are normally present in the oral cavity (10^3-10^4 colony forming units (CFU)/g), in the ileum (10^3-10^7 CFU/g) and the colon (10^4-10^8 CFU/g). An important concern with probiotics is the risk of sepsis. In humans, lactobacilli and bifidobacteria are extremely rare causes of infection and are estimated to be 0.05%-0.4% of cases of infective endocarditis and bacteremia [154, 155]. These cases of infection with lactobacilli occur mostly in patients with underlying conditions that are predominantly of a severe nature and lactobacillemia is a frequent marker of serious or fatal underlying disease [154-157]. One reported case is of a 74-year-old diabetic woman who developed liver abscess and pneumonia 4 months after starting daily LGG supplementation. The infective and probiotic strains were indistinguishable by pulse-filed electrophoresis of chromosomal DNA restriction fragments [158]. Another case showed the development of *L. rhamnosus* endocarditis (strain not specified) after a dental extraction in a 67-year-old man with mitral regurgitation who was taking probiotic capsules daily. The authors found no differences between the probiotic and the infective *L. rhamnosus* with the use of standard API 50 CH biochemical analysis and pyrolysis mass spectrometry [159]. Another study reported that two patients, who received LGG to treat antibiotic-associated diarrhea, developed subsequently bacteremia and sepsis attributable to *Lactobacillus* species [160]. Molecular DNA fingerprinting analysis showed that the *Lactobacillus* isolated from the blood sample was indistinguishable from the probiotic ingested by these two patients. This indicates that invasive disease can be associated with probiotic lactobacilli. However,

in these cases the individuals had a serious underlying disease, that made them more susceptible to sepsis.

In general, immune-compromised patients are more vulnerable to infection with pathogens and have a higher incidence of opportunistic infections. There is published evidence, showing that consumption of probiotic products containing lactobacilli or bifidobacteria increases the risk of opportunistic infection among these individuals. In a study described by Besselink et al. 296 predicted severe acute pancreatitis patients were allocated to receive either a probiotic mixture comprising six strains (four *Lactobacillus* and two *Bifidobacterium* strains) at 10^{10} CFU/day or placebo, starting within 72 hours of onset of symptoms for 28 days [161]. This mixture was found to diminish acute pancreatitis-induced intestinal barrier dysfunction in rats [162]. In the human study, the incidence of infectious complications was comparable between the groups (probiotics vs. placebo, 30% vs. 28%, respectively). However mortality rates were significantly higher in the probiotic group (16% vs. 6%) as was the incidence of bowel ischemia, which occurred in nine probiotic-treated patients compared to none in the placebo group. This study demonstrated that probiotics are not completely innocuous and should not be given to patients that have potential for increased bacterial translocation and risk of severe life threatening complications.

Two clinical studies in immune compromised patients (i.e. with HIV infection) showed that probiotic consumption is safe in these groups [163, 164]. There has been a marked increase in the use of the probiotic LGG since its introduction in Finland in 1990. Despite this increased use, no significant increase in *Lactobacillus* bacteremia or bacteremia attributable to probiotic strains has been observed in Finland [155, 156].

Pregnant women might be at increased risk of adverse immune stimulation. During pregnancy there is a shift in T cell responses toward a Th2 phenotype, which is thought to be important in maintaining fetal viability because Th1 cytokines are associated with pregnancy loss [165]. Some probiotic lactobacilli have been shown to down regulate Th2 cytokine responses *in vitro*, and in some human studies were also found to increase production of the Th1 cytokine IFN [166, 167]. Probiotics might skew thus the immune response into a Th1 phenotype which might be harmful to pregnancy viability. At present there is little support for the hypothesis that probiotics cause adverse immune development from empirical studies, but this is an area that warrants further investigation. However, a study by Kaliomaki et al. did not report any adverse effects when LGG, which induces Th1 cytokines, was supplemented to mothers during pregnancy [136].

Unpredictability of immune modulation through change in intestinal microbiota in certain disease states are also of safety concern. For instance in patients taking some probiotic formulations Crohn's disease worsened [168]. Another study showed that LGG worsened the indomethacin induced enteropathy in an animal model [169].

It is necessary that in the selection of new probiotic strains these strains are derived from the commensal microbiota of humans or from fermented foods that have been consumed for a long time by humans and that they should not carry antibiotic resistance genes that would prevent treatment in case of a rare probiotic infection. Many

strains of lactobacilli are naturally resistant to vancomycin and the vancomycin resistant genes of lactobacilli appear to be chromosomally located and are not easily transferable to other genera [170]. In case of lactobacillemia, vancomycin would not be used for treatment, therefore selected probiotic strains should be susceptible to two of the major antibiotics. At this moment it is difficult to predict the frequency of gene transfer from lactobacilli to other bacterial species *in vivo*, but the main concern is the transfer of resistance genes to pathogenic strains of *Enterococcus* species and *Staphylococcus aureus*.

Manufacturers of probiotics have to closely observe potential adverse events. Current evidence suggests that the risk of infection with probiotic lactobacilli or bifidobacteria is similar to that of a commensal strain. In healthy individuals the risk appears to be negligible, but despite a low risk of infection due to probiotics, carefulness should be maintained and isolates should be checked by molecular characterization and confirmation [171, 172]. The safety of probiotics should be confirmed in studies with humans, because the classical safety measurements such as the determination of toxicity or pathogenicity of probiotic bacteria will always be hampered by the fact that simplified animal models or cell assays will inadequately mimic the complex gene-environment interaction in genetically susceptible human populations that might be at risk for the development of chronic degenerative diseases such as autoimmune, atopic, neurodegenerative or metabolic disorders or complex acute diseases such as pancreatitis and sepsis. Although probiotics have an excellent overall safety record, they should be used with caution in certain individuals, such as pregnant women or individuals with immune deficiency.

Viability of probiotics: do they need to be alive to be beneficial?

The definition of probiotics states specifically that the bacteria should be alive during administration. For this reason it is for the industry a great challenge to deliver live, viable microorganisms. Moreover the culture method as well as the growth stage of the bacteria can induce differential responses in humans [148]. Physiological characteristics as well as the delivery format (for instance in a dairy product or pills) are important in maintaining the viability of probiotics. For example, dairy products have a naturally shorter shelf-life than freeze-dried probiotic products in the form of powders and pills. However, levels of oxygen, moisture and storage temperature play an important role in the stability of the bacteria even with dried formulations. If dead microorganisms could also retain their probiotic activity it might facilitate their formulation, enhance shelf life and increase standardization [173-175].

Inactivated microbes, although not defined as probiotics according to the generally accepted definition by WHO, have been investigated for beneficial effects. However in the published studies to date, viable probiotics appear to have superior efficacy to heat-inactivated microbes for both *in vitro* and clinical studies [176-178]. It should be recognized that the presence of dead or injured microbes in commercial products is unavoidable, as some death occurs during storage of products and the standard approach to

maintaining the target dosage of live probiotic entails addition of surplus probiotics to account for any death occurring during storage [179, 180].
Different results have been obtained in studies that compared directly the effects of live and killed bacteria. One study showed that both live and heat-killed LGG, ameliorates intestinal and other inflammation in infant rats, by decreasing LPS-induced pro-inflammatory mediators and increasing anti-inflammatory mediators [181]. Another study comparing UV killed bacteria and live bacteria showed similar PBMC responses [182]. Interestingly, a study has revealed adverse effects resulting from ingestion of heat-killed probiotics by Kirjavainen et al. One group that ingested live, viable LGG was compared with another group ingesting heat-killed LGG for the prevention of allergies in infants. The authors reported increased gastrointestinal symptoms and diarrhea in those ingesting the formula with the heat-killed product, which led the Review Ethics Board to prematurely discontinue the study [183].
To conclude, although it might be more attractive from a safety and commercial view to use inactivated bacteria, studies so far showed that the beneficial effects are not always comparable for live and dead probiotic strains.

Major conclusions and future research

Certain strains of probiotics have been shown to confer health benefits in clinical trials and experimental animal models but there have also been a number of inconclusive, negative and even contrasting outcomes from *in vivo* studies. It is clear from both the literature and the research described in this thesis, that the effects of probiotics are highly dependent on the strain properties. Thus probiotics should be selected on a strain by strain basis in comparative studies, because their effects on the immune system and other properties can vary considerably even within a species. Furthermore, our lack of knowledge concerning the precise mechanisms of different probiotics is hampering the rational selection of strains and the correlation of *in vitro* data with the outcomes *in vivo*. Nevertheless, recent studies including those presented in this thesis suggest that *in vitro* immune assays are useful in selecting candidate strains for effects on the host immune system.
In *Chapters 6* and *7*, different strains of lactobacilli were tested for their effects on the immune function in different mouse models. A study by Kwon et al. showed that a mixture of strains was more effective in enhancing Foxp3 levels in MLN cells compared to individual strains [56]. The use of multiple-species or multiple-strain probiotic mixture has been recently reviewed by Chapman et al. [184], suggesting that the evidence of using probiotic mixtures is limited especially in the area of immunomodulation. The comparison is also difficult, due to different doses and routes of application. More research is needed to show that multi-strain probiotics have a greater effectiveness, using standardized doses in the same target population. Interestingly, Chapman et al. also found that bifidobacteria might inhibit probiotic efficiency in the presence of other species within a multi-strain mixture. They suggested that different species might inhibit each

other, possibly by the production of antagonist agents, or by competition for either nutrients or binding sites within the gastrointestinal tract. Given the current interest in combining multiple strains [56, 184], some of the strains in this thesis could be tested in a mouse vaccination model (*Chapter 7*) for their antagonistic or synergistic effects, e.g. identified candidate strains WCFS1 (Th2 promoting) and LMG18021 (Th1 promoting) or WCFS1 and KOG18 (both Th2 promoting).

To have a better mechanistic understanding of the immunomodulation by lactic acid bacteria, it is important to determine the regions of the gastrointestinal tract where probiotic strains encounter immune cells (especially APCs) of the host. This could be achieved for example by the use of genetically tagged bacteria or by genetically modified bacteria expressing fluorescence molecules, using new techniques as 2-photon imaging in live tissue.

The probiotics used in our animal studies were applied intragastrically, to avoid the interaction with food components and passage through the stomach, however survival in the stomach is known to vary among species/ strains and growth phase. Additionally, the food industry is more interested to make functional food by adding probiotics in their products. This means that there can be interactions with dietary components, which can influence the efficiency of probiotic strains. It would be interesting to test in more depth how food components can influence the probiotic efficiency.

Many published studies on the immune system and probiotics have not described the immune properties of the strains *in vitro* or compared different strains. In the past this somewhat empirical approach may have limited progress in understanding of the mechanisms and importance of strain selection. However, over the past 5 years the field has progressed enormously, largely due to developments in mucosal immunology and new insights into the mechanisms controlling tolerance and immunity in mucosal tissues. More research is performed aimed at identifying the underlying mechanisms of probiotics which will ultimately enable the potential societal/health benefits of probiotics to be fully realized.

References

1. Ichinohe, T., et al., Microbiota regulates immune defense against respiratory tract influenza A virus infection. Proc Natl Acad Sci U S A, 2011. 108(13): p. 5354-9.
2. Round, J.L. and S.K. Mazmanian, The gut microbiota shapes intestinal immune responses during health and disease. Nature reviews. Immunology, 2009. 9(5): p. 313-23.
3. Rakoff-Nahoum, S., et al., Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell, 2004. 118(2): p. 229-41.
4. Hall, J.A., et al., Commensal DNA limits regulatory T cell conversion and is a natural adjuvant of intestinal immune responses. Immunity, 2008. 29(4): p. 637-49.
5. O'Mahony, C., et al., Commensal-induced regulatory T cells mediate protection against pathogen-stimulated NF-kappaB activation. PLoS pathogens, 2008. 4(8): p. e1000112.
6. Round, J.L. and S.K. Mazmanian, Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. Proceedings of the National Academy of Sciences of the United States of America, 2010. 107(27): p. 12204-9.
7. Niess, J.H., et al., Commensal gut flora drives the expansion of proinflammatory CD4 T cells in the colonic lamina propria under normal and inflammatory conditions. Journal of immunology, 2008. 180(1): p. 559-68.
8. FAO and WHO, Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria. 2001.
9. Mileti, E., et al., Comparison of the immunomodulatory properties of three probiotic strains of Lactobacilli using complex culture systems: prediction for *in vivo* efficacy. PLoS One, 2009. 4(9): p. e7056.
10. Licciardi, P.V., et al., Epigenome targeting by probiotic metabolites. Gut Pathog, 2010. 2(1): p. 24.
11. Fukuda, S., et al., Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature, 2011. 469(7331): p. 543-7.
12. Maslowski, K.M., et al., Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature, 2009. 461(7268): p. 1282-6.
13. Corr, S.C., et al., Bacteriocin production as a mechanism for the antiinfective activity of *Lactobacillus salivarius* UCC118. Proceedings of the National Academy of Sciences of the United States of America, 2007. 104(18): p. 7617-21.
14. Mack, D.R., et al., Probiotics inhibit enteropathogenic E. coli adherence *in vitro* by inducing intestinal mucin gene expression. The American journal of physiology, 1999. 276(4 Pt 1): p. G941-50.
15. Wehkamp, J., et al., NF-kappaB- and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by Escherichia coli Nissle 1917: a novel effect of a probiotic bacterium. Infection and immunity, 2004. 72(10): p. 5750-8.
16. Schlee, M., et al., Probiotic lactobacilli and VSL#3 induce enterocyte beta-defensin 2. Clinical and experimental immunology, 2008. 151(3): p. 528-35.
17. Walker, W.A., Mechanisms of action of probiotics. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 2008. 46 Suppl 2: p. S87-91; discussion S144-51.
18. Corthesy, B., H.R. Gaskins, and A. Mercenier, Cross-talk between probiotic bacteria and the host immune system. The Journal of nutrition, 2007. 137(3 Suppl 2): p. 781S-90S.
19. Gill, H.S., et al., Enhancement of immunity in the elderly by dietary supplementation with

the probiotic *Bifidobacterium lactis* HN019. Am J Clin Nutr, 2001. 74(6): p. 833-9.
20. Klijn, N., A.H. Weerkamp, and W.M. de Vos, Genetic marking of Lactococcus lactis shows its survival in the human gastrointestinal tract. Appl Environ Microbiol, 1995. 61(7): p. 2771-4.
21. Vesa, T., P. Pochart, and P. Marteau, Pharmacokinetics of *Lactobacillus plantarum* NCIMB 8826, *Lactobacillus fermentum* KLD, and *Lactococcus lactis* MG 1363 in the human gastrointestinal tract. Aliment Pharmacol Ther, 2000. 14(6): p. 823-8.
22. Tannock, G.W., Probiotic properties of lactic-acid bacteria: plenty of scope for fundamental R & D. Trends Biotechnol, 1997. 15(7): p. 270-4.
23. Gardiner, G.E., et al., Relative ability of orally administered *Lactobacillus murinus* to pre dominate and persist in the porcine gastrointestinal tract. Appl Environ Microbiol, 2004. 70(4): p. 1895-906.
24. Klingberg, T.D. and B.B. Budde, The survival and persistence in the human gastrointestinal tract of five potential probiotic lactobacilli consumed as freeze-dried cultures or as probiotic sausage. Int J Food Microbiol, 2006. 109(1-2): p. 157-9.
25. McGuckin, M.A., et al., Mucin dynamics and enteric pathogens. Nat Rev Microbiol, 2011. 9(4): p. 265-78.
26. Wang, M.L., et al., Immune-mediated signaling in intestinal goblet cells via PI3-kinase- and AKT-dependent pathways. American journal of physiology. Gastrointestinal and liver physiology, 2008. 295(5): p. G1122-30.
27. Johansson, M.E., et al., The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Proc Natl Acad Sci U S A, 2008. 105(39): p. 15064-9.
28. Johansson, M.E., K.A. Thomsson, and G.C. Hansson, Proteomic analyses of the two mucus layers of the colon barrier reveal that their main component, the Muc2 mucin, is strongly bound to the Fcgbp protein. J Proteome Res, 2009. 8(7): p. 3549-57.
29. Atuma, C., et al., The adherent gastrointestinal mucus gel layer: thickness and physical state *in vivo*. Am J Physiol Gastrointest Liver Physiol, 2001. 280(5): p. G922-9.
30. van der Waaij, L.A., et al., Bacterial population analysis of human colon and terminal ileum biopsies with 16S rRNA-based fluorescent probes: commensal bacteria live in suspension and have no direct contact with epithelial cells. Inflamm Bowel Dis, 2005. 11(10): p. 865-71.
31. Kim, S.K., Small intestine transit time in the normal small bowel study. Am J Roentgenol Radium Ther Nucl Med, 1968. 104(3): p. 522-4.
32. Lambert, J.M., et al., Functional analysis of four bile salt hydrolase and penicillin acylase family members in *Lactobacillus plantarum* WCFS1. Appl Environ Microbiol, 2008. 74(15): p. 4719-26.
33. Gebert, A., et al., The development of M cells in Peyer's patches is restricted to specialized dome-associated crypts. Am J Pathol, 1999. 154(5): p. 1573-82.
34. Onori, P., et al., Peyer's patches epithelium in the rat: a morphological, immunohistochemical, and morphometrical study. Dig Dis Sci, 2001. 46(5): p. 1095-104.
35. Sharma, R., U. Schumacher, and E. Adam, Lectin histochemistry reveals the appearance of M-cells in Peyer's patches of SCID mice after syngeneic normal bone marrow transplantation. J Histochem Cytochem, 1998. 46(2): p. 143-8.
36. Macpherson, A.J. and T. Uhr, Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. Science, 2004. 303(5664): p. 1662-5.
37. Tyrer, P., et al., Microbial pattern recognition receptors mediate M-cell uptake of a gram-negative bacterium. Infect Immun, 2006. 74(1): p. 625-31.
38. Gullberg, E., et al., Identification of cell adhesion molecules in the human follicle-associated epithelium that improve nanoparticle uptake into the

Peyer's patches. J Pharmacol Exp Ther, 2006. 319(2): p. 632-9.
39. Begue, B., et al., Microbial induction of CARD15 expression in intestinal epithelial cells via toll-like receptor 5 triggers an antibacterial response loop. J Cell Physiol, 2006. 209(2): p. 241-52.
40. Tyrer, P., et al., Validation and quantitation of an *in vitro* M-cell model. Biochem Biophys Res Commun, 2002. 299(3): p. 377-83.
41. Perdigon, G., et al., Study of the possible mechanisms involved in the mucosal immune system activation by lactic acid bacteria. J Dairy Sci, 1999. 82(6): p. 1108-14.
42. Perdigon, G., et al., Intestinal pathway of internalisation of lactic acid bacteria and gut mucosal immunostimulation. Int J Immunopathol Pharmacol, 2000. 13(3): p. 141-150.
43. Berg, R.D. and W.E. Owens, Inhibition of translocation of viable Escherichia coli from the gastrointestinal tract of mice by bacterial antagonism. Infect Immun, 1979. 25(3): p. 820-27.
44. Berg, R.D., Bacterial translocation from the gastrointestinal tract. Adv Exp Med Biol, 1999. 473: p. 11-30.
45. Berg, R.D., Bacterial translocation from the gastrointestinal tract. Trends Microbiol, 1995. 3(4): p. 149-54.
46. Gautreaux, M.D., E.A. Deitch, and R.D. Berg, Bacterial translocation from the gastrointestinal tract to various segments of the mesenteric lymph node complex. Infect Immun, 1994. 62(5): p. 2132-4.
47. Rossi, O., P. Van Baarlen, and J. Wells, Mammalian Intestinal Host-Microbe Relationships. Curr Top Microbiol Immunol, 2011.
48. Rescigno, M., et al., Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat Immunol, 2001. 2(4): p. 361-7.
49. Rescigno, M., et al., Dendritic cells shuttle microbes across gut epithelial monolayers. Immunobiology, 2001. 204(5): p. 572-81.
50. Niess, J.H., et al., CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. Science, 2005. 307(5707): p. 254-8.
51. Rimoldi, M., et al., Monocyte-derived dendritic cells activated by bacteria or by bacteria-stimulated epithelial cells are functionally different. Blood, 2005. 106(8): p. 2818-26.
52. Kalinski, P., et al., Prostaglandin E(2) is a selective inducer of interleukin-12p40 (IL-12p40) production and an inhibitor of bioactive IL-12p70 heterodimer. Blood, 2001. 97(11): p. 3466-9.
53. Smythies, L.E., et al., Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. J Clin Invest, 2005. 115(1): p. 66-75.
54. Zeuthen, L.H., L.N. Fink, and H. Frokiaer, Epithelial cells prime the immune response to an array of gut-derived commensals towards a tolerogenic phenotype through distinct actions of thymic stromal lymphopoietin and transforming growth factor-beta. Immunology, 2007. 123(2): p. 197-208.
55. Foligne, B., et al., Correlation between *in vitro* and *in vivo* immunomodulatory properties of lactic acid bacteria. World J Gastroenterol, 2007. 13(2): p. 236-43.
56. Kwon, H.K., et al., Generation of regulatory dendritic cells and CD4+Foxp3+ T cells by probiotics administration suppresses immune disorders. Proc Natl Acad Sci U S A, 2010. 107(5): p. 2159-64.
57. Snel, J., et al., Strain-specific immunomodulatory effects of *Lactobacillus plantarum* strains on birch-pollen-allergic subjects out of season. Clin Exp Allergy, 2011. 41(2): p. 232-42.
58. Meijerink, M. and J. Wells, Probiotic modulation of dendritic cells and T cell responses in the intestine. Beneficial microbes, 2010. 1(4): p. 317-326.
59. Meijerink, M., et al., Identification of genetic loci in *Lactobacillus plantarum* that modulate the immune response of dendritic cells using comparative genome hybridization.

PLoS One, 2010. 5(5): p. e10632.
60. van Hemert, S., et al., Identification of *Lactobacillus plantarum* genes modulating the cytokine response of human peripheral blood mononuclear cells Submitted, 2010.
61. Ghadimi, D., et al., Effects of probiotic bacteria and their genomic DNA on TH1/TH2-cytokine production by peripheral blood mononuclear cells(PBMCs) of healthy and allergic subjects. Immunobiology, 2008. 213(8): p. 677-92.
62. Hayden, H., et al., Cryopreservation of monocytes is superior to cryopreservation of immature or semi-mature dendritic cells for dendritic cell-based immunotherapy. J Immunother, 2009. 32(6): p. 638-54.
63. John, J., et al., Cryopreservation of immature monocyte-derived dendritic cells results in enhanced cell maturation but reduced endocytic activity and efficiency of adenoviral transduction. J Immunol Methods, 2003. 272(1-2): p. 35-48.
64. Lewalle, P., et al., Freezing of dendritic cells, generated from cryopreserved leukaphereses, does not influence their ability to induce antigen-specific immune responses or functionally react to maturation stimuli. J Immunol Methods, 2000. 240(1-2): p. 69-78.
65. Hori, S., et al., Freeze-thawing procedures have no influence on the phenotypic and functional development of dendritic cells generated from peripheral blood CD14+ monocytes. J Immunother, 2004. 27(1): p. 27-35.
66. Delcenserie, V., et al., Immunomodulatory effects of probiotics in the intestinal tract. Curr Issues Mol Biol, 2008. 10(1-2): p. 37-54.
67. Medina, M., et al., Differential immunomodulatory properties of *Bifidobacterium longum* strains: relevance to probiotic selection and clinical applications. Clin Exp Immunol, 2007. 150(3): p. 531-8.
68. van Hemert, S., et al., Identification of *Lactobacillus plantarum* genes modulating the cytokine response of human peripheral bloodmononuclear cells. BMC Microbiology, 2010. 10(1): p. 293.
69. Miettinen, 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(12): p. 6058-62.
70. Miettinen, M., J. Vuopio-Varkila, and K. Varkila, Production of human tumor necrosis factor alpha, interleukin-6, and interleukin-10 is induced by lactic acid bacteria. Infect Immun, 1996. 64(12): p. 5403-5.
71. Niers, L.E., et al., Identification of strong interleukin-10 inducing lactic acid bacteria which down-regulate T helper type 2 cytokines. Clin Exp Allergy, 2005. 35(11): p. 1481-9.
72. Lebeer, S., J. Vanderleyden, and S.C. De Keersmaecker, Genes and molecules of lactobacilli supporting probiotic action. Microbiol Mol Biol Rev, 2008. 72(4): p. 728-64,
73. Fernandez, E.M., et al., Anti-inflammatory capacity of selected lactobacilli in experimental colitis is driven by NOD2-mediated recognition of a specific peptidoglycan-derived muropeptide. Gut, 2011. 60(8): p. 1050-9.
74. Grangette, C., et al., Enhanced antiinflammatory capacity of a *Lactobacillus plantarum* mutant synthesizing modified teichoic acids. Proc Natl Acad Sci U S A, 2005. 102(29): p. 10321-6.
75. Mohamadzadeh, M., et al., Regulation of induced colonic inflammation by *Lactobacillus acidophilus* deficient in lipoteichoic acid. Proc Natl Acad Sci U S A, 2011. 108 Suppl 1: p. 4623-30.
76. Hemmi, H., et al., A Toll-like receptor recognizes bacterial DNA. Nature, 2000. 408(6813): p. 740-5.
77. Lee, J., et al., Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells. Nat Cell Biol, 2006. 8(12): p. 1327-36.

78. Jijon, H., et al., DNA from probiotic bacteria modulates murine and human epithelial and immune function. Gastroenterology, 2004. 126(5): p. 1358-73.
79. Ghadimi, D., et al., Effect of natural commensal-origin DNA on toll-like receptor 9 (TLR9) signaling cascade, chemokine IL-8 expression, and barrier integritiy of polarized intestinal epithelial cells. Inflamm Bowel Dis, 2010. 16(3): p. 410-27.
80. Katakura, K., et al., Toll-like receptor 9-induced type I IFN protects mice from experimental colitis. J Clin Invest, 2005. 115(3): p. 695-702.
81. Crabtree, T.D., et al., Preexposure of murine macrophages to CpG oligonucleotide results in a biphasic tumor necrosis factor alpha response to subsequent lipopolysaccharide challenge. Infect Immun, 2001. 69(4): p.2123-9.
82. Obermeier, F., et al., CpG motifs of bacterial DNA exacerbate colitis of dextran sulfate sodium-treated mice. Eur J Immunol, 2002. 32(7): p. 2084-92.
83. Liao, A.P., et al., Salmonella type III effector AvrA stabilizes cell tight junctions to inhibit inflammation in intestinal epithelial cells. PLoS One, 2008. 3(6): p. e2369.
84. Collier-Hyams, L.S., et al., Cutting edge: Salmonella AvrA effector inhibits the key proinflammatory, anti-apoptotic NF-kappa B pathway. J Immunol, 2002. 169(6): p. 2846-50.
85. Ye, Z., et al., Salmonella effector AvrA regulation of colonic epithelial cell inflammation by deubiquitination. Am J Pathol, 2007. 171(3): p. 882-92.
86. Boneca, I.G., et al., A critical role for peptidoglycan N-deacetylation in Listeria evasion from the host innate immune system. Proc Natl Acad Sci U S A, 2007. 104(3): p. 997-1002.
87. Fittipaldi, N., et al., Significant contribution of the pgdA gene to the virulence of *Streptococcus suis*. Mol Microbiol, 2008. 70(5): p. 1120-35.
88. Troy, E.B. and D.L. Kasper, Beneficial effects of Bacteroides fragilis polysaccharides on the immune system. Front Biosci, 2010. 15: p. 25-34.
89. Mosmann, T.R., et al., Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. 1986. J Immunol, 2005. 175(1): p. 5-14.
90. Mosmann, T.R., et al., Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol, 1986. 136(7): p. 2348-57.
91. Crome, S.Q., A.Y. Wang, and M.K. Levings, Translational mini-review series on Th17 cells: function and regulation of human T helper 17 cells in health and disease. Clin Exp mmunol, 2010. 159(2): p. 109-19.
92. Guarner, F., et al., Mechanisms of disease: the hygiene hypothesis revisited. Nat Clin Pract Gastroenterol Hepatol, 2006. 3(5): p. 275-84.
93. Shida, K., et al., *Lactobacillus casei* strain Shirota suppresses serum immunoglobulin E and immunoglobulin G1 responses and systemic anaphylaxis in a food allergy model. Clin Exp Allergy, 2002. 32(4): p. 563-70.
94. Kim, H., et al., Oral probiotic bacterial administration suppressed allergic responses in an ovalbumin-induced allergy mouse model. FEMS Immunol Med Microbiol, 2005. 45(2): p. 259-67.
95. Frossard, C.P., L. Steidler, and P.A. Eigenmann, Oral administration of an IL-10-secreting Lactococcus lactis strain prevents food-induced IgE sensitization. J Allergy Clin Immunol, 2007. 119(4): p. 952-9.
96. Kim, J.Y., Y.O. Choi, and G.E. Ji, Effect of oral probiotics (*Bifidobacterium lactis* AD011 and *Lactobacillus acidophilus* AD031) administration on ovalbumin-induced food allergy mouse model. J Microbiol Biotechnol, 2008. 18(8): p. 1393-400.
97. Kalliomaki, M., et al., Guidance for Substantiating the Evidence for Beneficial Effects of Probiotics: Prevention and Management of Allergic Diseases by Probiotics. J Nutr, 2010.
98. Schiavi, E., et al., Oral therapeutic administration of a probiotic mixture suppresses

established Th2 responses and systemic anaphylaxis in a murine model of food allergy. Allergy, 2011. 66(4): p. 499-508.
99. Peran, L., et al., Preventative effects of a probiotic, *Lactobacillus salivarius* ssp. *salivarius*, in the TNBS model of rat colitis. World J Gastroenterol, 2005. 11(33): p. 5185-92.
100. Zoumpopoulou, G., et al., *Lactobacillus fermentum* ACA-DC 179 displays probiotic potential *in vitro* and protects against trinitrobenzene sulfonic acid (TNBS)-induced colitis and Salmonella infection in murine models. Int J Food Microbiol, 2008. 121(1): p. 18-26.
101. Ivory, K., et al., Oral delivery of *Lactobacillus casei* Shirota modifies allergen-induced immune responses in allergic rhinitis. Clin Exp Allergy, 2008. 38(8): p. 1282-9.
102. Holt, P.G. and C.A. Jones, The development of the immune system during pregnancy and early life. Allergy, 2000. 55(8): p. 688-97.
103. Trammell, R.A. and L.A. Toth, Genetic susceptibility and resistance to influenza infection and disease in humans and mice. Expert Rev Mol Diagn, 2008. 8(4): p. 515-29.
104. Howell, W.M., P.C. Calder, and R.F. Grimble, Gene polymorphisms, inflammatory diseases and cancer. Proc Nutr Soc, 2002. 61(4): p. 447-56.
105. Yaqoob, P., E.A. Newsholme, and P.C. Calder, Comparison of cytokine production in cultures of whole human blood and purified mononuclear cells. Cytokine, 1999. 11(8): p. 600-5.
106. Schroder, N.W. and R.R. Schumann, Single nucleotide polymorphisms of Toll-like receptors and susceptibility to infectious disease. Lancet Infect Dis, 2005. 5(3): p. 156-64.
107. Ogura, Y., et al., A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature, 2001. 411(6837): p. 603-6.
108. Hampe, J., et al., Association between insertion mutation in NOD2 gene and Crohn's disease in German and British populations. Lancet, 2001. 357(9272): p. 1925-8.
109. Manicassamy, B., et al., Protection of mice against lethal challenge with 2009 H1N1 influenza A virus by 1918-like and classical swine H1N1 based vaccines. PLoS Pathog, 2010. 6(1): p. e1000745.
110. Boon, A.C., et al., Host genetic variation affects resistance to infection with a highly pathogenic H5N1 influenza A virus in mice. J Virol, 2009. 83(20): p.10417-26.
111. Sun, L., G.M. Nava, and T.S. Stappenbeck, Host genetic susceptibility, dysbiosis, and viral triggers in inflammatory bowel disease.Curr Opin Gastroenterol, 2011. 27(4): p. 321-7.
112. Abraham, C. and R. Medzhitov, Interactions between the host innate mmune system and microbes in inflammatory bowel disease. Gastroenterology, 2011. 140(6): p. 1729-37.
113. Saleh, M. and C.O. Elson, Experimental inflammatory bowel disease: insights into the host-microbiota dialog. Immunity, 2011. 34(3): p. 293-302.
114. Feng, T. and C.O. Elson, Adaptive immunity in the host-microbiota dialog. Mucosal Immunol, 2011. 4(1): p. 15-21.
115. Ivanov, II, et al., Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell, 2009. 139(3): p. 485-98.
116. Gaboriau-Routhiau, V., et al., The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses.Immunity, 2009. 31(4): p. 677-89.
117. Mazmanian, S.K., J.L. Round, and D.L. Kasper, A microbial symbiosis factor prevents intestinal inflammatory disease. Nature,2008.453(7195):p. 620-5.
118. Arumugam, M., et al., Enterotypes of the human gut microbiome. Nature, 2011. 473(7346): p. 174-80.
119. Morafo, V., et al., Genetic susceptibility to food allergy is linked to differential TH2-TH1 responses in C3H/HeJ and BALB/c mice. J Allergy Clin Immunol, 2003. 111(5): p. 1122-8.
120. Nishimura, T., et al., Involvement of IL-4-producing Vbeta8.2+ CD4+ CD62L- CD45RB- T cells in non-MHC gene-controlled predisposition toward skewing into T helper type-2 immunity in BALB/c mice. J Immunol, 1997. 158(12): p. 5698-706.

121. Fullner, K.J., W.I. Lencer, and J.J. Mekalanos, Vibrio cholerae-induced cellular responses of polarized T84 intestinal epithelial cells are dependent on production of cholera toxin and the RTX toxin. Infect Immun, 2001. 69(10): p. 6310-7.
122. O'Toole, P.W. and J.C. Cooney, Probiotic bacteria influence the composition and function of the intestinal microbiota. Interdiscip Perspect Infect Dis, 2008. 2008: p. 175285.
123. Petrosino, J.F., et al., Metagenomic pyrosequencing and microbialidentification. Clin Chem, 2009. 55(5): p. 856-66.
124. Goldberg, S.M., et al., A Sanger/pyrosequencing hybrid approach for the generation of high-quality draft assemblies of marine microbial genomes. Proc Natl Acad Sci U S A, 2006. 103(30): p. 11240-5.
125. Moore, M.J., et al., Rapid and accurate pyrosequencing of angiosperm plastid genomes. BMC Plant Biol, 2006. 6: p. 17.
126. Wicker, T., et al., 454 sequencing put to the test using the complex genome of barley. BMC Genomics, 2006. 7: p. 275.
127. Gharizadeh, B., et al., Large-scale pyrosequencing of synthetic DNA: a comparison with results from Sanger dideoxy sequencing. Electrophoresis, 2006. 27(15): p. 3042-7.
128. Peterson, D.A., et al., Metagenomic approaches for defining the pathogenesis of inflammatory bowel diseases. Cell Host Microbe, 2008. 3(6): p. 417-27.
129. Delgado, S., A. Suarez, and B. Mayo, Identification of dominant bacteria in feces and colonic mucosa from healthy Spanish adults by culturing and by 16S rDNA sequence analysis. Dig Dis Sci, 2006. 51(4): p. 744-51.
130. Lucke, K., et al., Prevalence of Bacteroides and Prevotella spp. in ulcerative colitis. J Med Microbiol, 2006. 55(Pt 5): p. 617-24.
131. Prindiville, T., M. Cantrell, and K.H. Wilson, Ribosomal DNA sequence analysis of mucosa-associated bacteria in Crohn's disease. Inflamm Bowel Dis, 2004. 10(6): p. 824-33.
132. Hold, G.L., et al., Assessment of microbial diversity in human colonic samples by 16S rDNA sequence analysis. FEMS Microbiol Ecol, 2002. 39(1): p. 33-9.
133. Hayashi, H., M. Sakamoto, and Y. Benno, Phylogenetic analysis of the human gut microbiota using 16S rDNA clone libraries and strictly anaerobic culture-based methods. Microbiol Immunol, 2002. 46(8): p. 535-48.
134. Suau, A., et al., Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl Environ Microbiol, 1999. 65(11): p. 4799-807.
135. Ryan, K.A., et al., Strain-specific inhibition of Helicobacter pylori by *Lactobacillus salivarius* and other lactobacilli. J Antimicrob Chemother, 2008. 61(4): p. 831-4.
136. Kalliomaki, M., et al., Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. Lancet, 2001. 357(9262): p. 1076-9.
137. Kalliomaki, M., et al., Probiotics and prevention of atopic disease: 4-year follow-up of a randomised placebo-controlled trial. Lancet, 2003. 361(9372): p. 1869-71.
138. Majamaa, H. and E. Isolauri, Probiotics: a novel approach in the management of food allergy. J Allergy Clin Immunol, 1997. 99(2): p. 179-85.
139. Isolauri, E., et al., Probiotics in the management of atopic eczema. Clin Exp Allergy, 2000. 30(11): p. 1604-10.
140. Viljanen, M., et al., Probiotics in the treatment of atopic eczema/dermatitis syndrome in infants: a double-blind placebo-controlled trial. Allergy, 2005. 60(4): p. 494-500.
141. Brouwer, M.L., et al., No effects of probiotics on atopic dermatitis in infancy: a randomized placebo-controlled trial. Clin Exp Allergy, 2006. 36(7): p. 899-906.
142. Folster-Holst, R., et al., Prospective, randomized controlled trial on *Lactobacillus rhamnosusin* infants with moderate to severe atopic dermatitis. Br J Dermatol, 2006.

155(6): p. 1256-61.
143. Gruber, C., et al., Randomized, placebo-controlled trial of *Lactobacillus rhamnosus* GG as treatment of atopic dermatitis in infancy. Allergy, 2007. 62(11): p. 1270-6.
144. Schabussova, I., et al., Distinctive anti-allergy properties of two probiotic bacterial strains in a mouse model of allergic poly-sensitization. Vaccine, 2011. 29(10): p. 1981-90.
145. Harata, G., et al., Intranasal administration of *Lactobacillus rhamnosus* GG protects mice from H1N1 influenza virus infection by regulating respiratory immune responses. Lett Appl Microbiol, 2010. 50(6): p. 597-602.
146. Kawase, M., et al., Oral administration of lactobacilli from human intestinal tract protects mice against influenza virus infection. Lett Appl Microbiol, 2010. 51(1): p. 6-10.
147. Boge, T., et al., A probiotic fermented dairy drink improves antibody response to influenza vaccination in the elderly in two randomised controlled trials. Vaccine, 2009. 27(41): p. 5677-84.
148. van Baarlen, P., et al., Differential NF-kappaB pathways induction by *Lactobacillus plantarum* in the duodenum of healthy humans correlating with immune tolerance. Proc Natl Acad Sci U S A, 2009. 106(7): p. 2371-6.
149. Li, X.M., et al., A murine model of peanut anaphylaxis: T- and B-cell responses to a major peanut allergen mimic human responses. J Allergy Clin Immunol, 2000. 106(1 Pt 1): p. 150-8.
150. van Wijk, F., et al., The CD28/CTLA-4-B7 signaling pathway is involved in both allergic sensitization and tolerance induction to orally administered peanut proteins. J Immunol, 2007. 178(11): p. 6894-900.
151. Lin, D.C., Probiotics as functional foods. Nutr Clin Pract, 2003. 18(6): p. 497-506.
152. Basu, S., et al., Efficacy of high-dose *Lactobacillus rhamnosus* GG in controlling acute watery diarrhea in Indian children: a randomized controlled trial. J Clin Gastroenterol, 2009. 43(3): p. 208-13.
153. Hammes, W.P. and P.S. Tichaczek, The potential of lactic acid bacteria for the production of safe and wholesome food. Z Lebensm Unters Forsch, 1994. 198(3): p. 193-201.
154. Gasser, F., Safety of lactic-acid bacteria and their occurrence in human clinical infections. Bulletin de L'Institut Pasteur, 1994. 92: p. 45–67.
155. Saxelin, M., et al., Lactobacilli and bacteremia in southern Finland, 1989-1992. Clin Infect Dis, 1996. 22(3): p. 564-6.
156. Salminen, M.K., et al., *Lactobacillus* bacteremia during a rapid increase in probiotic use of *Lactobacillus rhamnosus* GG in Finland. Clin Infect Dis, 2002. 35(10): p. 1155-60.
157. Husni, R.N., et al., *Lactobacillus* bacteremia and endocarditis: review of 45 cases. Clin Infect Dis, 1997. 25(5): p. 1048-55.
158. Rautio, M., et al., Liver abscess due to a *Lactobacillus rhamnosus* strain indistinguishable from *L. rhamnosus* strain GG. Clin Infect Dis, 1999. 28(5): p. 1159-60.
159. Mackay, A.D., et al., *Lactobacillus* endocarditis caused by a probiotic organism. Clin Microbiol Infect, 1999. 5(5): p. 290-292.
160. Land, M.H., et al., *Lactobacillus* sepsis associated with probiotic therapy. Pediatrics, 2005. 115(1): p. 178-81.
161. Besselink, M.G., et al., Probiotic prophylaxis in predicted severe acute pancreatitis: a randomised, double-blind, placebo-controlled trial. Lancet, 2008. 371(9613): p. 651-9.
162. Lutgendorff, F., et al., Probiotics prevent intestinal barrier dysfunction in acute pancreatitis in rats via induction of ileal mucosal glutathione biosynthesis. PLoS One, 2009. 4(2): p. e4512.
163. Wolf, B.W., et al., Safety and tolerance of *Lactobacillus reuteri* supplementation to a population infected with the human immunodeficiency virus. Food Chem Toxicol, 1998. 36(12): p. 1085-94.

164. Cunningham-Rundles, S., et al., Probiotics and immune response. Am J Gastroenterol, 2000. 95(1 Suppl): p. S22-5.
165. Wegmann, T.G., et al., Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? Immunol Today, 1993. 14(7): p. 353-6.
166. Pochard, P., et al., Lactic acid bacteria inhibit TH2 cytokine production by mononuclear cells from allergic patients. J Allergy Clin Immunol, 2002. 110(4): p. 617-23.
167. Pohjavuori, E., et al., *Lactobacillus* GG effect in increasing IFN-gamma production in infants with cow's milk allergy. J Allergy Clin Immunol, 2004. 114(1): p. 131-6.
168. Rolfe, V.E., et al., Probiotics for maintenance of remission in Crohn's disease. Cochrane Database Syst Rev, 2006(4): p. CD004826.
169. Kamil, R., et al., *Lactobacillus rhamnosus* GG exacerbates intestinal ulceration in a model of indomethacin-induced enteropathy. Dig Dis Sci, 2007. 52(5): p. 1247-52.
170. Tynkkynen, S., K.V. Singh, and P. Varmanen, Vancomycin resistance factor of *Lactobacillus rhamnosus* GG in relation to enterococcal vancomycin resistance (van) genes. Int J Food Microbiol, 1998. 41(3): p. 195-204.
171. Sanders, M.E., et al., Safety assessment of probiotics for human use. Gut Microbes, 2010. 1(3): p. 164-185.
172. Ezendam, J. and H. van Loveren, Probiotics: immunomodulation and evaluation of safety and efficacy. Nutr Rev, 2006. 64(1): p. 1-14.
173. Adams, C.A., The probiotic paradox: live and dead cells are biological response modifiers. Nutr Res Rev, 2010. 23(1): p. 37-46.
174. Kataria, J., et al., Probiotic microbes: do they need to be alive to be beneficial? Nutr Rev, 2009. 67(9): p. 546-50.
175. Ouwehand, A.C., S. Salminen, and E. Isolauri, Probiotics: an overview of beneficial effects. Antonie Van Leeuwenhoek, 2002. 82(1-4): p. 279-89.
176. Yan, F. and D.B. Polk, Probiotic bacterium prevents cytokine-induced apoptosis in intestinal epithelial cells. J Biol Chem, 2002. 277(52): p. 50959-65.
177. Olah, A., et al., Randomized clinical trial of specific *lactobacillus* and fibre supplement to early enteral nutrition in patients with acute pancreatitis. Br J Surg, 2002. 89(9): p. 1103-7.
178. Rayes, N., et al., Early enteral supply of *lactobacillus* and fiber versus selective bowel decontamination: a controlled trial in liver transplant recipients. Transplantation, 2002. 74(1): p. 123-7.
179. Drago, L., et al., Microbiological evaluation of commercial probiotic products available in the USA in 2009. J Chemother, 2010. 22(6): p. 373-7.
180. Wannaprasat, W., et al., Quality analysis of commercial probiotic products for food animals. Southeast Asian J Trop Med Public Health, 2009. 40(5): p. 1103-12.
181. Li, N., et al., Live and heat-killed *Lactobacillus rhamnosus* GG: effects on proinflammatory and anti-inflammatory cytokines/chemokines in gastrostomy-fed infant rats. Pediatr Res, 2009. 66(2): p. 203-7.
182. van Hoffen, E., et al., Exposure of intestinal epithelial cells to UV-killed *Lactobacillus* GG but not *Bifidobacterium breve* enhances the effector immune response *in vitro*. Int Arch Allergy Immunol, 2010. 152(2): p. 159-68.
183. Kirjavainen, P.V., S.J. Salminen, and E. Isolauri, Probiotic bacteria in the management of atopic disease: underscoring the importance of viability. J Pediatr Gastroenterol Nutr, 2003. 36(2): p. 223-7.
184. Chapman, C.M., G.R. Gibson, and I. Rowland, Health benefits of probiotics: are mixtures more effective than single strains? Eur J Nutr, 2011. 50(1): p. 1-17.

Summary

The aim of this thesis was to better understand the molecular mechanism of host responses to probiotics. Probiotics can be used to stimulate or regulate immune responses in epithelial and immune cells of the intestinal mucosa and generate beneficial effects on the immune system. Carefully selected probiotics are able to steer the activity of the immune response in a predetermined manner by increasing or decreasing the activity of different aspects of the immune system (e.g. development and activity of T helper subsets). Beneficial effects of strains of probiotics have been established in the treatment and prevention of various intestinal disorders, including allergic diseases and diarrhea. However the precise molecular mechanisms and the strain dependent factors involved are poorly understood. Here *in vitro* molecular studies and *in vivo* mechanistic studies were combined in different mouse models to generate new insights into the beneficial mechanisms of selected lactobacilli and identify novel bacterial genes influencing the immune response. A further aim was to investigate the predictive value of *in vitro* immune assays for the effects of probiotics *in vivo*.

Chapter 1 and **chapter 2** describe the current knowledge and understanding of the immunomodulatory effects of different probiotic species and strains on mucosal immune system, dendritic cells (DCs) and the adaptive immune system. The relevance and the implications of *in vitro* studies for clinical trials or mechanistic research in animal models are discussed.
Chapter 3 and **chapter 4** present new insights gained from research on the strain-dependent factors involved in probiotic immune modulation. Extensive variation was observed in the immune responses to 42 *L. plantarum* strains. These results were used to identify genetic loci that correlated with levels of induced cytokines (such as IL-10 or IL-12) following co-culture with DCs (**chapter 3**) or peripheral blood mononuclear cells (PBMCs) (**chapter 4**). This *in silico* "gene-trait matching" approach led to the identification of several candidate genes in the *L. plantarum* genome that might modulate the immune cytokine response to *L. plantarum*. Selective gene deletions mutants were constructed for the candidate genes in *L. plantarum* WCFS1 and compared to the wild-type strain in immune assays with PBMCs and DCs. The predicted phenotype of the genetic knock-out was confirmed for most of the candidate loci including genes encoding an N-acetyl-glucosamine/galactosamine phosphotransferase system, the LamBDCA quorum sensing system, a predicted transcriptional regulator gene (lp_2991) and components of the plantaricin (bacteriocin) biosynthesis and transport pathway. Transcriptome analysis and qPCR data showed that transcript level of *gtcA3*, which is predicted to be involved in the glycosylation of cell wall teichoic acids, was substantially increased in the *lp_2991* deletion mutant (44- and 29-fold respectively).
In vitro assays for pre-screening of candidate probiotics would benefit from standardized methods and cryopreservation techniques for immature DCs (iDCs) or precursor monocytes. Literature on the effects of cryopreservation and thawing of monocytes or monocyte-derived iDCs suggested that this strategy might be useful although bacteria had not been previously used as a stimulus. Thus in **chapter 5** we investigated

the effects of cryopreservation and thawing of precursor monocytes and iDCs on the maturation and immune response of DCs to potential probiotic strains and bacterial TLR agonists. Surface markers CD83 and CD86 were expressed at similar levels on iDCs generated from cryopreserved or freshly isolated monocytes. Cryopreservation of iDCs led to slightly decreased expression of CD86 and CD83 compared to freshly generated iDCs prepared from unfrozen cells but this did not affect the capacity of DCs to acquire fully mature characteristics after stimulation. In contrast the cytokine response to lipoteichoic acid and bacterial stimulation was altered by cryopreservation of monocytes or iDCs, particularly for IL-12 which was decreased up to 250 fold or even not detected at all. Cryopreservation also decreased TNF-α and IL-1β production in stimulated iDCs but to a lesser extent than for IL-12, depending on the maturation factors used. The amounts of IL-10 produced by stimulated iDCs were increased up to 3.6 fold when iDCs were cryopreserved, but decreased up to 90 fold when generated from cryopreserved monocytes. Immature DCs are often used to investigate the immunomodulatory properties of probiotics and here we showed for the first time that cryopreserved monocytes and cryopreserved iDCs have a skewed cytokine response to microbial stimulation. Therefore we consider that standardization of probiotic screening assays by the use of cryopreservation methods is currently not applicable. The detailed method for generating human monocyte derived DC described in **chapter 5** may however be useful for developing standardized immune assays.

In **chapter 6** we screened the immunomodulatory properties of 28 commercially available bacterial strains *in vitro* using human PBMCs and investigated selected strains for their *in vivo* immunomodulatory potential in an established mouse peanut allergy model. The 28 probiotic strains induced highly variable cytokine profiles in PBMCs. *L. salivarius* HMI001 (HMI001), *L. casei* Shirota (LCS) and *L. plantarum* WCFS1 (WCFS1) were selected for further investigation due to their distinct patterns of IL-10, IL-12 and IFN-γ induction. Prophylactic treatment with both HMI001 and LCS attenuated the Th2 phenotype in the mouse model (reduced mast cell responses and *ex vivo* IL-4 and/or IL-5 production). In contrast, WCFS1 augmented the Th2 phenotype (increased mast cell and antibody responses and *ex vivo* IL-4 production). *In vitro* PBMC screening was useful in selecting strains with anti-inflammatory and Th1 skewing properties. In the case of HMI001 (inducing a high IL-10/IL-12 ratio) and LCS (inducing high amounts of IFN-γ and IL-12) partial protection was seen in a mouse peanut allergy model. However, certain strains may worsen the allergic reaction as shown in the case of WCFS1. This approach indicated that pre-selection of candidate probiotics using *in vitro* immune assays is useful for selecting strains for translational research in humans.

Probiotics have been shown to increase the efficacy of different vaccines and can be easily consumed in food, and therefore probiotics might be useful in the improvement of current mucosal vaccines. In **chapter 7** we have investigated the mechanisms behind the effect of lactobacilli on humoral responses to an intranasal vaccine. In addition to *L. rhamnosus* GG we selected 6 strains of *Lactobacillus plantarum* which have strikingly different immunomodulatory properties *in vitro* and TLR-2/6 activating properties. This

selection was based on the approach outlined in **chapter 3** and **chapter 4** examining the *in vitro* immune responses of human monocyte derived DCs and PBMCs to 42 different *L. plantarum* strains. First we established an influenza vaccination model in Balb/c mice that would be sensitive to immunomodulation by lactobacilli, which allowed potential up- and down-regulation by the lactobacilli of the immune response. Strain WCFS1, that induced the lowest IL-10 to IL-12 cytokine ratio in DC co-culture significantly increased vaccine-specific antibody responses to the intranasal vaccine compared to the vaccine control group. Several *Lactobacillus* strains appeared to increase delayed-type hypersensitivity responses after vaccination compared to the vaccine control group indicating increased Th1-mediated vaccine responses. For strain LMG18021 this was also reflected in the significantly higher vaccine-specific IgG2a to IgG1 antibody ratio. LMG18021, CIP104448 and CIP104450 which have the highest IL-10 to IL-12 ratios of the strains tested, significantly enhanced the *ex vivo* vaccine-specific induction of IL-10, IL-17A, IL-6 and IL-4 in MLN cells. B1839 which was included as negative control, as it was a low cytokine inducer, did not enhance the vaccine-specific antibody or immune response indicating that the immune-stimulatory properties are important in mediating effects on the vaccine response. Further research is needed to demonstrate that these effects on the vaccine response impact on protection from influenza challenge and to validate the immunomodulatory mechanisms involved. Nevertheless, the *in vivo* studies described in this thesis support other publications proposing that *in vitro* immune assays can be useful for predicting which candidate probiotic strains will be most effective *in vivo*.

Chapter 8 completes this thesis with an overview of the most important findings of this thesis and discusses possible research limitations and future research perspectives. We stress the importance of proper strain selection using *in vitro* assays, and the use of strategies to identify novel immunomodulatory factors. The results described in this thesis support the rationale of using *in vitro* co-culture assays for selection of candidate probiotics for *in vivo* animal experiments or human trials.

Samenvatting

Het doel van dit proefschrift was om beter inzicht te krijgen in de moleculaire mechanismen van de gastheer reacties op probiotica. Probiotica kunnen worden gebruikt om het immuun systeem te stimuleren of reguleren in het epitheel van het darmslijmvlies en op die manier positieve effecten genereren op het immuunsysteem. Zorgvuldig geselecteerde probiotica zijn in staat zijn om de activiteit van de immuunrespons te sturen op een vooraf bepaalde manier door middel van het verhogen of verlagen van de activiteit van verschillende aspecten van het immuunsysteem (bijv. de ontwikkeling en activiteit van T-helper subsets). Gunstige effecten van stammen van probiotica zijn aangetoond in de behandeling en preventie van diverse darm aandoeningen, waaronder allergische ziekten en diarree. Echter, de precieze moleculaire werkingsmechanismen en de bacterie stam afhankelijke factoren die daarbij betrokken zijn, zijn nog niet geheel bekend. In dit proefschrift zijn *in vitro* moleculaire studies en *in vivo* mechanistische studies in verschillende muismodellen gecombineerd, om nieuwe inzichten krijgen in de positieve mechanismen van geselecteerde lactobacillen en om nieuwe bacteriële genen die invloed op de immuunrespons hebben te identificeren. Hiernaast is onderzocht of *in vitro* immuun testen een voorspellende waarde voor de effecten van probiotica *in vivo*.
Hoofdstuk 1 en **hoofdstuk 2** geven een overzicht van de huidige kennis en de werking van de immunomodulerende effecten van verschillende probiotische soorten en stammen op het mucosale immuunsysteem, dendritische cellen (DCs) en het adaptieve immuunsysteem. Daarnaast worden de relevantie en de implicaties van *in vitro* studies voor mechanistisch onderzoek in dierlijke modellen en in klinische studies besproken.
Hoofdstuk 3 en **hoofdstuk 4** tonen nieuwe inzichten aan verkregen uit onderzoek naar de stam-afhankelijke factoren die betrokken zijn bij probiotische immuunmodulatie. De inductie van de immuunrespons varieerde aanzienlijk tussen de 42 verschillende *L. plantarum* stammen. Deze resultaten werden gebruikt om genetische loci te identificeren die correleerden met de hoeveelheid geïnduceerde cytokines (zoals IL-10 of IL-12) na co-kweek met DCs (**hoofdstuk 3**) of perifere mononucleaire bloedcellen (PBMCs) (**hoofdstuk 4**). Deze *in silico* 'gen-eigenschap matching' aanpak heeft geleid tot de identificatie van een aantal kandidaat-genen in het genoom van *L. plantarum*, die cytokine respons van het immuunsysteem op *L. plantarum* zouden kunnen moduleren. Selectieve gen deletie mutanten werden gemaakt voor de kandidaat-genen in *L. plantarum* WCFS1 en vergeleken met de wild-type stam in immuun assays met PBMCs en DCs. De voorspelde fenotypes van de genetische deletie mutanten werden bevestigd voor de meeste van de kandidaat-loci, waaronder genen waren die coderen voor een N-acetylglucosamine/galactosamine fosfotransferase systeem, de LamBDCA quorum sensing-systeem, een geannoteerde transcriptionele regulator gen (*lp_2991*) en onderdelen van de plantaricin (bacteriocine) biosynthese en transport route. Transcriptoom analyse en qPCR data laten zien dat transcript niveau van *gtcA3*, waarvan voorspeld wordt dat deze betrokken is bij de glycosylering van celwand teichoine zuren, aanzienlijk toegenomen is in de *lp_2991* deletie mutant (44 - en 29-voud respectievelijk).
In vitro testen voor het pre-screenen van kandidaat-probiotica zouden gebaat kunnen zijn bij gestandaardiseerde methoden en technieken voor cryopreservatie van

ongerijpte DCs (iDCs) of precursor monocyten. Literatuur over de effecten van cryopreservatie en ontdooiing van monocyten of monocyt-afgeleide iDCs suggereren dat deze strategie nuttig zou kunnen zijn, hoewel bacteriën nog niet eerder gebruikt zijn als een stimulans. Daarom hebben we in **hoofdstuk 5** de effecten van cryopreservatie en ontdooiing van de voorloper van monocyten en iDCs op de rijping en immuunrespons van de DCs onderzocht met potentiële probiotische stammen en bacteriële TLR-agonisten. Een vergelijkbare expressie in de oppervlakte markers CD83 en CD86 werd gemeten bij iDCs gegenereerd uit gecryopreserveerde of vers geïsoleerde monocyten. Cryopreservatie van iDCs leidde tot een verhoging van de expressie van CD86 en CD83 in vergelijking met vers gegenereerd iDCs, maar dit had geen invloed op de capaciteit van de DCs om kenmerken te verwerven van volledig gerijpte cellen na stimulatie. In tegenstelling hier op was de cytokine respons tegen lipoteichoïnezuur en bacteriële stimulatie wel veranderd door cryopreservatie van monocyten of iDCs, met name voor IL-12 secretie dat 250-voud verminderd was of zelfs helemaal niet gedetecteerd kon worden. Cryopreservatie verminderde ook TNF-α en IL-1β productie in gestimuleerde iDCs, maar in mindere mate dan voor IL-12, en afhankelijk van de gebruikte rijpingsfactoren. De hoeveelheden IL-10 geproduceerd door gestimuleerde iDCs werden tot 3,6 keer verhoogd wanneer iDCs waren ingevroren, maar daalde 90-voudig wanneer zij gegenereerd waren uit gecryopreserveerd monocyten. Onrijpe DCs worden vaak gebruikt om de immunomodulerende eigenschappen van probiotica te onderzoeken, en hier hebben we voor de eerste keer laten zien dat gecryopreserveerde monocyten en gecryopreserveerde iDCs een veranderde cytokine respons tegen microbiële stimulatie geven. Daarom zijn wij van mening dat de standaardisatie van probiotische screeningsteesten door het gebruik van cryopreservatie methodes momenteel nog niet gebruikt kan worden. De beschreven gedetailleerde methode voor het genereren van DC uit menselijke monocyten beschreven in **hoofdstuk 5** kan echter nuttig zijn voor het ontwikkelen van gestandaardiseerde immuun assays.

In **hoofdstuk 6** zijn de immunomodulerende eigenschappen van 28 commercieel beschikbare bacteriestammen *in vitro* bepaald, gebruikmakend van menselijke PBMCs. Daaruit zijn stammen geselecteerd en onderzocht voor hun *in vivo* immunomodulerende potentieel in een geoptimaliseerd pinda-allergie model in de muis. De 28 probiotische stammen induceerden zeer variabele cytokine profielen in PBMCs. De stammen *L. salivarius* HMI001 (HMI001), *L. casei* Shirota (LCS) en *L. plantarum* WCFS1 (WCFS1) werden geselecteerd voor nader onderzoek op basis van hun verschillende patronen van IL-10, IL-12 en IFN-γ inductie. Profylactische behandeling met zowel HMI001 en LCS verzwakten het Th2-fenotype in het muis model (minder mestcel degranulatie en verminderde *ex vivo* pinda geinduceerde IL-4 en / of IL-5 productie). In tegenstelling daartoe verhoogde WCFS1 het Th2-fenotype (verhoogde mestcel degranulatie en pinda specifiek antilichaam reacties, en verhoogde *ex vivo* pinda geïnduceerde productie van IL-4). *In vitro* PBMC screening was nuttig bij het selecteren van stammen met anti-inflammatoire en Th1 sturende eigenschappen. In het geval van HMI001 (inductie van een hoge IL-10/IL-12-ratio) en LCS (inductie van grote hoeveelheden van IFN-γ en IL-

12) behandeling werd gedeeltelijke bescherming aangetoond in het muis pinda-allergie model. Echter sommige stammen kunnen de allergische reactie verergeren, zoals in het geval van de stam WCFS1. Dit hoofdstuk laat zien dat de pre-selectie van kandidaat-probiotica met behulp van *in vitro* immuun assays bruikbaar is voor het selecteren van stammen voor translationeel onderzoek bij de mens.

Van probiotica is aangetoond dat deze de effectiviteit van verschillende vaccins kunnen verhogen. Aangezien probiotica eenvoudig geconsumeerd kunnen worden in voeding, kunnen ze nuttig zijn in de verbetering van de huidige mucosale vaccins. In **hoofdstuk 7** hebben we onderzoek gedaan naar de mechanismen achter het effect van lactobacillen op de humorale reacties met een intranasaal vaccin. Naast *L. rhamnosus* GG, hebben we zes stammen van *Lactobacillus plantarum* geselecteerd, die verschillende immunomodulerende eigenschappen hadden *in vitro* en in hun TLR-2/6 activerende eigenschappen. Deze selectie was gebaseerd op de data verkregen in **hoofdstuk 3** en **hoofdstuk 4** waarin respectievelijk de *in vitro* immuunrespons van DCs afgeleid van menselijke monocyten en PBMCs tegen 42 verschillende *L. plantarum* stammen werden getest. Allereerst hebben we een griep vaccinatie model opgezet in Balb/ c muizen die gevoelig genoeg moest zijn voor immunomodulatie door lactobacillen. Het model moest gevoelig zijn voor zowel de potentiële op- als de neer-regulatie van het immuun systeem door de geselecteerd lactobacillen. Behandeling met stam WCFS1, die de laagste IL-10/ IL-12 ratio ïnduceerde in een DC co-kweek, leidde tot een sterk toegenomen vaccin-specifiek antilichaam reactie tegen het intranasale vaccin in vergelijking met de controle vaccin groep. Verschillende *Lactobacillus* stammen verhoogden de vertraagd-type overgevoeligheids respons na de vaccinatie ten opzichte van de controle vaccin groep, wat aangeeft dat in deze gevallen de Th1-gemedieerde vaccin-reacties waren verhoogd. Voor stam LMG18021 kwam dit ook tot uiting in de aanzienlijk hogere vaccin-specifieke IgG2a tot IgG1 antilichaam verhouding. LMG18021, CIP104448 en CIP104450, die de hoogste IL-10/ IL-12 ratios hadden van de geteste stammen, verbeterden aanzienlijk de *ex vivo* vaccin-specifieke inductie van IL-10, IL-17A, IL-6 en IL-4 in mesenteriale lymfeklier cellen. B1839 werd meegenomen als negatieve controle, aangezien deze stam een lage cytokine induceerder was. Deze stam liet geen verbetering van de vaccin-specifieke antilichaam reactie en de vaccin-specifieke immuunrespons zien. Dit toont aan dat immuun-stimulerende eigenschappen van stammen belangrijk zijn in het mediëren van de probiotische effecten op de vaccin reactie. Er is echter meer onderzoek nodig om aan te tonen of deze effecten ook leidt tot betere bescherming tegen influenza blootstelling en om de betrokken immunomodulerende mechanismen te valideren.

Hoofdstuk 8 sluit dit proefschrift af met een overzicht van de belangrijkste bevindingen van dit proefschrift, en bediscusseerd de mogelijke onderzoeks-beperkingen en toekomstige onderzoek perspectieven. Wij benadrukken het belang van een goede stam selectie met behulp van *in vitro* testen, en het gebruik van strategieën om nieuwe immunomodulerende factoren te identificeren. De resultaten beschreven in dit proefschrift ondersteunen het gebruik van *in vitro* co-cultuur assays voor de selectie van kandidaat-probiotica voor *in vivo* dierproeven en klinische studies.

Acknowledgements

Acknowledgements

This is it! My work over the last 4 years, collected in one thesis. It was a wonderful experience, which was very special, challenging, but mostly a lot of fun. Now it is time to express my gratitude and appreciation to all the people, who joined me during this PhD journey.

First of all, I want to show my deepest gratitude to my promotor. Jerry, I want to thank you for the opportunity to become part of the Host Microbe Interactomics group for the last 4 years. I want to thank you for all the nice meetings we had to discuss results and brainstorm about new ideas, and for correcting all my manuscripts. I also want to thank you for your nice company on the many trips we made together, it was a great experience to have my first ski lessons with you! Thank you for your trust, and your stimulating and inspiring talks.

My second promotor, Huub. During my study Biology, you made me enthusiastic for science during the Cell Biology and Health course and introduced me to the world of science. First in the project of Prescilla and Mirko, and later in the Tanzania project with Hans, Jacobien and Erasto, which made me even more interested in Cell Biology and Immunology. It was also you, who introduced me to Jerry, which was the start of this important journey. Thank you for your enthusiasm and your support during my Msc Biology and my PhD.

My co-promotor, Jolanda, thank you for all the support I got from you. For the help and opportunities you gave me within TNO and the nice girl-talks we had about all the man in the world. I really enjoyed our monthly meetings, where you really stimulated me. You helped me with my first big animal experiments, and without you this would have been really tough. Thank you for helping with the DEC proposals, dealing with political stuff, and all the opportunities to learn more about mice work.

My first project leader, Maria. Thank you for hiring me (together with Jerry) on this very interesting project. Unfortunately you already left after half a year. I hope we will collaborate in one way or another later in the future.
My second projectleader, Paul. You became part of the C-1002 team after half a year, after Maria left to America. Thank you for your trust and all the opportunities you gave me to present our work all over the world. To attend conferences to meet new people and to increase my scientific knowledge. You made everything always possible.

My “right hand”, Nico. Thank you so much that you joint me on this trip. It was a great pleasure to work with you and hopefully also will be in the future. We both learned a lot from each other. Thank you for your endless energy and your clear thoughts about data. But also thank you, to be a friend of mine. I am really happy that you will be my

paranymph during my PhD defence.

And the people working at HMI. It was a great pleasure to work with you and I am glad that in my Postdoc job most of you will join me as well. Linda, it was really nice to share our PhD experiences the last few years and I enjoyed our talks a lot. You were a great help also in more difficult moments. Ellen, although you talked too much in the morning ;-), it is really nice you joint the HMI group two years ago to show me the beautiful world of qPCR. Irene, unfortunately you accepted a job in Enschede, however I always enjoyed working with you and the little stay-overnight-parties. The ladies from the HMI secretariat, Sylvia and Trudy, thank you for always helping (re)scheduling meetings with Jerry and/ or Huub and the nice conversations. Laura thank you for sharing an office, it was a great pleasure. Peter, Jurgen, Oriana, Rosaria, Susana, Eduardo, thank you for all the nice coffee breaks and the nice conversations. Our HMI guests, Dulantha, Maria S., Marcella and Dudu, I enjoyed you were also there during my PhD. My Msc students, Rogier and Geraldine. Supervising both of you was a great experience, of which I learned a lot. It is really nice to see you both found your ways and started your own PhD projects.

During my PhD I spent a lot of time in the human culture lab. Gerco, Anja and Yvonne most of the time I spent with you and I want to thank you for all the nice time we spent together, discussing data or just sharing the same flow cabinet. Also all the other CBI people. Joeri, Carla, Tosca, Maria, Danilo, Nathalie, Geert, Harry, Mark, Joop, Trudy, Hilda, Edwin, Lidy, Prescilla, Hans and Jacobien. Thanks for all the help in the past 4 years!

The C-1002 team. Thank you all for the nice collaborations. Michiel K. for all your scientific input during meetings and for the manuscript. Daniela, thanks for all you help with cloning and the nice conversations. Peter thank you for all the nice conversations on the phone, trying to understand each other from the Microbiology and Immunology side. Roger and Iris, thanks for all the help and that I could sometimes come to NIZO to be supported by both of you. Maaike, thank you for joining the team later as an immunologist between all the other microbiologists. Saskia, Michiel W., Hermien, Miao Miao and Fabrizia, it was a pleasure to work with you in the TIFN project. The Program Director of Program 3, Jan Sikkema, thanks for all the trust.

The TNO team. Marcel, Mary Lène, Bianca, Geertje, Andre, Robert, Tim and Darryl, you all made it possible that the in vivo studies went fine. I really appreciate all you help, inputs and discussions. I always felt welcome in your lab by everyone of you.

The last part is for my family and therefore I will proceed in Dutch. Lieve pa en ma, bedankt voor al jullie vertrouwen en altijd motiverende peptalks. Jullie waren en zullen er altijd voor mij zijn. Ik weet dat jullie trots op mij zijn, maar ik ben ook heel trots om zulke lieve ouders te hebben! Mijn broer en zus. Rowin, jij hebt me laten zien hoe het is om een promotieonderzoek te doen en hebt me altijd gemotiveerd om het onderste

uit de kan te halen. Ilse, ik ben blij dat ik je getuige was op een zeer belangrijke dag voor jou. Daarom vind ik het super dat jij mijn getuige/ paranimf bent op één van de belangrijkste dagen voor mij. Menno, Samora, Mila, Julian, Chris, Betty, Carin, Martin, Jorrit, Wouter en Eva, bedankt voor het fijne gezelschap in de afgelopen jaren, maar vooral in de toekomst. Ik vind het erg fijn dat jullie er allemaal bij zijn!

Mijn allerliefste Koen, als laatste wil ik jou bedanken. We hebben elkaar leren kennen door mijn promotieonderzoek bij HMI/ TIFN, toen was je een expert bij onze project meetings. Maar na een tijdje ben je ook mijn vriend, mijn huisgenoot en mijn partner geworden. Bedankt voor alle rust die je me hebt gegeven, de discussies, voor alle last minute correcties van mijn proefschrift, maar vooral voor alle lieve woorden, je liefde en de vele knuffels die je me de afgelopen tijd hebt gegeven. Ik ben blij deze tijd met je te mogen delen! Ik hou van je lieverd!

Marjolein

Personalia

Curriculum vitae
List of publications

Education certificate

Curriculum Vitae

Marjolein Meijerink was born in Enschede, on 27th of September 1982. She attended Gymnasium at the 'Ichthus College' in Enschede, graduating in 2001. She obtained her degree of Master of Science in Biology from Wageningen University, in 2007. During this study she performed her major thesis at the Cell Biology and Immunology Group, under supervision of Dr. P. Jeurink, Dr. M. Bollen and Prof. Dr. Ir. H.F.J Savelkoul. She studied the cross reactivity effects of birch pollen Bet v 1 specific IgE with food related allergens Dau c 1 from carrot and Api g 1 from celery. In addition, she combined her minor thesis with her internship also at the Cell Biology and Immunology Group, to study the effects of zinc deficiency at the T cell response to malaria within the WOTRO/ NWO project "Micronutrients and Child Health Study". The experiments were partly performed at the Wageningen University and at the Kilimanjaro Christian Medical Centre, Moshi, Tanzania, under the supervision of Dr. J. Veenemans, Dr. E. Mbugi, Dr. H. Verhoef and Prof. Dr. Ir. H.F.J. Savelkoul.

Thereafter, the author started as a PhD student on the project entitled "Fermentation enhanced probiotic function" funded by the Top Institute Food and Nutrition. The project was carried out at the Host Microbe Interactomics Group, under the supervision of Prof. Dr. J. M. Wells, Prof. Dr. Ir. H.F.J. Savelkoul, and Dr. J van Bilsen.

Currently, the author is employed as a Postdoc in the Host Microbe Interactomics Group in a collaboration with Dr. A. Mercenier.

List of publications

Meijerink M, Wells JM, Taverne N, Zeeuw Brouwer M, Rappard B, Venema K and van Bilsen J. A comparative study of the immunomodulatory properties of potential probiotics *in vitro* and *in vivo* using a mouse model of peanut allergy. *Submitted*

Meijerink M, Loonen L, Taverne N, Kranenbarg E, Rappard B , de Vos P, Pels Rijcken R, Savelkoul HFJ, van Bilsen J[#] and Wells JM[#]. Immunomodulating effects of orally administered *L. plantarum* strains in a mouse vaccination model. *Submitted* [#]*equally contributed*

Meijerink M, Ulluwishewa D, Anderson RC and Wells JM. Cryopreservation of monocytes or differentiated immature DCs leads to altered cytokine response to TLR agonists and microbial stimulation. Journal of Immunological Methods **2011**. *In press*

Wells JM, Rossi O, **Meijerink M**, van Baarlen P. Epithelial crosstalk at the microbiota-mucosal interface. Proc Natl Acad Sci U S A. **2011** Mar 15;108 Suppl 1:4607-14. Epub 2010 Sep 8. Review.

Meijerink M, Wells JM. Probiotic modulation of dendritic cells and T cell responses in the intestine. Beneficial microbes, **2010**. 1(4): p. 317-326. Review.

van Hemert S, **Meijerink M**, Molenaar D, Bron PA, de Vos P, Kleerebezem M, Wells JM, Marco ML. Identification of *Lactobacillus plantarum* genes modulating the cytokine response of human peripheral blood mononuclear cells. BMC Microbiol. **2010** Nov 16;10:293.

Mbugi EV, **Meijerink M**, Veenemans J, Jeurink PV, McCall M, Olomi RM, Shao JF, Chilongola JO, Verhoef H, Savelkoul HF. Effect of nutrient deficiencies on *in vitro* Th1 and Th2 cytokine response of peripheral blood mononuclear cells to *Plasmodium falciparum* infection. Malar J. **2010** Jun 14;9:162.

Mbugi EV, **Meijerink M**, Veenemans J, Jeurink PV, McCall M, Olomi RM, Shao JF, Verhoef H, Savelkoul HF. Alterations in early cytokine-mediated immune responses to *Plasmodium falciparum* infection in Tanzanian children with mineral element deficiencies: a cross-sectional survey. Malar J. **2010** May 17;9:130.

Meijerink M, van Hemert S, Taverne N, Wels M, de Vos P, Bron PA, Savelkoul HF, van Bilsen J, Kleerebezem M, Wells JM. Identification of genetic loci in *Lactobacillus plantarum* that modulate the immune response of dendritic cells using comparative genome hybridization. PLoS One. **2010** May 13;5(5):e10632. **WIAS publication prize**

Patent application

Van Hemert S, **Meijerink M**, Bron PA, Marco M, Kleerebezem M, Vos P de, Wels MWW, Wells JM Improved immunomodulation by probiotics. Patent no, 09176131.2, **2009**

Overview of completed training activities

Discipline specific activities

Courses

ELISA basic understanding and trouble shooting, WIAS	2007
HMI workshop in Sardinia	2008
Light in the intestinal tract (Helsinki) (poster presentation), VLAG	2009
Advanced visualization, integration and biological interpretation of ~omics data	2009
RNAi & the world of small RNA molecules Spring school (poster presentation)	2010
Advanced course guide to scientific artwork	2010

Meetings

4th International Yakult Symposium, Verona, Italy	2007
WE day TIFN autumn, Wageningen	2007
Gut day Wageningen	2007
Annual Meeting Dutch Society (Winter meeting), NVVI, Noordwijkerhout	2007
Annual Meeting Dutch Society (Spring meeting), NVVI, Lunteren	2008
Microbiota, Probiotics and Host, Seeon, Germany	2008
WE Days TIFN, Helsinki, Finland	2008
TNO Beneficial Microbe Conference, Amsterdam	2008
European Mucosal Immunology Group (EMIG) meeting, Milan, Italy (poster presentation)	2008
Gut dag Utrecht	2008
Microbiota, Probiotics and Host, Seeon, Germany (poster presentation)	2009
WE day TIFN autumn (oral presentation)	2008
Innate adaptive and regulatory responses to microbiota, Taos, New Mexico (poster presentation)	2009
Keystone meeting Dendritic cells, Banff, Canada	2009
5th International Yakult Symposium, Amsterdam (poster presentation)	2009
2nd TNO beneficial microbes conference (oral presentation)	2010
3rd Joint Conference of the DGHM and VAAM, Hannover, Germany (oral presentation)	2010
First TIFN annual conference (oral presentation)	2010
International conference Probiotics and Probiotics, Kosice, Slovakia (oral presentation)	2010
Gut day Gent (poster presentation)	2010
Keystone meeting mucosal biology, Vancouver, Canada	2011
Microbiota, Probiotics and Host, Seeon, Germany (oral presentation)	2011
MACS User day, Gent, Belgium (oral presentation)	2011
Cross talk meeting, Wageningen (oral presentation)	2011
26th National Microbiology Conference, Brasil (oral presentation)	2011

General courses

PhD week, VLAG	2008
Project- and Time Management, WGS	2008
PhD Competence Assessment, WGS	2008
Effective publishing strategies and networking workshop, TIFN	2008
Statistics for the Life Sciences, WIAS	2008
Advanced statistical course, WIAS	2009
Design of animal experiments, WIAS	2009
Writing and presenting a scientific paper, WIAS	2010
Philosophy and Ethics of Food, VLAG	2011

Optional activities

Preparing PhD proposal	2007
TIFN project meetings	2007-2011
Journal club, HMI	2009-2011
Labmeetings, HMI	2007-2011

Design and layout: Marjolein Meijerink
Cover: Dendritic cells, artwork (David Mack) supplied by SCIENCE PHOTO LIBRARY
Printed by: GVO & designers B.V. Ponsen & Looijen