Doctoral Thesis
at the Medical University of Vienna
for obtaining the PhD-degree

Immune-modulatory effects of lactic acid bacteria on human peripheral blood mononuclear cells through the barrier of intestinal epithelial cells (Caco-2) in a trans-well system

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Vienna, October 19th 2007

DANKE!

Allen, die an der Entstehung dieser Arbeit im engeren und weiteren Sinn beteiligt waren, möchte ich hiermit meinen herzlichen Dank aussprechen:

Meinem Betreuer, Herrn Univ. Prof. Dr. Zsolt Szépfalusi und den Mitgliedern meines Dissertationskomitees, Frau Univ. Prof. Dr. Erika Jensen-Jarolim und Frau aoUniv. Prof. Dipl. Ing. Dr. Barbara Bohle, sowie Herrn Univ. Prof. Dr. Radvan Urbanek.

Dem Allergielabor der Kinderklinik, besonders Elke Kampl, Christine Loibichler und Thomas Eiwegger.

Unseren Kooperationspartnern am Department für Lebensmittelwissenschaften und –technologie der Universität für Bodenkultur Wien, Herrn Univ. Prof. Dr. Wolfgang Kneifel und Herrn Dr. Konrad Domig, sowie Herrn Univ. Prof. DDr. Apostolos Georgopoulos (Univ.-Klinik f. Innere Medizin I, Klin. Abt. f. Infektionen u. Tropenmedizin, Mikrobiologische Laboratorien, Medizinische Universität Wien).

Herrn Dr. Rainer Englisch (THP Medical Products, Wien) und Frau Katalin Nagy-Bojarszky (Klinisches Institut für Pathologie, Medizinische Universität Wien).

Meiner Familie und meinem Partner Franz Roitner.

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AP	alkaline phosphatase
APC	antigen presenting cell
CFU	coloncy forming unit
CPM	counts per minute
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
CD	cluster of differentiation
DC	dendritic cell
Der p	*Dermatophagoides pteronyssinus*
DMEM	Dulbecco's modified minimal essential medium
E.	*Escherichia*
GM-CSF	Granulocyte-macrophages colony-stimulating factor
GRO	Growth related oncogene
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	Immunoglobulin
IL	interleukin
IEC	intestinal epithelial cell
IU	international unit
L.	*Lactobacillus*
LPS	lipopolysaccharide
MCP	monocyte chemoattractant protein
MHC	major histocompatibility complex
NF	nuclear factor
NK	natural killer cell

PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cells
PHA	phytohaemagglutinine
S.	*Streptococcus*
TEER	transepithelial electric resistance
TGF	Transforming growth factor
Th	T-helper cell
TLR	Toll-like receptor
TNF	tumor necrosis factor
T-reg	T-regulatory cell

1 ABSTRACT - English

Modulation of the host's immune system is one of the most commonly attributive effects of probiotic preparations, mainly deduced from *in vitro* PBMC cultures. Intestinal epithelial cells seem to contribute to immune responses to non-pathogenic bacteria, though. The aim of this thesis was to investigate immune-modulatory properties of probiotic bacterial strains in an *in vitro* co-culture model of human intestinal epithelial cells (Caco-2 cells) and peripheral blood mononuclear cells (PBMC). Three probiotic strains and a non-fermenting control strain were evaluated for their impact on interleukin 6, 8, and 10 secretion of Caco2-PBMC cocultures and PBMC cultures. The effect of inactivation treatment of these strains on cytokine profiles was tested by applying live, heat- and formalin-inactivated bacteria. To investigate the contribution of M-cells of Peyer's patches on Caco-2-PBMC cytokine responses, Caco-2 cells were converted into M-cells using Raji cells, human B-cell lymphoma cells. The final goal was to test whether probiotic bacteria could revert an established allergen specific T-helper cell type 2 cytokine response. All tested bacteria induced strain-specific cytokine secretion indicating immune-modulatory features, although the Caco-2 barrier had suppressive effects on cytokine production compared to PBMC cultures and inhibited lymphoproliferation. Live microorganisms seemed to be more potent inducers of cytokine production than non-viable strains, but differences to inactivated bacteria were not statistically significant. Heat- and formalin-inactivation induced comparable cytokine patterns. M-cells did not alter cytokine responses, strengthening the general assumption that M-cells strictly act as antigen-sampling system in the intestinal epithelium. The experimental system was not applicable to analyse modulatory features of probiotic strains on allergen-specific cytokine responses, because allergen-induced cytokine production was not significant. Of note, Caco-2 transwell cultures remained hypo-responsive to bacterial or allergen stimulation in the absence of PBMC, supporting the concept of enterocyte-leucocyte cross-talk in cytokine responses to antigenic stimuli in the gut epithelium.

2 ABSTRACT – Deutsch

Bestimmten Milchsäure-Bakterienstämmen, sogenannten Probiotika, wird eine immunmodulatorische Wirkung zugeschrieben, was hauptsächlich auf Daten aus PBMC *in vitro* Kulturen basiert. Darmepithelzellen scheinen jedoch auch an Immunantworten gegenüber apathogenen Bakterien beteiligt zu sein. Ziel dieser Dissertation war es, immunmodulatorische Effekte von Probiotika an einem *in vitro* Modell von Darmepithelzellen (Caco-2) und mononukleären Zellen des peripheren Blutes (PBMC) zu untersuchen. Es wurde die Wirkung dreier probiotischer und eines nicht-fermentierenden Kontrollstamms auf die Sekretion von Interleukin 6, 8 und 10 in Caco-2-PBMC Transwell Co-Kulturen und PBMC Kulturen untersucht, um den Einfluss der Darmepithelzellen auf die Zytokinantworten zu analysieren. Lebende, Hitze- und Formalin-inaktivierte Bakterien wurden verglichen, um den Effekt von Viablität und Inaktivierung der Bakterien auf die Zytokininduktion zu testen. Weiters wurde die Beteiligung von M-Zellen, spezialierten Zellen aus Peyerschen Plaques, an der Bakterien-induzierte Zytokinantwort von Caco-2-PBMC Co-Kulturen untersucht. Hierfür wurden Caco-2 Zellen durch die Co-Inkubation mit Raji Zellen, humanen B-Zell Lymphomzellen, in M-Zellen umgewandelt. Das Endziel dieser Arbeit war zu testen, ob eine etablierte, allergen-spezifische T-Helferzell-Typ 2 Zyotkinantwort durch Probiotika umgekehrt werden kann. In unseren Experimenten zeigten alle untersuchten Stämme immunmodulatorische Wirkung im Sinne einer spezifischer Zytokininduktion. Der Caco-2 Monolayer verminderte jedoch die Zytokinantwort im Vergleich zur direkten Stimulation von PBMC ohne Caco-2 Zellen und unterdrückte Lymphoproliferation. Lebende Mikroorganismen waren etwas stärkere Stimuli für die Zytokinsekretion, die Unterschiede zu inaktivierten Stämmen waren jedoch nicht statistisch signifikant. Hitze- und Formalin-Inaktivierung der Bakterien veränderten die induzierten Zytokinprofile nicht. M-Zellen hatten keinen Einfluss auf die Zytokinproduktion, was die allgemeine Annahme unterstützt, dass dieser Zelltyp ausschließlich der Antigenaufnahme im Darmepithel dient. Unser experimentelles System eignete sich nicht zur Analyse des Effekts von Probiotika auf allergen-spezifische Zyotkinprofile, da keine statistisch signifikanten allergen-induzierten Zyotkinantworten ausgelöst werden konnten. Bakterielle oder Allergen-Stimulation von Caco-2 Transwell-Kulturen ohne PBMC führte zu keiner Zytokinsekretion. Unsere Daten unterstützen das vor kurzem publizierte Konzept von „Cross-Talk“ zwischen Darmepithelzellen und PBMC bei Zytokinantworten gegen Antigene im Darmepithel.

3 INTRODUCTION

3.1 Probiotic bacteria – history and definition

3.1.1 The hygiene hypothesis

The hygiene hypothesis was first proposed in the late 1980s to explain the increase in allergic disorders in industrialized countries. The rapid change in the prevalence of allergies and atopic sensitizations in distinct parts of the world was likely influenced by environmental factors or the host's response to environmental factors.

Strachnan et al first suggested the concept that infections and "unhygienic conditions" especially in early childhood might protect from the development of allergic illnesses. The idea originated from epidemiological observations showing an inverse correlation between family size and the prevalence of allergic rhinitis (*142*). This observation led to the more generalized hypothesis that infections acquired from older siblings or domestic exposure to environmental lipopolysaccharides (LPS), a cell wall component of Gram-negative bacteria, might protect from allergic illnesses, such as allergic rhinoconjuncitivitis, allergic asthma, or atopic eczema (*11*, *34*, *35*, *158*).

3.1.1.1 Immunologic concepts of the hygiene hypothesis

3.1.1.1.1 Re-addressing the Th1/Th2 balance

The T-helper (Th) 1/Th2 paradigm of the adaptive immune response initially provided the immunologic backbone of the hygiene hypothesis. Based on cytokine profiles, T-cell responses can be divided into two opposed sub-types as initially

described by Mosmann and Coffman (*98*). Intracellular microbial infections are believed to elicit a Th-1 immune response, while the Th2-type is assigned to the atopic phenotype (*124*). A predominance of Th2 cells was found at sites of allergic inflammatory reactions, such as in bronchoalveolar lavages of asthmatic patients (*123*) and atopic dermatitis lesional skin (*155*). Th1 responses, or "inflammatory responses" have been generally thought to counterbalance Th2 type immunity. Relating to the hygiene hypothesis, the Th1/Th2 paradigm has been used to argue that reduced contact with microbes and infectious agents decreases Th1 responses resulting in overwhelming Th2 activity, characteristic for allergic diseases. The perinatal period and early infancy are thought to be the crucial window of opportunity.

In utero, a Th2-driven immunologic environment is thought to prevail to prevent rejection of the allograft (*116*, *161*). At the same time, transplacental transfer of allergens and prenatal priming of the human immune system occurs, as was shown in our laboratory (*77*, *145*). In healthy infants, the Th2-skewed immune response is thought to be re-directed and matured during the first year of life. In children who develop atopy, the Th1/Th2 balance is not reached, and Th2-responses are further augmented (*115*).

3.1.1.1.2 Regulatory T-cells and the hygiene hypothesis

In recent years, doubts have emerged whether the Th1/Th2 paradigm sufficiently explains the hygiene hypothesis. Paradoxically, helminth infections, which usually provoke strong Th2 responses, seem to decrease the risk of atopy (*154*, *166*). In a similar manner, if the Th1/Th2 paradigm holds true, one could assume that with the rise in atopic diseases there would be a decrease in the prevalence of

autoimmune diseases, which are considered to be driven by exaggerated Th1 responses. Studies however have shown, that there is no inverse relationship between these two entities (*48*, *65*, *140*).

In addition, the complex interplay of the innate immune system in directing adaptive immune responses became apparent (*157*, *163*), and the dichotomic Th1/Th2 classification was broken by the discovery of a regulatory T-cell subset (T-reg) pivotal in acquisition and maintenance of mucosal and systemic tolerance (*39*, *149*, *167*, *171*).

This T-cell population comprises different subsets, such as T regulatory cells 1 (Tr1) and Th3 cells featuring distinct cytokine profiles (*1*). Coherent data are indicating that interleukin (IL)-10 and transforming growth factor (TGF) beta, attributed to the Tr1 and the Th3 subtype respectively, are the most important cytokines in establishing and maintaining tolerance at mucosal surfaces and consecutively systemically (*16*, *28*, *56*, *162*, *168*). The two cytokines appear to function synergistically, with one favouring the synthesis of the other.

These findings changed the view of the immunologic background of the hygiene hypothesis. It is now believed that constant anti-inflammatory and regulatory action is needed to counter-regulate both branches of T-cells responsiveness, and that lack of the tolerogenic activity results in either Th1-dominated autoimmune-diseases or Th2 driven atopic illnesses. The central cell type in this process is thought to be T-reg.

3.1.1.2 Microbial stimulation at mucosal surfaces and the hygiene hypothesis

Investigating immune responses towards microbes at mucosal surfaces, the intimate interplay between the innate and the adaptive immune system and the gate-keeping immune-regulatory function of the intestinal immune system became evident.

Upon intestinal exposure to micro-organisms, the immunologic cascade is initiated by the innate immune system and directs adaptive immunity. Macrophages and monocytes produce TGF-beta, which induces TGF-beta secreting T-cells and influence antigen presenting cells. IL-10 induced in parallel acts on dendritic cells to bias T-cells in favour of the development of T-reg (reviewed by Rautava et al. (*119*)). Regarding effects on the mucosal milieu, TGF-beta seems to affect B-cells and secures mucosal barrier function by inducing secretory immunoglobulin (Ig) A antibodies (*67*). IL-10 is furthermore considered pivotal in inducing tolerance to commensal intestinal microbiota (*66*, *95*). Lack in IL-10 responses in the intestine may add to the development of inflammatory bowel disease (*75*) and asthma (*9*). Accordingly, the intestine has been attributed a gate keeper function for maintenance of immunologic homeostasis and the pathogenesis of immunologically-driven diseases.

The perinatal period is still considered the crucial window of opportunity for establishing immune balance, as barrier functions of mucosal surfaces are not fully developed, and most importantly, the immune system is immature. In infancy, colonisation of the intestine takes place and elicits IgA antibody responses, which constitute a central part of the immunological barrier function of the gut (*38*). Derived from animals raised under germfree conditions, it has been suggested that

the endogenous intestinal microbiota represents an important maturation stimulus for the immune system (*152*). The commensal gut microbiota has been found to direct the diversification and expansion of the antibody repertoire in mucosal lymphoid tissues in newborn piglets (*13*). It also seems to have implications in oral tolerance. Germfree mice were found to have reduced numbers of T-cells in Peyer's patches, which was related to the failure to induce oral tolerance (*82*). Constant stimulation from the indigenous microflora and/or probiotics, may hence contribute to the functional maturation of the immune system. Poor microbial stimuli may thus lead to dysregulation and diseases.

Taken together, at present, the immunologic mechanism underlying the hygiene hypothesis is thought to be as follows: microbial stimulation via mucosal surfaces induces IL-10 and TGF-beta secreting T-reg, which mediate the establishment and maintenance of a general anti-inflammatory and tolerogenic milieu, suppressing both, Th1 and Th2-driven immune reactions, like chronic inflammatory bowel disease and atopic diseases. In Western societies, microbial exposure is reduced through improved hygienic conditions, consecutively, the vigorous anti-inflammatory activity is down-regulated and the tolerogenic environment is softened.

3.1.2 The hygiene hypothesis and non-pathogenic bacteria – the evolving of the concept of probiotics

Long before the hygiene hypothesis emerged, Metchnikoff presented the idea of lactic acid bacteria as health-promoting environmental factors, and recommended

to “absorb large quantities of microbes amongst which lactic bacilli have an honourable place”. Lactic acid bacilli have been part of the daily diet for hundreds of years as they were used in fermentation of food to improve storage. The development of new conservation methods for food in the past century lead to a reduction of the daily intake of Lactobacilli. As at the same time, the incidence of allergies and other immune-mediated disorders rose, Metchnikoff’s idea of health-promoting effects of non-pathogenic bacteria used in food fermentation raised enormous interest to clarify the interrelation between intestinal microbiota and immune-mediated diseases (reviewed by Vaarala (*153*)). Large efforts have been made by researchers and industry ever since to identify microbial strains that are capable of modifying clinical diseases, in particular allergic disorders.

3.1.3 Definition of the term “probiotic”

The word “probiotic” means “for life”. Probiotics are defined as: “microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host” (from (*153*)).

Among microbial strains used for food fermentation, mainly *Lactobacilli* and *Bifidobacteria* have been classified as probiotic. Both species are Gram-positive, acid- and bile-resistant and capable of adhering to the intestinal epithelium and colonizing the human gastrointestinal tract. In addition, they can be used safely in clinical trials and functional food, as there are minimal safety risks like with Gram-negative strains (i.e. because of LPS) or pathogenic enteral viruses.

Today, the most prominent representative of probiotics is *Lactobacillus rhamnosus GG* (*L. rhamnosus GG*), a probiotic strain well studied in clinical trials (*60*).

3.2 Clinical applications of probiotic microbes, focussing on Lactobacilli

3.2.1 Observational studies

The initial, epidemiology-based hygiene hypothesis claimed that the risk of allergic illnesses is modified by microbial infections in infancy (*142*). Numerous studies suggested, that the exposure to environmental LPS early in life decreased the risk of atopic disorders by promoting to the maturation of immune system in the first years of life (*11*, *34*, *35*, *158*).

However, the microbial milieu the host is exposed to does not only consist of the outer environment, but includes inner surfaces of the body as well. The human intestine probably is the strongest, constant source of microbial stimulation. It is heavily colonized by bacteria starting immediately after birth, and the composition of the indigenous intestinal microbiota is determined by genetic factors, environmental exposure, diet, and diseases. Colonization starts with facultative anaerobics, e.g. enterobacteria, coliforms, and lactobacilli, followed by bifidobacteria and lactic acid bacteria (reviewed by Rautava et al. (*119*), Figure 1).

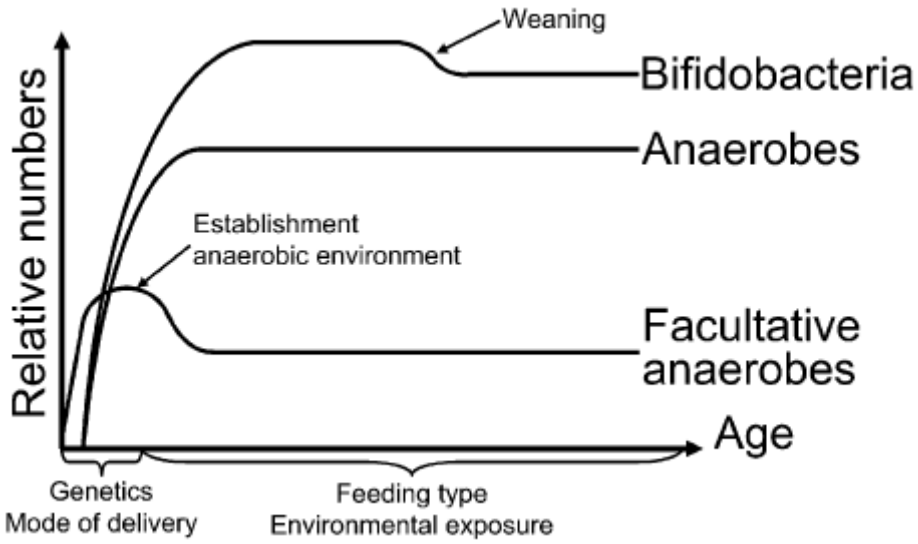


Figure 1. The stepwise compositional development of the gut microbiota. From Rautava et al. J. Pediatr. Gastroenterol. Nutr. 2004 (*119*).

Accordingly, it has been hypothesized that the composition of indigenous microbiota may also differ between healthy individuals and those with an immune-mediated disease. When investigating the microbiota of Swedish and Estonian children, representing a population with high and low prevalence of allergy, differences in the composition was found. Estonian children were more commonly colonized with Lactobacilli (*6*, *137*). In Swedish infants, higher levels of *Clostridium difficile*-derived caproic acid were found in allergic individuals than in non-allergic children (*10*). In prospective studies, the faeces of Swedish children who developed allergic diseases less often contained Enterococci and Bifidobacteria during the first year of life, but higher levels of Clostridia and Stapylococci, in comparison to children who did not develop allergies (*7*). These findings were confirmed in populations from other nations (*59*, *160*). Similarly, data from several groups suggested that the composition of the faecal microbiota of patients suffering from inflammatory bowel disease differs from healthy

controls. Patients seem to have less Lactobacilli and Bifidobacteria and increased levels of *Bacteroides* species (reviewed by Dotan et al. (*29*)).

Nevertheless, some authors also emphasize that the host immune system itself may modify colonization of the intestinal tract, and the faecal microbial composition may reflect the characteristics of the immune system prone to atopy (*153*)

Other orofaecal microbes that seemed to protect from allergic diseases are *Helicobacter pylori* and *Hepatitis A* virus (*71*, *89*), but initially promising findings could not be confirmed by other authors (*54*, *68*).

The initial composition of the gut microbiota is now believed as a key determinant in the establishment and maintenance of normal gut barrier function and immunologic balance of the host. Disturbances of the initial colonization as a result of modern living conditions and improved hygiene might open the gates for pathologic Th1 and Th2 immune responses.

3.2.2 Clinical interventional studies

Today, modification/re-balancing of the intestinal microbiota by administration of probiotic bacteria is a promising therapeutic option for the treatment and prevention of certain conditions, such as gastrointestinal infections, atopic diseases, and chronic inflammatory bowel disease (*29*, *104*, *153*).

3.2.2.1 Lactic acid bacteria in acute diarrhoea of various aetiologies

Beneficial effects of lactic acid bacteria have been reported for all aetiologies of diarrhoea. The intestinal microflora is essential to maintain a barrier against invasion of pathogenic bacteria, and fermented foods, like yogurt or fermented

milk, have been suggested as healthy foods to control diarrhoeal diseases for many decades. Positive effects of certain strains have been studied extensively, but drawing conclusions is difficult. Many variables are likely to be involved in the positive effects observed, study designs greatly differ, and many studies are performed in a non-controlled design. In addition, the importance of the viability of bacterial strains in fermented milk products used for the treatment of diarrhoeal diseases is not yet clarified.

In 1996, a review concluded that the administration of certain microorganisms has no proven effect in the treatment and prevention of intestinal infections (*30*). A recently published meta-analysis however compared 34 masked, randomised, placebo-controlled trials investigating effects of probiotics on antibiotics-associated and travellers diarrhoea as well as acute diarrhoea of different causes. Probiotics significantly reduced antibiotic-associated diarrhoea by 52% (95% CI 35-65%), the risk of travellers' diarrhoea by 8% (-6 to 21%), and that of acute diarrhoea of diverse causes by 34% (8-53%). The authors did not find significant differences in the protective effects among different probiotic strains, among them *L. rhamnosus GG*, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, used alone or in combinations. The authors conclude that there is sufficient evidence for a role of probiotics in the prevention of acute diarrhoea, especially in antibiotics-associated diarrhoea. The beneficial effects seemed to be more pronounced in children than in adults. However, the authors state that before giving global recommendations, community-based studies, especially in developing countries are needed (*133*).

3.2.2.1.1 Acute viral diarrhoea

Treatment of acute viral enteritis in children by live microbial supplementation has probably been the first and by now best documented field of application of probiotic strains.

L. rhamnosus GG has been shown to shorten the course of and promote recovery from acute rotavirus infection in children (*52*, *84*). Live *L. rhamnosus GG* seemed to be more effective than inactivated (*57*). The group found that *L. rhamnosus GG* promoted specific IgA response to rotavirus and suggested that it is thereby augmenting the local immune defense. In addition they hypothesized that the induction of specific IgA is possibly relevant in protection against re-infections (*58*, *84*). Similarly, a couple of studies from other groups from various countries showed beneficial effects of *L. rhamnosus GG* on time course and severity of children acute viral diarrhoea (*41*, *42*, *103*, *108*).

A protective effect on the development of viral diarrhoea and shedding of rotavirus in the faeces has been shown for *Bifidobacterium bifidum*- and *Streptococcus thermophilus*-supplemented infant milk formulas in comparison to control formula (*130*). In 2002, a meta-analysis of randomized, controlled studies showed that a reduction in diarrhoea duration of 0.7 days and a decrease in diarrhoea frequency of 1.6 stools on day 2 of therapy were achieved in subjects who received *Lactobacillus* in comparison to the placebo group (*156*).

In a study comparing effects of live versus heat-inactivated L. *rhamnosus* GG on the time course of rotavirus diarrhoea, both preparations were effective in shortening the course of the disease. However, with live L. *rhamnosus* GG, rotavirus specific serum IgA levels were higher than with heat-inactivated bacteria (*57*).

3.2.2.1.2 Antibiotics-associated diarrhoea

Likewise positive data are available for antibiotics-associated diarrhoea. A lower incidence of diarrhoea has been reported in children and infants treated with amoxicillin who received Lactobacilli in parallel (*23*). There is evidence that even *Clostridium difficile* colitis, a severe complication of antibiotics therapy, can be treated successfully with *L. rhamnosus GG* (*5*).

Two recently published meta-analyses (n= 767 and n=707) including 6 randomized, controlled studies showed significant benefits of probiotics over placebo in antibiotics-associated diarrhoea (*55*, *144*). However, Johnston et al. also performed intention-to-treat analysis, which did not reveal significant results. Thus the authors conclude that before routine use is recommended, further studies are needed. *L. rhamnosus GG*, *L. sporogens,* and *S. boulardii* were found to be the most effective strains, used at 5-40 x 10^9 CFUs daily.

For the prevention of antibiotics-associated diarrhoea, the viability of the microbes administered was regarded to be crucial, as only patients receiving *L. rhamnosus GG* but not those taking pasteurized yogurt experienced beneficial effects (*138*).

3.2.2.1.3 Lactose malabsorption

Some studies even found beneficial effects of probiotics in diarrhoea due to lactose malabsorption (*26*, *87*, *131*). Bacterial beta-galactosidase contained in yogurt, seems to account for these effects. It presumably resists luminal hydrolysis and can hydrolyze lactose in parts of the small intestine where a pH of 6-8 allows its enzymatic activity. Another mechanism could be slowing down of the gastrointestinal transit by yogurt, facilitating the contact between lactose and

enterocytes in the lumen. Viability of microbial strains does not seem to be prerequisite in this diarrhoeal disease (reviewed by Heyman (*50*)).

3.2.2.2 Inflammatory bowel disease and probiotics

In inflammatory bowel disease, aberrant commensal gut microbiota seems to trigger disease-causing immune dysregulation in individuals with a distinct genetic susceptibility. The rationale for the use of probiotics in inflammatory bowel disease is the re-balancing of the "dysbiosis" between beneficial and harmful microorganisms in the intestinal microbiota. A relation between "dysbiosis" and mucosal inflammation in inflammatory bowel disease has been demonstrated in observational studies (reviewed by Tamboli et al. (*146*)). In a murine model, IL-10 deficient mice have been shown to develop a gut barrier dysfunction and a Th1 type inflammation against their own commensal gut microbiota (*81*). Interestingly, these mice have been found to have decreased numbers of Lactobacilli in their neonatal period, and normalizing the level of Lactobacilli prevents intestinal inflammation (*80*).

So far, the clinical effect of probiotics in the treatment of inflammatory bowel disease seems to be modest. The most convincing results have been found in the prevention and maintenance of remission of pouchitis (*36*, *37*, *96*). In ulcerative colitis, probiotic treatment (*E. coli* Nissle 1917) has been reported to be as effective as standard mesalazine therapy in maintaing remission (*73*). Other authors found that adding bifidobacteria or the probiotic preparation VSL#3 to patients' salazine treatment was superior to adding placebo in the treatment of active ulcerative colitis within an 8- and 12-months follow-up, respectively (*62*,

151). In a 6-weeks study, the probiotic preparation was found to induce remission of mild to moderate active ulcerative colitis in 53% (18/32) of patients (*4*).

For Crohn's disease, very few studies are available, and data are contradictory. In a pilot study involving four male children with active Crohn's disease, application of *L. rhamnosus GG* resulted in "significant improvement of symptoms" (*40*), whereas no effects in terms of inducing or maintaining remission were seen in an adult population (*135*).

3.2.2.3 Lactic acid bacteria in the treatment and prevention of atopic diseases

A large field of research on probiotics-containing functional food is the prevention and treatment of atopic diseases.

Food allergy, one of the earliest and mostly transient manifestation of atopic disorders is nowadays believed to result from a breakdown of oral tolerance during a presumed window of immunologic immaturity (*15*). Animal studies raised evidence that the resident gut microbiota might also influence the mucosal immune response towards food allergens, and that intestinal colonization after birth hence is essential for developing oral tolerance (*13*, *143*).

Another experiment showed that the transient breakdown of tolerance towards ovalbumin could be recovered by the gut microbiota (*33*). These studies suggest that the intestinal colonization influences the development of the gut immune system and the induction of tolerance towards food antigens. These findings in animals gave rise to the hypothesis that probiotics during the first years of life could be beneficial in preventing food allergies or other atopic disorders.

One of the most re-known studies on the prevention of atopic disease by probiotics was performed by Kalliomaki et al. in Finland. In a randomised, placebo-controlled trial, capsules of *L. rhamnosus GG* were given daily to mothers of children with a high risk for atopy for 2-4 weeks before the expected delivery, and for 6 months thereafter, either to mother when they were breastfeeding, or into the infant's formula. The prevalence of atopic eczema was evaluated at age 2, and was half that of the placebo-group (23% versus 46%), with a relative risk of 0.51 (95% CI 0.32-0.84). However, the two subgroups did not differ in skin prick test positivity or total and specific IgE levels (*60*). 109 children were also available for a follow up at the age of 4 years. By this time, 26% of the initially *L. rhamnosus GG*-treated group and 46% of the placebo-treated group had developed atopic eczema (relative risk, 0.57, 95% CI 0.33-0.97), diagnosed by a questionnaire and clinical examination. Still, no differences in skin prick test reactivity were found between groups (*61*).

Effects of probiotics in the treatment of infants suffering from atopic dermatitis were studied by Majamaa et al. After one month feeding of *L. rhamnosus GG*-supplemented hydrolysed formula, the clinical score of atopic dermatitis improved significantly. Fecal anti-1-antitrypsin and TNF-alpha levels decreased at the same time (*83*).

Isolauri et al. studied the effects of *L. rhamnosus GG* and *Bifidobacterium lactis* Bb12-supplemented formula in the management of atopic eczema in infants who developed eczema during exclusive breastfeeding. After two months of treatment, the severity score for atopic eczema, SCORAD, significantly decreased in infants who received the probiotics-supplemented formula in comparison to children receiving un-supplemented hydrolysed formula. Levels of soluble CD4 in serum and eosinophilic protein X in urine reduced in parallel (*51*).

A recently published report investigated the effect of *Lactobacillus acidophilus* given during the first 6 months of life on the development of atopic dermatitis in 178 children with a maternal history of allergy. At the end of intervention, infants in the probiotic group showed similar rates of atopic dermatitic than in the placebo group, but higher intestinal colonization rates with the probiotic microorganism. At 12 months of age, still no difference in atopic dermatitis was found, but children in the probiotic group who had developed atopic dermatitis showed higher sensitization rates in skin prick test (*147*). In the same study population, levels of circulating CD4+CD25+CTLA4+cells and expression of FOXP3, markers for T-reg, were not altered by probiotics. FOXP3 levels following stimulation with house dust mite allergen and ovalbumin were higher in children with atopic dermatitis. This was however found to be a predictor of atopic dermatitis rather than probiotic treatment (*148*).

The role of probiotic supplementation in allergy prevention remains under debate though.

3.2.3 Recommendations for the clinical use of probiotics by March 2006

Based on clinical studies, abstracts of meetings, and the knowledge of the expert panel, Floch et al. published recommendations for the clinical use of probiotics for physicians in March 2006 (Table 1). The authors conclude that at present, there is sufficient data to sustain their use in acute diarrhoea, antibiotic-associated diarrhoea, and pouchitis. In general, live specific probiotics are recommended for clinical applications. The use of probiotics in immunocompromised status should not be undertaken in routine. They also emphasize the importance of strain- and dose specificity.

Clinical Condition	Clinical Effectiveness*	Organisms**
Adult and childhood diarrhea		*Lactobacillus reuteri*[13,14]
Prevention	B	*Lactobacillus* GG[15,16] *L. casei*,[17,18] *L. acidophilus*,[19–21] *S. boulardii*[21,22]
Treatment	A	Bifodobacteria[24,25]
Antibiotic-associated diarrhea	A	*S. boulardii*,[25–27] L GG[28–30]
Radiation	C	VSL no. 3[31]
Vaginosis	C	*Lactobacillus acidophilus*[32,33]
H pylori	C	*L. johnsonii*[34,35]
Ulcerative colitis	C	*E. coli* (Nissle),[36,37] Bifodobacteria and Lactobacillus,[38] VSL no. 3[39,40]
Crohn disease	C	*E. coli* (Nissle),[41] *S. Boulardii*,[42,43] L GG (variable)[44]
Pouchitis	A	VSL no. 3[45–47]
Irritable bowel syndrome	C	*L. plantarum*,[48,49] VSL no. 3,[50] *B. infantis*[51]
Prevention of cardiovascular disease	C	Lactobacillus in milk and yogurts[52,53]
To improve immune response	B	*L. acidophilus*,[54] *L. Plantarum*,[55] *B. Lactis*,[56,57] L GG,[58,59] *L. Johnsonii*[60,61]

Table 1. Guidelines for Probiotic Use*A indicates strong evidence; B, suggestive evidence; C, inadequate studies to be certain.**Exact dosage used is in appropriate reference.VSL no. 3 indicates *Lactobacillus casei*, *L. plantarum*, *L. acidophilus*, *L. delbreueckii*, *Bifidobacterium longum*, *B. breve*, *B. infantis*, and *Streptococcus salivarius*. From: Floch MH et al., J. Clin. Gastroenterol. 2006 (*32*)

3.3 Immunologic mechanisms of probiotics as modulators of immune responses

The mechanisms by which specific microbial cells and -components exert beneficial health effects are poorly understood. Local as well as systemic effects have been described. Suggested local effects are the induction of secretory IgA and IgM (*2*, *58*, *84*), inhibition of pathogenic bacterial adherence and translocation (*85*), as well as trophic effects on the intestinal epithelium, thereby reducing gut permeability to allergens and pathogens (*2*, *53*).

Systemically, non-pathogenic bacteria are thought to be capable of modifying host's immune responses. There is evidence that probiotics stimulate B-cell antibody production, in particular secretory and serum IgA responses in humans and mice (*38*, *58*, *157*), and modulate peripheral blood mononuclear cells' (PBMC) cytokine profiles, the two branches of the adaptive immune system. These systemic immunologic effects will be focussed on from now on.

3.3.1 Immune responses and immuno-regulation initiated by intestinal microbiota

Recognition of intestinal microbiota by the immune system is mediated by a complex interplay of the innate and the adaptive immune system. The primary effector arm, the innate immune-system, recognizes the bacterial stimulus. Macrophages, monocytes, and dendritic cells function as antigen-presenting cells and initiate and regulate the antigen-specific adaptive immune system, comprising T- and B-cells.

The innate immune system reacts to microbial antigens by recognizing conserved pathogen-associated molecular pattern (PAMPs) through Toll-like receptors (TLR) and CD14 (*169*). PAMPs are for instance unmethylated CpG motifs characteristic of bacterial DNA, which are recognized by TLR 9 and induce Th1 type immune responses (*72*), or LPS of Gram-negative bacteria, which signals via TLR2 and TLR4 (*18*, *165*). Both, TLR 2 and 4 were found to be essential in responding to *Lactobacillus* in the murine intestinal epithelium (*157*). CD14 acts as co-receptor for LPS, but also recognizes peptidoglycan, a cell wall component of all bacteria (*76*).

Once activated, TLR induce a signalling cascade via the transcription factor NF-kappa-B pathway (*165*, *169*) resulting in the expression of NF-kappa-B controlled genes for inflammatory cytokines like IL-1, IL-6, and IL-8, and the expression of co-stimulatory molecules required for the activation of T-cells of the adaptive immune system (*91*).

In contrast, non-pathogenic intestinal microbiota have been shown to inhibit the NF-kappa-B pathway (*99*). However, activation of the NF-kappa-B pathway similarly to virulent pathogens has also been described, indicating that some

members of the commensal microbiota provide stimulatory signals to the intestinal immune system (*46*, *94*).

3.3.2 Effects of probiotics on peripheral blood mononuclear cells (PBMC)

3.3.2.1 Impact on Th1/Th2 cytokine production

In *in vitro* studies as well as following *in vivo* oral administration, distinct strains, especially Lactobacilli and Bifidobacteria, have been shown to alter PBMC cytokine profiles.

Pochard et al. discovered that pre-incubation of PBMC from house dust mite allergic and healthy donors with Lactobacilli, before stimulating them with *Dermatophagoides pteronyssinus* (Der p) and staphylococcal superantigen significantly decreased the secretion of the Th2 cytokines IL-4 and IL-5. Interferon (IFN) -gamma and IL-12 production were amplified at the same time, and blocking these two cytokines restored IL-4 production (*112*).

Another group reported the induction of tumor necrosis factor (TNF) -alpha, IL-6, IL-12, IL-18, and low amounts of IL-10 in PBMC after *in vitro* stimulation with Lactobacilli, suggesting a stimulatory potential of lactic acid bacteria on non-specific immunity (*94*, *95*).

Analyzing PBMC of infants (16-18 months) participating in a randomized controlled clinical trial testing the effect of probiotics (*Lactobacillus fermentum* PCC^{TM}) on atopic dermatitis, Prescott et al. found an increase in IFN-gamma responses to the polyclonal stimuli phytohaemagglutinine (PHA) and *Staphylococcus aureus* enterotoxin B, the latter being directly proportional to the improvement of atopic dermatits. The authors interpreted this result as a surrogate

for the bacteria-induced maturation of the immune system, which is thought to be delayed in atopic children, as previously described by the same group (*115*). TNF-alpha production to heat killed *Lactobacillus* and heat-killed *Staphylococcus aureus*, representing the intestinal and the skin flora, significantly increased as well. However, no changes in IL-10 or TGF-beta secretion were observed (*114*). Taken together, these results provide evidence that probiotic strains have the potential to modulate Th1 and Th2 cytokine profiles (summerized in Figure 2)

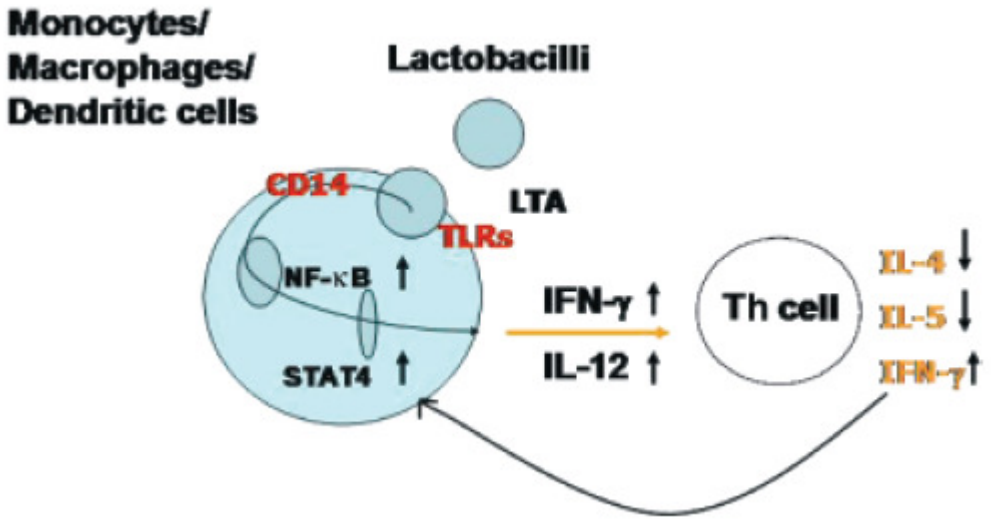


Figure 2. Effects of lactobacilli on immune cells according to *in vitro* studies. Lactobacilli may modify the innate and adaptive immune response by activating IFN-gamma and IL-12 production in monocyte lineage cells. This is mediated by activation of NF-kappa B and STAT signalling pathways. The cytokines produced by monocytes/macrophages inhibit the production of IL-4 and IL-5 but support IFN-gamma production by T cells. Thus, lactobacilli enhance the immune response and modify the polarization of T cell response by down-regulating Th2 immune response. From: Vaarala O, Clin. Exp. Allergy 2003 (*153*).

However, other mononuclear fractions are affected by probiotic stimuli as well. Haller and co-workers showed evidence for natural killer (NK) cells as primary targets of immunostimulatory effects of non-pathogenic bacteria. Investigating different fractions of PBMC, NK cells were identified as the only ones up-regulating the IL-2 receptor alpha chain (CD25) and proliferating upon direct

bacterial challenge, whereas purified T-cells (CD4+ and CD8+) and B-cells (CD19+) remained unresponsive (*44*). Marin et al. tested the cytokine-induction potential of different *Streptococcus thermophilus* and Bifidobacterium strains on a macrophage - (RAW 264.7 cells) and a T-cell model (EL4.IL-2 thymoma cells). They found that the tested strains could differentially induce cytokine synthesis in macrophages, but that effects on T-cells required co-stimulatory signals (*86*).

3.3.2.2 Induction of regulatory mechanisms

There is accumulating evidence that a key feature of probiotics is the activation of regulatory mechanisms, such as the induction of T-reg and stimulation of IL-10 and TGF-beta production.

Recent data suggest that micro-organisms recognized by the immune system as "harmless" prime immune regulatory responses rather than aggressive inflammatory responses (reviewed by Rook et al. (*125*)). In a murine model, Lactobacilli induced a distinct type of dendritic cells (DCs), that retained the ability to induce T-reg (*20*). DCs are professional antigen presenting cells (APCs) that developed efficient mechanisms to monitor their antigenic environment (*92*). DCs have a unique ability to elicit primary T cell responses, thereby representing a central element in conducting the immune system. In human monocyte-derived dendritic cells, *Lactobacillus reuteri* and *Lactobacillus casei* primed the development of T-reg cells. These T-reg cells produced increased levels of IL-10 and inhibited the proliferation of bystander T cells in an IL-10-dependent fashion (*139*). In a recent study by Pochard and co-workers, a direct regulatory activity of *Lactobacillus plantarum* NCIMB 8826 on monocyte-derived DC from *Der p* 1 allergic individuals was seen. Incubation with the strain induced maturation of

DCs, even if pulsed with the allergen. Accordingly, Th2 cytokine production of naïve or memory autologous T-cells upon co-culture with *Der p* 1-pulsed DCs was highly reduced in the presence of *L. plantarum*. Interestingly, T-cells once stimulated with DCs pulsed with *Der p* 1 and *L. plantarum* failed to produce any Th2 cytokines in response to stimulation with *Der p* 1-pulsed DCs (*113*). Hart et al. described a potent induction of IL-10 from dendritic cells from blood and intestinal tissue upon *in vitro* culture with the probiotic preparation VSL#3, together with a down-regulation of the co-stimulatroy molecule CD80. Th1 cytokines were inhibited and LPS-induced IL-12 production was diminished (*49*).

It has thus been hypothesized that the increased activity of regulatory DC and T-reg accounts for immunoregulatory mechanisms mediated by IL-10 and TGF-beta. Hence, constant stimulation of the intestinal immune system by the indigenous non-pathogenic microbiota and externally applied probiotics may cause continuous activation of T-reg, resulting in a constant suppressive and tolerogenic milieu in the gut.

Further supporting this assumption, Pessi et al. measured increased serum- as well as mitogen-induced IL-10 levels in a clinical study in children after ingestion of *Lactobacillus GG* (*110*).

Niers et al. characterized a variety of potentially probiotic lactic acid strains according to their potential to induce IL-10 in PBMC. The authors found a distinct profile of high levels of IL-10 and low amounts of IL-12p70, IL-5, and IL-13 in the tested strains. In PHA-stimulated PBMC cultures, these strains decreased the production of Th2 cytokines. Neutralizing IL-10 production resulted in restoration of Th2 cytokine production and concurred with an increase in pro-inflammatory cytokines such as IL-12p70 and TNF-alpha. However, in this study, the main source of IL-10 were CD14+ cells and not T-cells (*101*).

3.4 Ways of cross-talk between probiotic bacteria and the host immune system

3.4.1 M-cell mediated uptake of non-pathogenic bacteria

Non-invasive bacteria are able to cross the intestinal barrier via adhesion to microfold (M)-cells in the follicle associated epithelia (FAE) in Peyer's Patches to gain access to the local immune system (*136*). M-cells are specialized epithelial cells with a high capacity in vesicular transcytosis for macromolecules and microorganisms that act as antigen sampling system in the intestinal barrier. M-cells differ from intestinal epithelial cells (IEC) by the absence of a typical brush border, reduced alkaline phosphatase (AP) activity, a marker of the brush border, loss of glycocalix, and a specialized cytoskeleton, all together permitting adherence and transcytosis and of antigens and pathogens (reviewed in (*74*)). M-cells are the major sites of antigen sampling, invasion of microorganisms, and subsequent delivery to the underlying mucosal immune system (*100*, *107*). M-cell transport immediately elicits mucosal immune responses. Transcytosed bacteria are captured by DC of the subepithelial dome region and taken to local lymph nodes. There they trigger local immune responses, like IgA induction, which helps to prevent penetration of commensal bacteria (*79*). M-cells represent a gap in the intestinal barrier which is used by many pathogens to cross the healthy epithelium and seem to play a key role during breakdown of the epithelial barrier during inflammatory conditions in the intestine. However, it is believed that through their gate keeper function M-cells keep a balance between antigen up-take and inflammatory responses to luminal antigens.

3.4.2 Dendritic cells sample microorganisms from the intestinal lumen

DC developed efficient mechanisms to constantly monitor their antigenic environment (*92*). When resident in the lamina propria of the intestinal mucosa, DCs reach through the epithelial cell layer with periscope-like protrusions to read the antigenic environment of the intestinal lumen (*102*, *120*). DCs are able to open tight junctions to send out projections into the intestinal lumen through which they constantly monitor their antigenic environment. It has been shown that this way non-invasive bacteria are sampled from the intestinal epithelium and shuttled to the basolateral side of the epithelial layer (*121*). Shuttled microbes can either be captured by subepithelial DCs or directly taken to local lymphnodes by the shuttling DCs. DCs of the lamina propria are highly endocytotic. Upon exposure to innate stimuli (e.g., TLR-ligands), DCs undergo a process of functional maturation and activation that includes the termination of antigen uptake, the migration from the periphery to secondary lymphoid organs, the upregulated surface expression of major histocompatibility (MHC) antigens and costimulatory molecules, and the secretion of cytokines. Mature DCs are capable to productively present previously captured antigenic material to naïve T cells and initiate immune responses (*3*, *17*).

3.4.3 Cross-talk between non-pathogenic bacteria and enterocytes

Recent studies suggest that intestinal epithelial cells (IEC) contribute to immune-modulation by non-pathogenic bacteria as well, rendering bacterial trans-epithelial migration unnecessary. Using Caco-2 cells, a human intestinal epithelial-like cell line which is widely used as an *in vitro* model of polarized, fully differentiated human enterocytes (*93*), it has been demonstrated that lactic acid bacteria and

Bifidobacteria not only adhere to IEC in a strain-dependent manner (*97*, *150*), but also induce specific cytokine responses in Caco-2 cells (*97*). There is emerging evidence for a "cross talk" between intestinal microorganisms, IEC, and cells of the immune-system resulting in differential immunological responses in the gut mucosa.

3.5 The Caco-2 transwell culture system: an in vitro model of human enterocytes to study immune-modulating effects of probiotic bacteria

3.5.1 The Caco-2 transwell model

The human enterocyte-like Caco-2 cell line was established in the early eighties of the 20th century, as an attempt to study cancer mechanisms and mechanisms of related therapies in gastrointestinal tumors.

This cell line, derived from a human colon adenocarcinoma of a male, 72 year-old Caucasian, showed spontaneous differentiation in long-term culture. The first studies revealed that these cells upon differentiation express morphological and biochemical characteristics of small intestinal ileal enterocytes. They were found to grow in a functionally polarized monolayer, express microvilli on their apical side, form tight junctions, and express small intestinal enzymes on their apical membrane. In addition, polarized expression of membrane receptors for growth factors and various transport activities have been described (*111*). Other groups confirmed the enterocytic differentiation, but a co-existence of enterocytic and colonocytic characteristics has been described as well (*31*).

To better mirror the conditions in vivo, Caco-2 cells were grown on permeable filter inserts to allow free flux of ions and nutrients to either sides of the epithelial layer. Since then, the transwell model has been extensively used for transport and toxicity studies (reviewed by Meunier et al. (*93*)).

3.5.2 M-cell-like cells in the Caco-2 model

In recent years, a modification of the Caco-2 model has been established, which allows mimicking of human M-cells of Peyer's patches.

By the addition of Raji-cells, a human B-cell lymphoma cell line, Caco-2 cells could be converted into cells expressing morphological and functional M-cell properties. Caco-2-Raji cocultures showed potential human M-cell properties, like enhanced trans-epithelial microparticle transport, reduced microvilli and alkaline phosphatase activity, Sialyl Lewis antigen up-regulation, increased binding of lectins, as well as wheat germ, up-regulation of peanut agglutinin, intercellular adhesion molecule (ICAM)-1, and vascular cell adhesion molecule (*43*, *63*, *64*).

3.5.3 Non-pathogenic bacteria in the Caco-2 transwell model – evidence for intestinal epithelial cell/leucocyte crosstalk

To mimic the site of natural exposure to non-pathogenic bacteria, the intestinal epithelium, effects of non-pathogenic bacteria and probiotics have been investigated in the Caco-2 transwell model. Zhang et al. observed that live strains of *L. rhamnosus GG* induced IL-8 in Caco-2 mono-cultures, and that both, live and heat-killed *L. rhamnosus GG* inhibited IL-8 production induced by addition of TNF-alpha to the cultures by affecting the NF-kappa-B/I-kappa B pathway in Caco-2 cells (*170*). In an other experiment, live and heat-killed Lactobacilli and

Bifidobacteria have been shown to adhere to Caco-2 cells but induce only marginal IL-6 and IL-8 production by these cells (*97*). Haller et al. discovered that Caco-2 cells remain unresponsive to stimulation with non-pathogenic bacteria unless PBMC are present in the culture. Similar observations were made by Parlesak et al. (*109*). Hence, the importance of intestinal epithelial cell/leucocyte cross-talk in responding to non-pathogenic bacteria became evident. The intestinal epithelial cell was no longer considered a simple physical barrier, but seemed to play a crucial, active part in the response to bacteria.

Leucocytes sensitised Caco-2 cells secreted IL-1 beta and TNF alpha, while IL-10 was exclusively produced by PBMC. In a consecutive study, monocytes were identified as source of IL-10 (see below). Caco-2-leucocyte cocultures showed a discriminative response to the different strains tested. *Lactobacillus johnsonii*, an intestinal isolate, for instance, was found to induce a strong TGF-beta and IL-10 response in cocultures, while eliciting only low levels of pro-inflammatory cytokines (*45*).

In a subsequent study, performed in Caco-2 cells and HT29/MTX cells, the presence of *E. coli* or *Bacteroides vulgatus* induced endogenous I-kappa-B alpha phosphorylation, indicating the induction of the NF-kappa-B pathway, whereas the latter strain failed to induce NF-kappa B signalling and pro-inflammatory gene expression in the presence of PBMC. This phenomenon was interpreted as a mechanism assuring hyporesponsiveness of the intestinal epithelium to commensally intestinal bacteria, like *Bacteroides vulgatus*. It further supported the concept of cellular cross-talk of intestinal epithelial cells and leucocytes when initiating responses towards non-pathogenic bacteria (*46*). The authors further demonstrated that in the presence of Caco-2 cells as well as Caco-2 monolayers stimulated with non-pathogenic *E. coli* and lactobacilli, CD 14^{high} PBMC acquired

a $CD14^{low}$ $CD16^{low}$ phenotype, featuring immunosuppressive functions. They were found to predominantly secret IL10 and IL-1Ra and have a diminished potential to trigger allogeneic lymphocyte responses (*47*).

Rimoldi and co-workers observed that human monocyte-derived DCs incubated with supernatants of bacteria-treated Caco-2 monolayers generated an anti-inflammatory environment through the release of regulatory IL-10 and CCL22, a chemokine known to preferentially recruit Th2 and T-reg, and the induction of Th2 cytokines. In contrast, co-incubation of DC, Caco-2 and bacteria led to a more inflammatory response, characterized by IL-12 release and the induction of Th1 as well as Th2 cytokines (*122*).

These studies strengthened the hypothesis of an active influence of intestinal epithelial cells on regulating immune responses in the intestinal epithelium and securing mucosal immune homeostasis and immunologic tolerance towards commensal bacteria.

3.6 Summary

There is accumulating evidence that modification of the intestinal microbiota by ingestion of distinct bacterial strains, so-called probiotic bacteria, is beneficial for well-being and health. Clinical studies even suggest that probiotic preparations could be a promising approach for treatment and prevention of specific diseases, such as gastrointestinal infections, atopic diseases, and chronic inflammatory bowel disease. However, our knowledge of the mode of action of probiotic strains is still limited. Beside local effects like the enhancement of the mucosal barrier and induction of secretory IgA, one putative mechanism is modifying the host's immune system. It has been hypothesized that probiotics may modulate PBMC

cytokine profiles which are central in orchestrating immune responses. Distinct strains of Lactobacilli and Bifidobacteria have been shown to increase the production of pro-inflammatory cytokines like IFN-gamma, TNF-alpha, IL-6, and IL-12 and the regulatory cytokine IL-10(*139*), while down-regulating Th2-cytokines such as IL-4 and IL-5 in *in vitro* PBMC models. Another player in the effect of probiotics is the intestinal epithelium. Based on findings in the Caco-2-PBMC transwell coculture system, there is emerging evidence for a "cross talk" between intestinal microorganisms, intestinal epithelial cells, and cells of the immune-system resulting in differential immunological responses in models of the gut mucosa. Lamina propria dendritic cells are also thought to be involved in capturing luminal microbes and inducing immune responses via presentation to naïve T-cells in local lymph nodes.

A schematic representation of the complex interplay of probiotic bacteria and the host immune system is given in Figure 3 (from (*24*)).

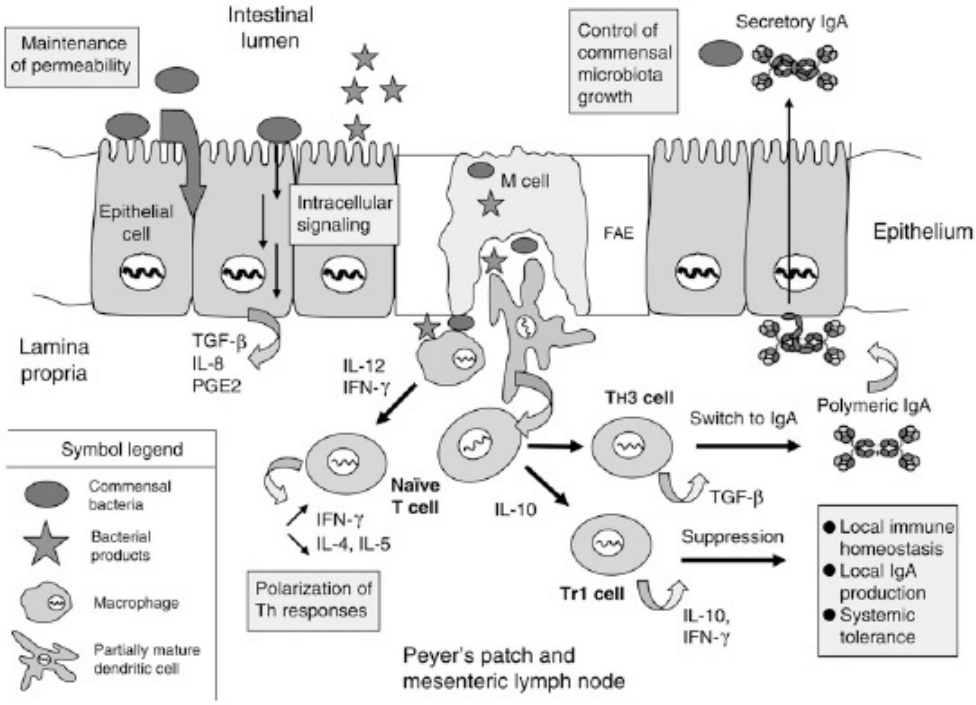


Figure 3. Schematic representation of the multiple consequences of the cross-talk between the probiotic bacteria and the intestinal mucosa. At the intestinal epithelial level, probiotic bacteria may allow beneficial effects through transient colonization and/or release of bioactive compounds. This translates into reinforcement of the intestinal barrier as well as direct modulation of epithelial cell functions including cytokine and chemokine release. Although a limited event, translocation of bacteria to the lamina propria may affect innate and adaptive immunity by activating production of cytokines by monocytes/macrophages. Sampling by M cells in Peyer's patches (*PP*) and subsequent engulfment by dendritic cells (*DC*) of the innate immune system may contribute to present microbial antigens to naïve T cells in the PP and mesenteric lymph nodes (*MLN*). This allows IgA antibody-mediated mucosal response to take place against the bacterium to prevent overgrowth and spreading beyond MLN […]. Remarkably, the same processing pathway plays a critical role in the shaping of the mucosal immune system toward a noninflammatory, tolerogenic pattern that takes place through the induction of regulatory T cells. *Author's caution:* The scheme is a simplified synthesis obtained from data collected in vivo and in vitro in various experimental models; the specific effects of a particular probiotic on the development of local and systemic responses must be considered on a case-by-case basis. From: Corthésy B et al., J. Nutr. 2007 (*24*).

4 AIMS of the THESIS

The **purpose of this thesis** was to investigate immune-modulatory properties of probiotic bacterial strains in an *in vitro* co-culture model of human intestinal epithelial cells (Caco-2 cells) and peripheral blood mononuclear cells (PBMC).

The mechanisms by which probiotics exert "beneficial health effects", which is the general definition of "probiotic", are not fully understood. Several *in vitro* studies suggest that probiotic strains alter PBMC cytokine patterns in favor of pro-inflammatory and regulatory cytokines. Recently, intestinal epithelial cells have been found to actively take part in the "cross talk" between microbiota and cells of the immune-system leading to strain-specific immune responses.
Using the Caco-2-PBMC transwell model, we **first** tested whether the barrier of the Caco-2 monolayer changes bacteria-induced cytokine responses of PBMC. We directly compared cytokine secretion upon bacterial stimulation in Caco-2-PBMC cocultures and PBMC cultures.

It is unclear, whether the viability of applied bacteria is relevant. Even though live specific probiotics are recommended for clinical use, inactivated strains may also have beneficial effects in certain indications. However, literature on *in vitro* immunologic effects of viable and non-viable probiotic bacteria is contradictory, to the point of reversal of effects on cytokine production in Caco-2 cultures. Therefore, **second**, we analyzed whether inactivation of probiotics impacts their immune modulating properties in the enterocytes-PBMC co-culture system. We investigated live, heat-, and formalin-inactivated probiotic bacteria in terms of

their capacity to induce cytokine responses in the Caco-2-PBMC coculture transwell model.

In a **third** approach, we aimed to investigate the role of M-cells of Peyer's patches in "cross talk" between probiotic bacterial strains, IEC, and PBMC in our *in vitro* model. In the intestinal epithelium, M-cells are the major sites of antigen sampling and invasion of microorganisms. They represent a central gate for antigens to reach the underlying mucosal immune system. We hypothesized that M-cells not only act as an antigen entry site in the GI mucosa, but also affect bacteria-IEC-leucocyte interactions, thereby altering cytokine responses. For this set of experiments, we used a modification of the Caco-2 system, the Caco-2 ^{Raji} model, allowing the simulation of M-cells in the enterocyte monolayer.

The **final aim** was to test the hypothesis that probiotic bacteria can counterbalance allergen-specific, Th2-prone cytokine-responses, as in allergic diseases, taking into account the contribution of the intestinal epithelial cell layer. In PBMC-cultures, probiotic strains have been shown to re-balance the allergic Th2-prone cytokine-milieu towards a Th-1- or regulatory T-cell response. Here, we aimed to test immune modulatory features of distinct probiotic strains on allergen-specific cytokine responses in the Caco-2-PBMC coculture model.

5 MATERIALS and METHODS

5.1 Bacterial strains

Microbiota used in these experiments included *Lactobacillus rhamnosus GG (L. rhamnosus GG*), *Lactobacillus rhamnosus* (*L. rhamnosus*), *Streptococcus thermophilus* (*S. thermophilus*), and *Escherichia coli* BL 21-DE3 (*E. coli*).

L. rhamnosus GG is a probiotic strain well studied in clinical trials (*51*, *58*, *60*, *126*, *127*). Type strain *L. rhamnosus* (*22*) served as control strain of the same species. *S. thermophilus* (*134*) is part of the yogurt microbiota (*141*). Non-pathogenic, gram negative *E. coli* was used as non-fermenting control strain.

5.1.1 Specification and source

L. rhamnosus GG (ATCC 53103) was kindly provided by Valio, Helsinki, Finland. *L. rhamnosus* (type strain, ATCC 7469) and *S. thermophilus* (type strain, ATCC 19258) were obtained from the strain collection, Division of Food Microbiology and Hygiene, Department of Food Science and Technology, BOKU, University of Natural Resources and Applied Life Sciences, Vienna, Austria. *E. coli* was kindly provided by A. Mercenier, Nestlé Research Centre, Lausanne, Switzerland.

5.1.2 Growth conditions

L. rhamnosus GG and *L. rhamnosus* were cultured anaerobically (80 % N_2, 10 % CO_2, 10 % H_2) in MRS broth (Merck, Darmstadt, Germany) at 37 °C. *S. thermophilus* was cultured aerobically at 37 °C in M17 broth (Merck, Darmstadt, Germany). *E. coli* was grown in LB Medium (Merck, Darmstadt, Germany) at the

same conditions. Stock cultures were kept in broth containing 20 % (v/v) glycerol (AppliChem, Darmstadt, Germany) at -80 °C. Strains were grown for 14 hours and were then harvested by centrifugation (2500 g, 10 min). Bacterial cells were washed three times with phosphate-buffered saline (PBS) (Oxoid, Basingstoke, England). The number of viable cells was determined by plating-technique on media described above, containing 1.5 % agar (Oxoid, Basingstoke, England). Based on the number of colony-forming units (CFU) the amount of cell-suspension for the experiments was calculated. The washed cells were either stored at 4 °C until use (at most 4 hours) or subjected to heat- or formalin-inactivation.

5.1.3 Inactivation procedures

In some experiments bacteria were used either heat- or formalin-inactivated. For heat-inactivation cells were re-suspended in tissue culture medium (Dulbecco`s modified Eagle medium, Gibco BRL, Paisley, Scotland, UK) and heat-treated in a water bath (85 °C, 10 min). Inactivation by formalin was carried out by suspending the washed bacterial cells in 4 % para-formaldehyde (AppliChem, Darmstadt, Germany) for 30 min, followed by 3 washing-steps with PBS and final resuspension in tissue culture medium (modified from Pochard et al. (*112*)). Inactivation was confirmed for both inactivation methods by absence of viable cells by plate counts. Aliquots of the inactivated bacterial cells were stored at -20 °C until use.

5.2 Caco-2 cell transwell cultures

The human enterocyte-like adenocarcinoma cell line Caco-2 (ATCC HTB-37, passage 30-40) was kindly provided by E. Jensen-Jarolim, Center for Physiology and Pathophysiology, Department of Pathophysiology, Medical University of Vienna, Austria. Cells were propagated in Dulbecco modified Eagle's minimal essential medium containing 25 mM HEPES, 1mM sodium pyruvate (both Sigma Sciences, St. Louis, MO), 2mM L-glutamine (Invitrogen, Carlsbad, CA), 10% heat-inactivated fetal calf serum (FCS, Invitrogen), 1% MEM non-essential amino acids, and 1% penicillin-streptomycin (both Gibco BRL, DMEM+) at 37^{o} C, 5% CO_2. For transwell cultures, 5x10^{5} cells were seeded onto the bottom side of 22mm cell culture inserts (0,4 µm membrane pore size, BD Falcon, Franklin Lakes, NJ, USA) and allowed to attach for 6 hours at 37^{o} C, 5% CO_2. Filter inserts were then transferred into 12-well flat bottom culture plates (BD Falcon, Becton Dickinson, San José, CA, USA) in their normal orientation. Culture medium in both compartments was changed every other day.

5.2.1 Monolayer integrity

Tightness of the Caco-2-monolayer was controlled by measuring transepithelial electric resistance (TEER, Millicell ERS Voltmeter/Ohmmeter, Millipore, Eschborn, Germany) and trans-epithelial transport of [2-3H]-D-Mannitol, a leakage marker, as described by Blais et al. (*8*). Briefly, Caco-2 covered filter inserts were washed with PBS and transferred into 12-well flat bottom culture plates containing transfer buffer (NaCl 127 mM, $Na_2HPO_4x2H_2O$ 10 mM, KCl 4.7 mM, $MgCl_2$ 1.2 mM, $CaCl_2$ 1.8 mM,, HEPES 20 mM, $NaHCO_3$ 2.2 g/l, all Sigma Sciences, and L-glutamine 1 mM, Invitrogen, adjusted to pH 7.2 at room

temperature). 500µl [2-3H]-D-Mannitol (ICN Biomedicals, Irvine, CA, USA) was applied to the upper compartment of the transwell system. After 1 hour at 37°C, [2-3H]-D-Mannitol in both compartments was detected using a liquid scintillant (Betaplate Scint, Wallac, Perkin Elmer, Waltham, MA, USA). The [2-3H]-D-Mannitol-transfer rate into the lower compartment was calculated as follows: $^{cpm\ (lower\ compartment)}/_{cpm\ (upper\ compartment)}$ x 100, (cpm = counts per minutes).

5.2.2 Immunhistochemical stainings of filter inserts

Formaldehyde-fixed sections of Caco-2 covered transwell inserts were stained for the tight-junction marker Occludin. 2 µm sections of formalin-fixed, paraffin-embedded filters were dried over night at 56°C. Sections were de-paraffined with Xylol and 100% ethanol (both Sigma Chemicals), then re-hydrated in a decreasing ethanol-series, and heated in sodium citrate buffer 0.01% twice for 5 minutes in a microwave at 800 Watt, before treating them with peroxide 3% for 10 minutes. Rabbit anti-Occludin antibody (Zymed Laboratories Inc., San Francisco, CA, USA) was applied at 1:200 in PBS 1% FCS, and the IDetect™ Superstain System HRP (ID-Lab Inc., London, Ontario, Canada) was used according to the manufacturer's instructions with AEC (ID-Lab Inc., London, UK) as chromogen. Sections were counter-stained with haemalaun.

5.3 Caco-2 cultures with Raji-cell-induced M-cells (Caco2 ^{Raji})

B-cell lymphoma Raji cells were kindly provided by E. Jensen-Jarolim. Cells were maintained in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated FCS, Invitrogen, 2mM L-glutamine (Invitrogen), and 170 mg/L gentamycin-sulphate, Sigma Sciences (RPMI+).

Caco-2-Raji cocultures were performed as described by Roth-Walther et al. (*129*). Raji cells were seeded at a density of $2x10^6$/ml to the basolateral side of of Caco-2 monolayers (upper compartment of the experimental system) at day 21. Medium was changed every day in the upper compartment, and every other day in the lower compartment, using DMEM+/RPMI+ 1:2 (Figure 4B).

At day 28, monolayer integrity was measured as described above, and transformation of Caco-2 into cells with M-cell-like morphology was confirmed by measuring apical AP activity, a potential human M-cell marker, adapted from Gullberg et al.: 200 µl alkaline phosphatase substrate (Sigma Sciences) was applied to the apical surface of Caco-2 and Caco-2-Raji covered filter inserts after washing three times with PBS/0,05% Tween 20. After 30 Minutes, substrate was transferred into 96-well plates (Iwaki, Tokyo, Japan) and substrate conversion was measured at 405 nm using an ELISA plate reader. Successful transformation of Raji-treated Caco-2 cells into M-cells was shown by reduced substrate conversion in comparison to Caco-2 cells without Raji-cells (*43*).

Before challenge experiments, Raji-cells were removed from filter inserts by extensive washing with PBS.

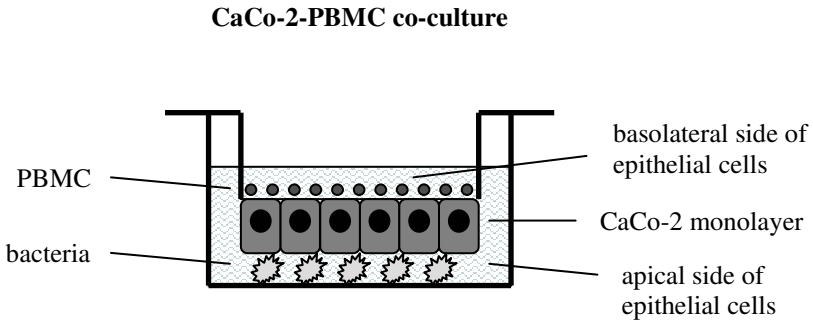
5.4 Isolation of human peripheral blood mononuclear cells (PBMC)

PBMC from healthy volunteers and peanut allergic individuals were obtained by density gradient centrifugation of heparinized peripheral venous blood over Ficoll-Paque (Pharmacia, Uppsala, Sweden, 300g, 30 minutes) as described previously (*70*). Cells were gradually frozen and stored in liquid nitrogen at –180° C.

5.5 Bacterial challenge of Caco-2-PBMC, Caco-2 ^{Raji}-PBMC cocultures, and PBMC cultures

Caco-2- or Caco-2 ^{Raji} covered cell culture inserts were washed twice with DMEM+/RPMI+ 1:2 and transferred into fresh 12-well flat bottom culture plates containing DMEM+/RPMI+ 1:2. $2x10^6$ PBMC/ml were added to the upper compartment of the transwell system, corresponding to the basolateral side of the epithelium. The apical surface of the epithelial monolayer (lower compartment) was challenged by 10^6 CFU/ml live, heat- or formalin-inactivated *L. rhamnosus GG*, *L. rhamnosus*, and *S. thermophilus*, and *E. coli* for 40 hours (previously determined to be optimal, Figure 4A and B). Challenge with viable bacteria was performed in the absence of antibiotics. After 4 hours, 150 µg/ml gentamycin-sulphate (Sigma Sciences) was added to prevent overgrowth of *S. thermophilus*. Caco-2-PBMC cultures without bacteria served as controls. Supernatants from both compartments were harvested and cell debris was removed by centrifugation (5 minutes, 300g). Supernatants were stored at -20° C for cytokine detection. Integrity of Caco-2 monolayers during the experiments was monitored by measuring [2-3H]-D-Mannitol translocation before and after bacterial challenge. Viability of PBMC was ascertained by Trypan blue, which does not stain live cells (Trypan blue exclusion method).

A



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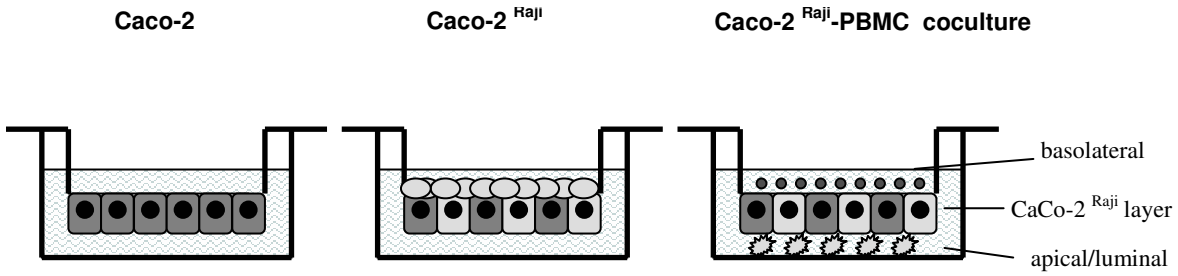

Figure 4. Experimental design. (A) Caco-2 cells were grown on permeable filter inserts until they formed a confluent, polarized epithelial monolayer (d28). PBMC were added to the basolateral side of the epithelial layer. CaCo-2-PBMC cocultures were challenged with live, heat- and formalin-inactivated bacteria or peanut allergen extract for 40 hours on the apical side. Supernatants from both sides were harvested for cytokine measurements. (B) Raji-induced M-cells. Caco-2 cells were grown on permeable filter inserts for 21 days (left panel). Raji cells were added to the basolateral side of the epithelial layer for 7 days and induced the conversion of Caco-2 into M-cells (middle panel). At day 28, Raji cells were removed by extensive washing, and CaCo-2 ^{Raji}-PBMC cocultures were challenged with heat- and formalin-inactivated bacteria for 40 hours on the apical side (right panel). Supernatants were harvested for cytokine measurements.

In parallel to Caco-2-PBMC cocultures, $2x10^6$ PBMC/ml DMEM+/RPMI+ 1:2 were stimulated with 10^6 CFU/ml live, heat- or formalin-inactivated bacteria for 40 hours, or cultured without stimulus in the absence of Caco-2 cells. Supernatants were harvested and processed as Caco-2-leucocyte coculture.

Experiments to compare cytokine responses of Caco-2- and Caco-2 ^{Raji}-PBMC coculture were performed simultaneously, to ensure equal culture conditions.

All experiments were performed in triplicates.

5.6 Allergen challenge of Caco-2-PBMC^A coculture and PBMC^A cultures

PBMC were obtained from adult individuals with a doctor's diagnosed peanut allergy (peanut specific serum IgE and a clear history, PBMC^A, n=5). Caco-2-PBMC^A cocultures were challenged apically with peanut allergen extract (kindly provided by Jean-Michel Wal, Laboratoire d'Immuno-Allergie Alimentaire, INRA-CEA Saclay, France) at 0.5 mg/ml, 0.05 mg/ml, and 0.005 mg/ml for 40 hours in triplicates following the same procedure as in bacterial challenge experiments. Supernatants were harvested, processed, and stored for cytokine measurements.

Direct allergen-stimulation of PBMC^A without the Caco-2 barrier was performed at the same time identically.

5.7 Cytokine assays

5.7.1 Cytokine screening

The cytokine panel was compiled after qualitative screening of basolateral supernatants with RayBio® Cytokine antibody array 1.1 (Ray Biotech, Norcross, GA) following the manufacturer's protocol. Briefly, anti-cytokine coated membranes were blocked for 30 minutes in Blocking Buffer before incubation with 1 ml of culture supernatants for 2 hours at room temperature. Membranes were washed three times in Wash Buffer I, followed by 2 times Wash Buffer II at room temperature. Biotin-Conjugated Anti-Cytokines Mix was added to each membrane for 2 hours at room temperature to detect bound cytokines. After washing, membranes were covered with HRP-conjugated streptavidin (1:1000) for

2 hours, washed, and incubated with Detection Buffer for 1 minute. Membranes were exposed to x-ray film (Kodak X-omat AR film) and signal was detected using film developer.

5.7.2 Cytometric bead array

IL-6, IL-8, and IL-10 were determined in supernatants simultaneously using a multiplex cytometric bead array system, designed in a capture sandwich immunoassay format (Bio-Plex™ Cytokine Assay, Bio-Rad Laboratories Inc., Hercules, CA). Briefly, anti-IL-6, -IL-8, and -IL-10 conjugated colour-coded 5.5 µm polystyrene beads were added to pre-wetted assay plates. Standards and samples pooled from triplicate cultures were incubated in duplicates for 45 minutes at room temperature to allow binding of the respective cytokines to the capture antibody-coupled beads. Plates were washed three times before the biotinylated cytokine detection antibody was added. After incubation for 30 minutes at room temperature, plates were washed and the reaction was detected by the addition of streptavidin-phycoerythrin. The bead suspension was measured using a BioPlex Luminex Bead Analyzer equipped with software version 4.0 (Bio-Rad Laboratories Inc.), and each specific cytokine was identified and quantified based on bead color and fluorescence. Detection limits of the multiplex assay were 43.36 pg/ml for IL-6, 36.27 pg/ml for IL-8, and 45.38 pg/ml for IL-10.

5.7.3 Enzyme-linked immuno-sorbent assay (ELISA)

Cytokines in supernatants of peanut-challenged Caco-2 cultures were determined by ELISA using matched antibody pairs. IL-5-, IFN-gamma- (both Bender Med Systems, Vienna, Austria), and IL-10 ELISA-kits (HybriDomus™, Nota Bene

Scientific, Hellebek, Denmark) were performed according to the manufacturers instructions. The detection limits were 7.8 pg/ml for IL-5, 4.7 pg/ml for IFN-gamma, and 12.5 pg/ml for IL-10.

5.8 Limulus Amoeboid Lysate Assay

Potential trans-epithelial migration of bacterial products into the basolateral compartment was controlled by apical application of LPS (0.1, 1, 10 µg/ml, Serotype O111:B4, Sigma Sciences). Basolateral LPS-concentrations were measured after 40 hours of incubation using the Endochrom^{TM} assay (Carles River Endosafe, Charlston, SC, USA) following the manufacturer's protocol.

5.9 Lymphoproliferation assays with conditioned medium

Supernatants (150 µl) freshly harvested from the basolateral and the apical sides of Caco-2-PBMC cocultures after 40 hours +/- stimulation with heat- and formalin-killed bacterial strains were incubated with $1x10^5$ PBMC from the same donor in triplicates. PBMC cultured in non-conditioned medium (DMEM+/RPMI+ 1:2) and DMEM+/RPMI+ 1:2 with 25IU/ml human recombinant interleukin 2 (IL-2, Roche Diagnostics, Basel, Switzerland) served as controls. At day 3, [3H]-Thymidin (Amersham Int, Amersham, Little Chalfont, Buckinghamshire, UK, 0.5 mCi/well) was added and lymphoproliferation was measured by scintillation counting after 16 hours (*69*). Results are given in cpm.

5.10 Statistical analysis

Data were analyzed using SPSS for windows (SPSS Inc., Chicago, IL, USA). Since data were not normally distributed, non-parametric tests were applied and medians (25^{th} – 75^{th} percentile in tables) are presented. Cytokine levels of bacteria- or allergen-stimulated cocultures were compared with those of unchallenged control cultures using Wilcoxon matched-pairs signed-rank test. To compare IL-6, IL-8, and IL-10 induction by live versus heat- and formalin-inactivated microorganisms, cytokine induction in Caco-2PBMC cocultures versus PBMC cultures, and Caco-2-PBMC versus Caco-2 ^{Raji} /PBMC cocultures, relative fold changes of these cytokines were compared by Mann-Whitney-U test. A p-value less than 0.05 was considered significant.

6 RESULTS

6.1 Designing the experimental set-up

6.1.1 Initial experiments

A tight, differentiated Caco-2 epithelial cell layer was a condition precedent to study non-pathogenic bacteria-Caco-2-PBMC interactions in the transwell system. As markers of intercellular leakage, TEER and [2-3H]-D-Mannitol transfer were monitored regularly during Caco-2 culture. The development of a confluent epithelial layer was also followed by haemalaun-stained filter inserts sections in initial experiments (Figure 5A). TEER peaked at 350 to 450 $\Omega x cm^2$ which usually corresponded to a [2-3H]-D-Mannitol flux across the monolayer of $\leq$2% per hour at day 28. Expression of the tight junction marker occludin at the same time indicated a confluent, fully differentiated cell layer (Figure 5B). Accordingly, a culture time of 28 days was chosen for experiments.

6.1.2 Monolayer Integrity during bacterial challenge

To ascertain intact barrier function of Caco-2 monolayers during bacterial challenge, [2-3H]-D-Mannitol transfer was re-evaluated after co-culture experiments. Translocation rates did not increase after 40 hours of bacterial challenge but rather decreased, confirming integrity of the monolayer (1.9+/-0.17%/h before experiments, 1.25+/-0.1%/h after bacterial challenge of Caco-2-PBMC cocultures, 1.4+/-0.1%/h in Caco-2-PBMC cocultures without bacteria, mean +/-SD of 5 independent experiments). In addition, LPS (0.1, 1, 10 µg/ml) added to the apical compartment was not detected on the basolateral side after 40

hours, suggesting that no transepithelial transport of immuno-stimulatory gram-negative bacterial products across the epithelial barrier occurred during the experiments (Limulus Amoeboid Lysate assay, data not shown).

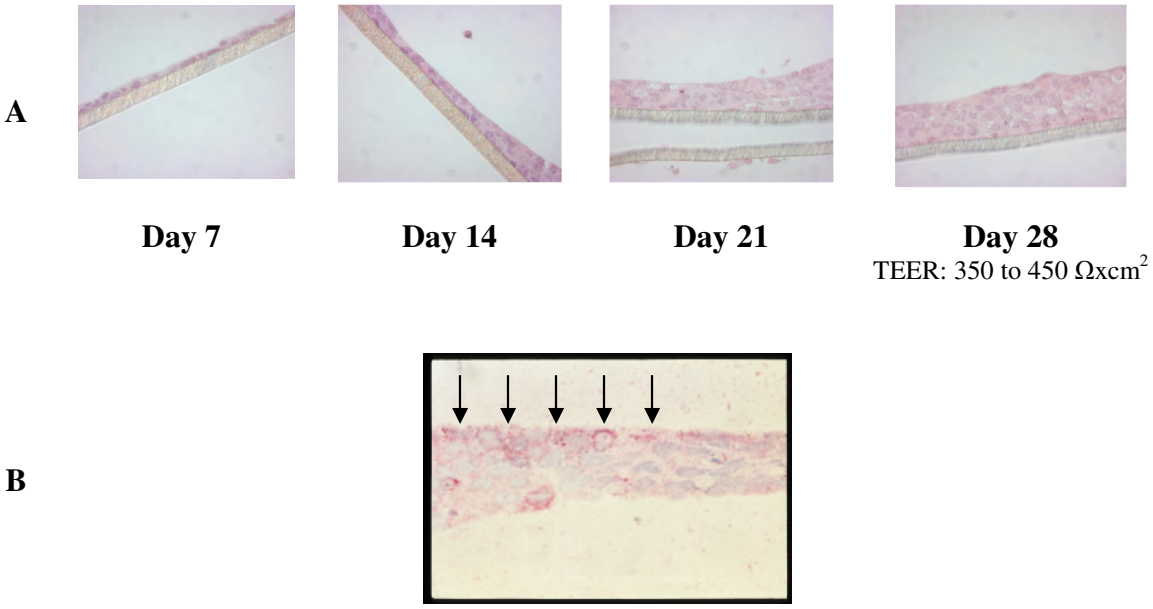

Figure 5. Development of a confluent, differentiated Caco-2 layer. Caco-2 cells were grown on permeable filter inserts in DMEM+, 37°C, 5% CO_2. The formation of a confluent epithelial layer was monitored by measuring TEER, which peaked at 350 to 450 Ωxcm^2 by day 28. Sections of haemalaun-stained filter inserts reflect the development of the cell layer (A). The expression of the tight junction marker occludin at day 28 indicates a fully differentiated intestinal epithelial cell layer (B, arrows mark occludin staining).

6.1.3 Caco-2-PBMC cocultures produce a limited panel of cytokines

To compile a cytokine panel allowing to measure immune modulation in our experimental system, we first screened culture supernatants for cytokines that contribute to acute inflammation (IL-1a, IL-2, IL-3, IL-6, IL-7, IL-8, IL-15, IFN-gamma, TNF-alpha, TNF-beta), Th-2-associated cytokines (IL-5, IL-13) and immune-regulatory cytokines (IL-10, TGF-beta 1). Of these, CaCo2-leucocyte cocultures were found to secret a limited pattern, dominated by IL-6, IL-8, and IL-10. The composition of the cytokine profile was unaffected by the presence and absence of bacteria or peanut extract. Besides, the chemotactic factors GRO, GRO-alpha, MCP 1 + 2, RANTES, and GM-CSF were detected (Figure 6).

Since our results were consistent with published data of non-pathogenic strains affecting IL-6, IL-8, and IL-10 responses of PBMC and Caco-2 cells *in vitro*, we hereupon focussed on these three cytokines as markers for immune-modulation.

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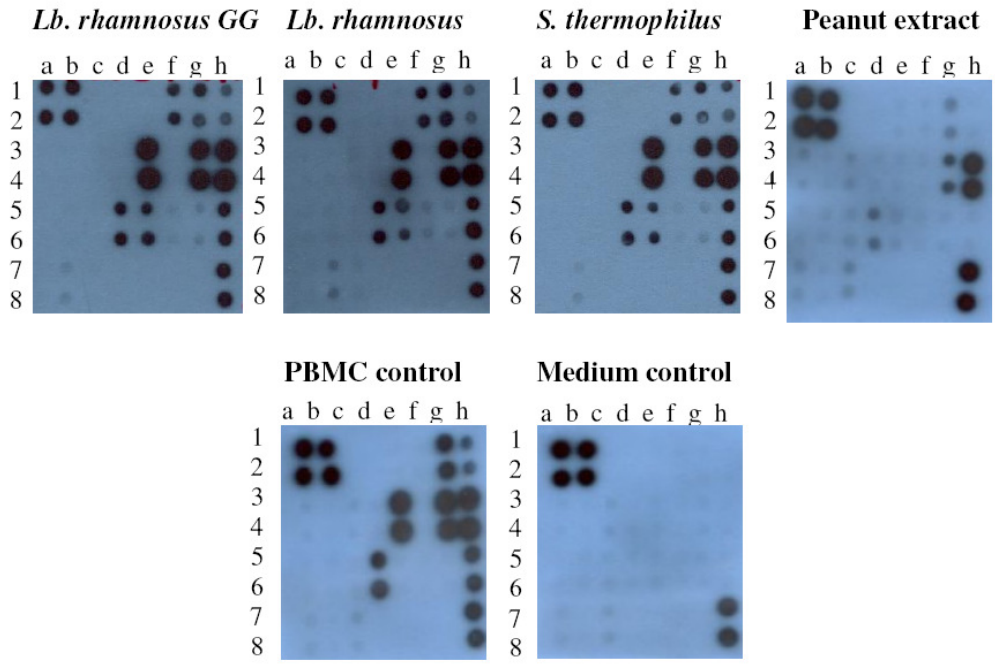


B

	a	b	c	d	e	f	g	h
1	Pos	Pos	Neg	Neg	GCSF	GM-CSF	GRO	GRO-α
2	Pos	Pos	Neg	Neg	GCSF	GM-CSF	GRO	GRO-α
3	IL-1α	IL-2	IL-3	IL-5	IL-6	IL-7	IL-8	IL-10
4	IL-1α	IL-2	IL-3	IL-5	IL-6	IL-7	IL-8	IL-10
5	IL-13	IL-15	IFN-γ	MCP-1	MCP-2	MCP-3	MIG	RANTES
6	IL-13	IL-15	IFN-γ	MCP-1	MCP-2	MCP-3	MIG	RANTES
7	TGF-β1	TNF-α	TNF-β	Blank	Blank	Blank	Blank	Pos
8	TGF-β1	TNF-α	TNF-β	Blank	Blank	Blank	Blank	Pos

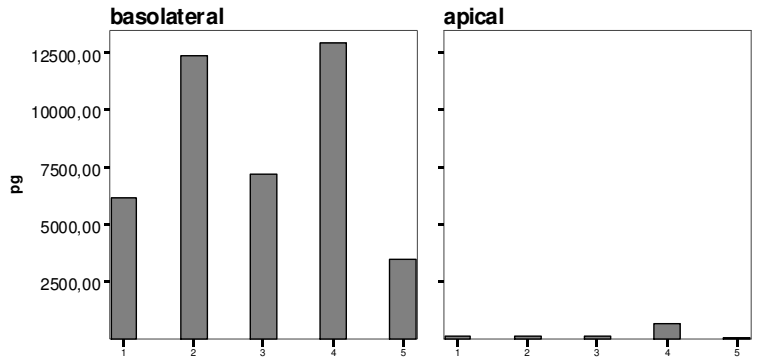
Figure 6. Composing the cytokine panel. Supernatants of Caco-2-PBMC cocultures were screened for cytokines using RayBio® Cytokine antibody array 1.1 Representative screenings of basolateral supernatants from transwell cultures challenged with formalin-killed bacteria or peanut allergen extract and unchallenged transwell cultures (PBMC control) are depicted. Cell culture medium (DMEM+) served as negative control (A). Legend to the assay from http://www.raybiotech.com (B).

6.1.4 Characteristics of cytokine secretion in the Caco-2-PBMC transwell system

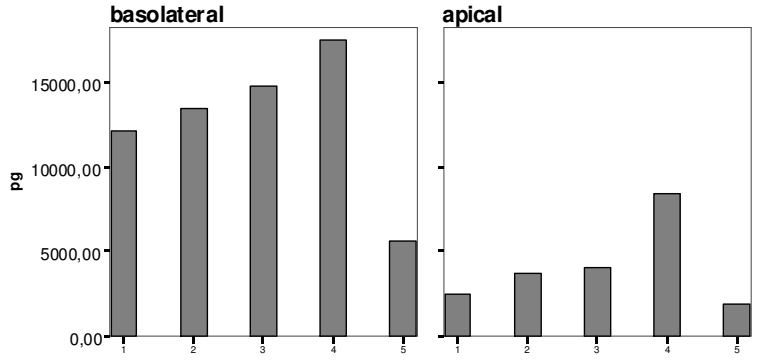
Cytokine secretion by Caco-2-PBMC cocultures was polarized towards the basolateral side of the epithelial line, and only marginal levels were detected in the apical compartment of the culture system (Figure 7). No cytokines were detected in the absence of PBMC (data not shown). Bacterial challenge did not decrease the viability of PBMC (Trypane-blue exclusion method, not shown).

Cytokine responses of cocultures highly varied between individual PBMC-donors even in controls without bacteria (IL-6 <43.36-5338 pg/ml, IL-8 <36.27-69654 pg/ml, IL-10 854-7938 pg/ml). Therefore, values upon bacterial challenge were expressed as relative fold changes compared to the respective control for comparison of different experimental conditions. The detection limit of each cytokine was used instead of zero when results were below the minimum cytokine value detectable.

IL-6



IL-8



IL-10

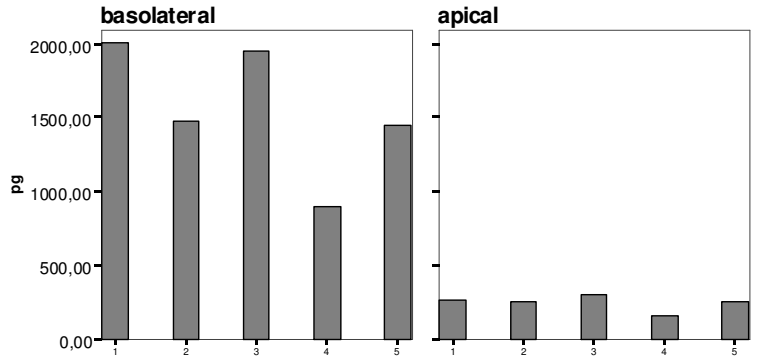


Figure 7. Cytokine secretion by Caco-2-PBMC transwell cultures is polarized towards the basolateral side. Compartmentalized Caco-2-PBMC cocultures were challenged with live *L. rhamnosus GG* (1), *L. rhamnosus* (2), *S. thermophilus* (3), and *E. coli* (4). Unchallenged cocultures (5) served as controls. After 40 hours, IL-6, IL-8, and IL-10 secretion was determined in supernatants from the apical and the basolateral compartment using a cytometric bead array. Bars depict medians of 5 independent experiments, performed in triplicates.

6.2 Bacteria-induced cytokine responses in Caco-2-PBMC cocultures versus PBMC-cultures

To address the question of whether or not the barrier of intestinal epithelial cells affects PBMC cytokine production induced by probiotic bacteria, we measured IL-6, IL-8, and IL-10 secretion by live, heat-, and formaldehyde-treated probiotic strains (*L. rhamnosus GG*, *L. rhamnosus, S. thermophilus*) and non-probiotic, non-pathogenic *E. coli*. We compared bacteria-induced cytokine profiles in Caco-2-PBMC cocultures, where the Caco-2 monolayer separated bacteria from PBMC (Figure 4A, Materials and Methods), and cultures where PBMC had direct contact to the applied microorganisms.

In both systems, all live, heat-, and formalin-treated strains significantly increased IL-6 production compared to controls without bacterial stimulation. In compartmentalized Caco-2-PBMC cultures, live *L. rhamnosus GG*, *L. rhamnosus*, *S. thermophilus*, and *E. coli* enhanced IL-6 synthesis 2.13-, 2.32-, 2.24-, and 3.87-fold, heat-treated strains 1.58-, 1.36-, 1.9-, and 2.52-fold, and formalin-inactivated bacteria 1.41-, 1.22-, 2.45-, and 1.4-fold (Wilcoxon matched-pairs signed-rank test, Figure 8 and Table 2). Direct bacterial stimulation boosted IL-6 production of PBMC 16.56-, 26.21-, 6.53-, and 12.31-fold for live *L. rhamnosus GG*, *L. rhamnosus*, *S. thermophilus*, and *E.coli*, and 17.87-, 11.46-, 5.46-, and 31.0-fold for the respective inactivated strains. Formalin-killed bacteria amplified IL-6 levels 16.31-, 14.66-, 9.3-, and 24.77-fold (live *L. rhamnosus GG*, *L. rhamnosus*, *S. thermophilus*, and *E.coli*, respectively).

Significant IL-8 values were induced by live *L. rhamnosus GG, L. rhamnosus*, and *E. coli* in the co-culture system (1.48-, 2.67-, and 2.22-), and by live *E. coli* and

heat-killed *S. thermophilus* in PBMC-cultures (1.66 and 3.15- fold increase). All other strains caused slightly weaker responses, not reaching the level of significance.

IL-10-secretion was significantly enhanced only by live and formalin-inactivated *L. rhamnosus GG* (1.43- and 1.23-) as well as live *S. thermophilus* (1.46-fold increase) in transwell cultures. In direct PBMC-cultures, all inactivated strains significantly raised IL-10 production (heat-inactivated: *L. rhamnosus GG* 35.25-, *L. rhamnosus* 20.56-, *S. thermophilus* 12.18-, *E. coli* 81.43-, formalin-inactivated: *L. rhamnosus GG* 38.42-, *L. rhamnosus* 27.23-, *S. thermophilus* 14.71-, *E. coli* 56.19-fold). So did both live *Lactobacilli* (*L. rhamnosus GG* 3.73- and *L. rhamnosus* 5.60-fold increase).

In all experiments, the presence of the Caco-2 barrier seemed to diminish cytokine production. However, when comparing bacteria-induced IL-6, IL-8, and IL-10 responses of the two culture systems statistically, we found that bacteria-induced IL-6 patterns of Caco-2-PBMC cocultures mirrored direct PBMC cultures with no significant differences. IL-8-responses were generally low in the absence and the presence of the Caco-2 monolayer, revealing no statistically significant differences either. IL-10 induction versus unstimulated controls was however significantly stronger when PBMC had direct contact to either of the heat- and formalin-killed strains, as well as live *L. rhamnosus* and *S. thermophilus* (Mann-Whitney-U test, respectively Figure 8).

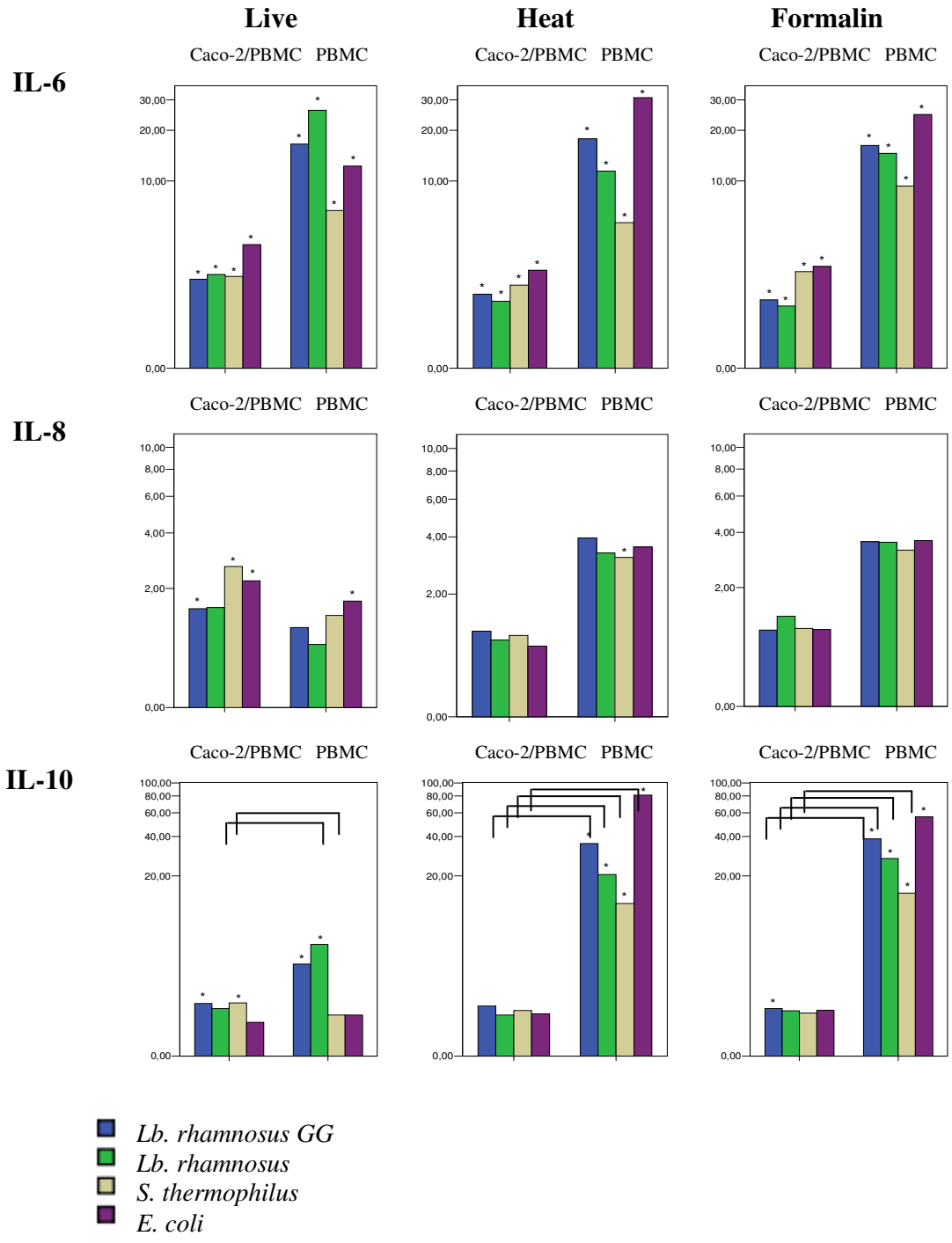


Figure 8. Bacteria-induced cytokine secretion in Caco-2-PBMC cocultures and PBMC cultures. Triplicates of Caco-2-PBMC and PBMC cultures were challenged apically with live, heat- and formalin-inactivated bacteria for 40 hours. IL-6, IL-8, and IL-10 levels were measured in supernatants from the basolateral compartments by cytometric bead array. Cytokine values upon bacterial challenge are expressed as relative fold increase versus unchallenged cultures. * significant cytokine induction versus respective controls ($p<0.05$, Wilcoxon matched-pairs signed-rank test). ⌜⌝ $p< 0.05$, Mann Whitney U test. Bars represent medians of 5 PBMC-donors**.**

6.3 The impact of inactivation of bacteria on cytokine patterns in Caco-2-PBMC cocultures

The mode of inactivation of probiotic bacteria may profoundly affect their immune-modulatory properties up to reversal of effects in *in vitro* human Caco-2 cultures. To further investigate the influence of inactivation treatment on cytokine production in Caco-2-PBMC transwell cultures, we compared IL-6, IL-8, and IL-10 secretion induced by live, heat-, and formalin-treated probiotic strains (*L. rhamnosus GG*, *L. rhamnosus*, *S. thermophilus*) and the control strain *E. coli*.

In our experiments, the tested strains induced comparable levels of IL-6, IL-8, and IL-10 in the Caco-2-PBMC co-culture system, irrespective of viability or mode of inactivation (Mann-Whitney-U test, Table 2). Although it seemed that heat- and formalin-inactivated strains were less effective in inducing cytokine responses than viable mircroorganisms, no statistically significant differences were found (Mann-Whitney-U test). Bacterial challenge enhanced IL-6, IL-8, and IL-10 production in the majority of experiments, and we did not observe any consistent suppressive effects on Caco-2-PBMC cytokine responses subject to mode of inactivation of bacterial strains.

bacterial stimulation	IL-6			IL-8			IL-10		
	Live	**Heat**	**Formalin**	**Live**	**Heat**	**Formalin**	**Live**	**Heat**	**Formalin**
L. rhamnosus GG	2.13 (1.25-73.33)	1.58 (1.38-128.95)	1.41 (1.21-136.46)	1.48 (1.465-26.32)	1.15 (0.99-286.86)	1.03 (0.92-2631.43)	1.43 (1.17-1.65)	1.33 (0.94-2.03)	1.23 (1.07-1.63)
L. rhamnosus	2.32 (1.97-150.97)	1.36 (1.12-148.86)	1.22 (1.044-154.94)	1.51 (1.21-39.04)	0.99 (0.77-273.39)	1.3 (0.95-288.53)	1.23 (0.9-1.87)	1 (0.84-1.65)	1.15 (0.95-1.55)
Streptococcus thermophilus	2.24 (1.76-84.78)	1.9 (1.36-120.70)	2.45 (1.22-134.25)	2.67 (1.28-20.17)	1.07 (0.87-221.12)	1.06 (0.92-278.26)	1.46 (1.23-1.89)	1.16 (0.97-1.60)	1.06 (0.93-1.51)
E. coli	3.87 (2.58-88.0)	2.52 (2.0-181.62)	1.41 (2.08-223.53)	2.22 (1.93-81.77)	0.88 (0.85-307.94)	1.04 (0.83-272.79)	0.77 (0.52-1.11)	1.04 (0.80-1.73)	1.18 (0.98-1.94)

Table 2. IL-6, IL-8, and IL-10 induced by live, heat-, and formalin-inactivated bacteria in Caco-2-PBMC cocultures. Cytokine values upon bacterial challenge are expressed as relative fold increase versus Caco-2-PBMC cocultures without bacteria. Live, heat-, and formalin-inactivated strains induced comparable IL-6, IL-8, and IL-10 secretion into the basolateral compartments (Mann-Whitney-U test). Medians (25th 75th percentile) of 5 independent experiments, each performed in triplicates after 40 hours of bacterial stimulation are shown.

6.4 Bacterial challenge of PBMC via M-cells in the Caco-2^{Raji} model

6.4.1 The Caco-2 M-cell model (Caco-2 ^{Raji})

To investigate whether M-cells participate in interactions of apathogenic bacteria with the Caco-2-PBMC system, the Caco-2 ^{Raji} coculture model was used (Figure 4B, Materials and methods). Conversion of Caco-2 cells into M-cell-like cells was monitored by measuring apical AP activity. Raji-treated Caco-2 cultures showed a decrease in the activity of AP by 23-65% compared with Caco-2 monocultures. Cultures containing Raji-induced M-cell-like cells showed similar TEER but slightly higher [2-3H]-D-Mannitol-permeability than Caco-2 mono-cultures (2,2+/- 0,29% in Caco2 ^{Raji} versus 1,91+/- 0,11% in Caco-2 cultures, mean +/-SD of 5 independent experiments). Like in Caco-2 monocultures, no LPS was transported across the Raji-cell treated epithelium (data not shown).

6.4.2 Bacterial challenge of Caco-2 ^{Raji}- PBMC cocultures

Caco-2 ^{Raji}-PBMC cocultures were challenged apically with heat- and formaldehyde-inactivated (10^6 CFU/ml) for 40 hours. Live bacteria were omitted because inactivation did not affect cytokines responses of cocultures in previous experiments (Table 2).

All tested heat- and formalin inactivated strains elicited comparable cytokine secretion by Caco-2 ^{Raji}-PBMC and Caco-2-PBMC transwell cultures and no significant differences were observed (Figure 9, Mann-Whitney-U test). As in Caco-2-PBMC cocultures, broad inter-individual variations in cytokine secretion were observed. Baseline production of unstimulated cocultures did not

significantly differ whether or not Raji-cells had been present during Caco-2 culture (Mann-Whitney-U test, data not shown).

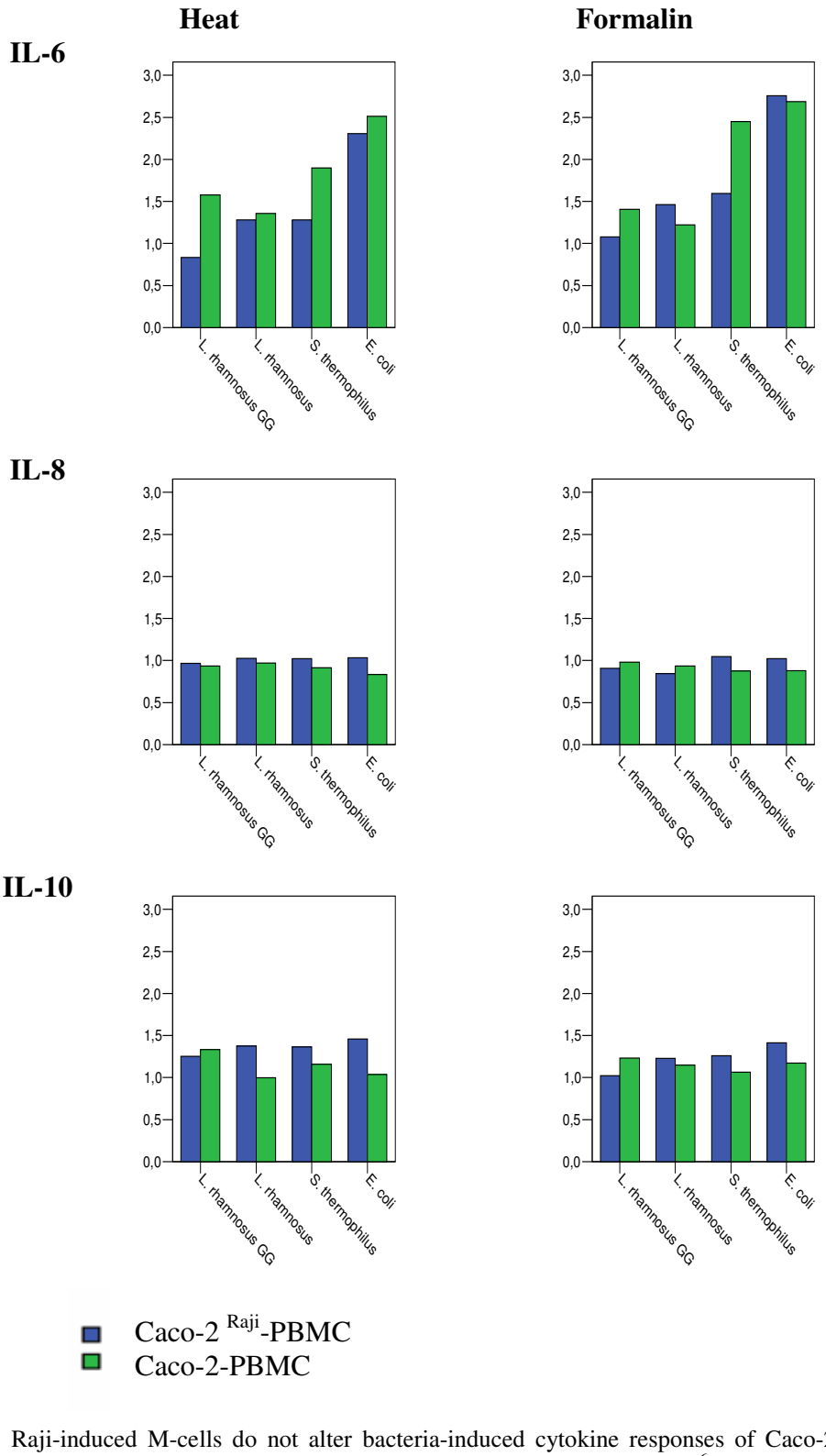


Figure 9. Raji-induced M-cells do not alter bacteria-induced cytokine responses of Caco-2-PBMC transwell cultures. Transformation of Caco-2 into M-cells was induced by addition of $2x10^6$/ml Raji cells to the basolateral compartment of Caco-2 transwell cultures at day 21. At day 28, triplicates of Caco-2 ^{Raji}-PBMC and Caco-2-PBMC cocultures were challenged apically with heat- and formalin-inactivated bacteria for 40 hours. Supernatants from the basolateral compartments were assayed for IL-6, IL-8, and IL-10. Cytokine values upon bacterial challenge are expressed as relative fold increase versus unchallenged Caco-2 ^{Raji}-PBMC (blue bars) or Caco-2/PBMC co-cultures (green bars). Bars represent medians of 5 PBMC-donors.

6.5 Allergen-specific cytokine responses of Caco-2-PBMC cocultures

Clinical and experimental data indicate that distinct probiotic strains, among them the well characterized *L. rhamnosus GG*, are capable of re-balancing the allergic phenotype. Based on *in vitro* experiments on PBMC it has been assumed that these strains act by shifting the Th2-prone cytokine-milieu towards a Th1- or regulatory T-cell response. The final aim of this thesis was to test whether the selected probiotics can counterbalance an allergen-specific Th2-response in the Caco-2-leucocyte coculture model.

To set up the experimental system for measuring allergen-specific responses by Caco-2-PBMC cocultures, PBMC of five patients with a doctor's diagnosed peanut allergy ($PBMC^A$) were challenged with three concentrations of peanut allergen extract (0.5 mg/ml, 0.05 mg/ml, and 0.005 mg/ml). Like upon bacterial challenge, Caco-2 cells remained unresponsive to stimulation in the absence of PBMC and cytokine secretion was polarized towards the basolateral compartment of the co-culture system (data not shown).

When PBMC were challenged in the absence of the Caco-2 monolayer, a clear-cut Th2-response could be detected: peanut allergen extract at 0.05 mg/ml induced significant IL-5 production while 0.5 mg/ml peanut allergen extract significantly suppressed IFN-gamma secretion by $PBMC^A$ (Wilcoxon matched-pairs signed rank test). IL-10 production was not affected by allergen stimulation.

Allergen-specific effects in the Caco-2-$PBMC^A$ coculture system did not reach significance (Figure 10).

Since we were not able to induce significant allergen-specific cytokine production in the Caco-2-PBMC^{A} transwell system, we refrained from direct co-incubation with bacteria and allergen. Our next approach to examine immune modulatory properties of the bacterial strains was to incubate allergen-pulsed PBMC^{A} with conditioned supernatants from bacteria-challenged Caco-2-PBMC^{A} cultures of the same PBMC-donor.

However, conditioned supernatant from every Caco-2 culture +/- PBMC turned out to strongly suppress lymphoproliferation of PBMC^{A} in comparison to non-conditioned medium. The presence of bacteria in Caco-2-PBMC cocultures had no specific effect (Figure 11, inactivated bacteria are depicted). Supernatants did not reduce viability of PBMC as determined by trypan exclusion method (data not shown).

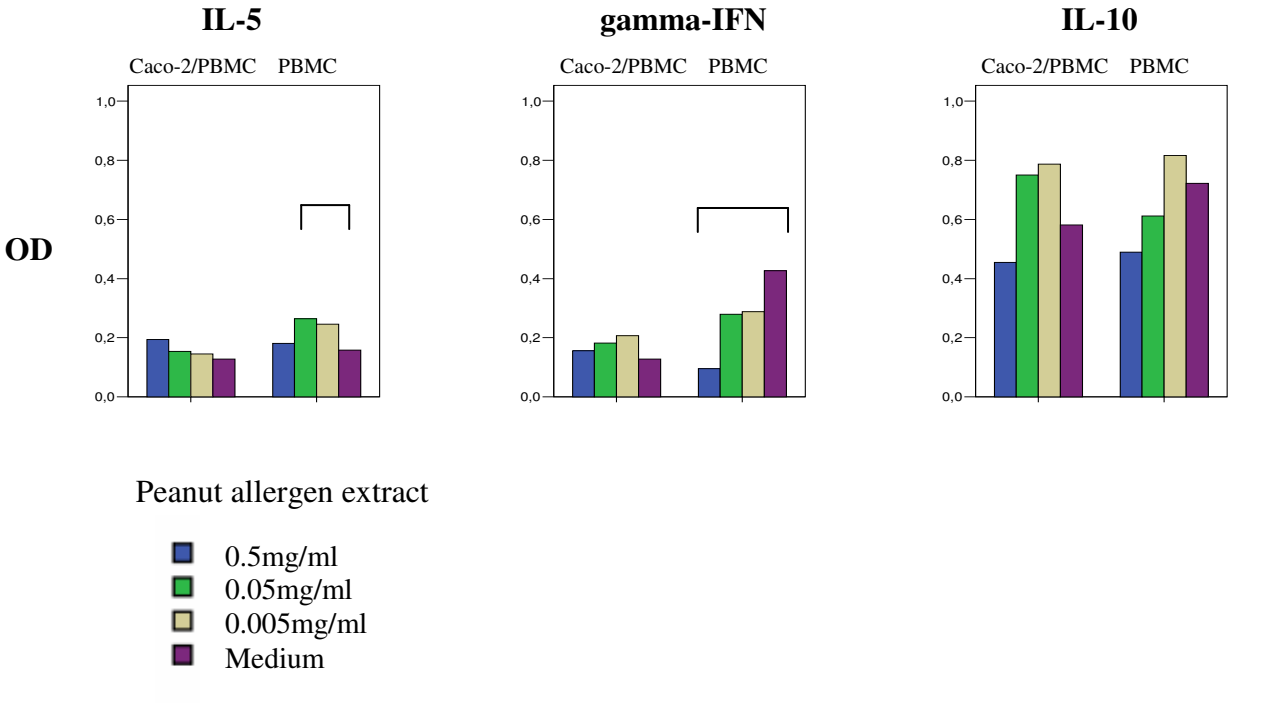


Figure 10. Allergen-specific cytokine panels in Caco-2-PBMC cocultures and PBMC-cultures. PBMC of peanut-allergic individuals were stimulated with three concentrations of peanut allergen extract (0.5mg/ml, blue bars, 0.05mg/ml, green bars, 0.005mg/ml, grey bars) or medium (purple bars) in the presence (left group of bars in each graph) or the absence of Caco-2 (right group of bars in each graph). After 40 hours of coculture, IL-5, gamma-IFN, and IL-10 were detected in supernatants recovered from PBMC cultures and basolateral compartments of Caco-2-PBMC cocultures in ELISA. Bars represent median OD of 5 independent experiments, each performed in triplicates. For IL-10 measurements, supernatants of Caco-2/PBMC co-cultures were diluted by the factor of 50. ⌜⌝ $p<0,05$ (Wilcoxon matched-pairs signed rank test).

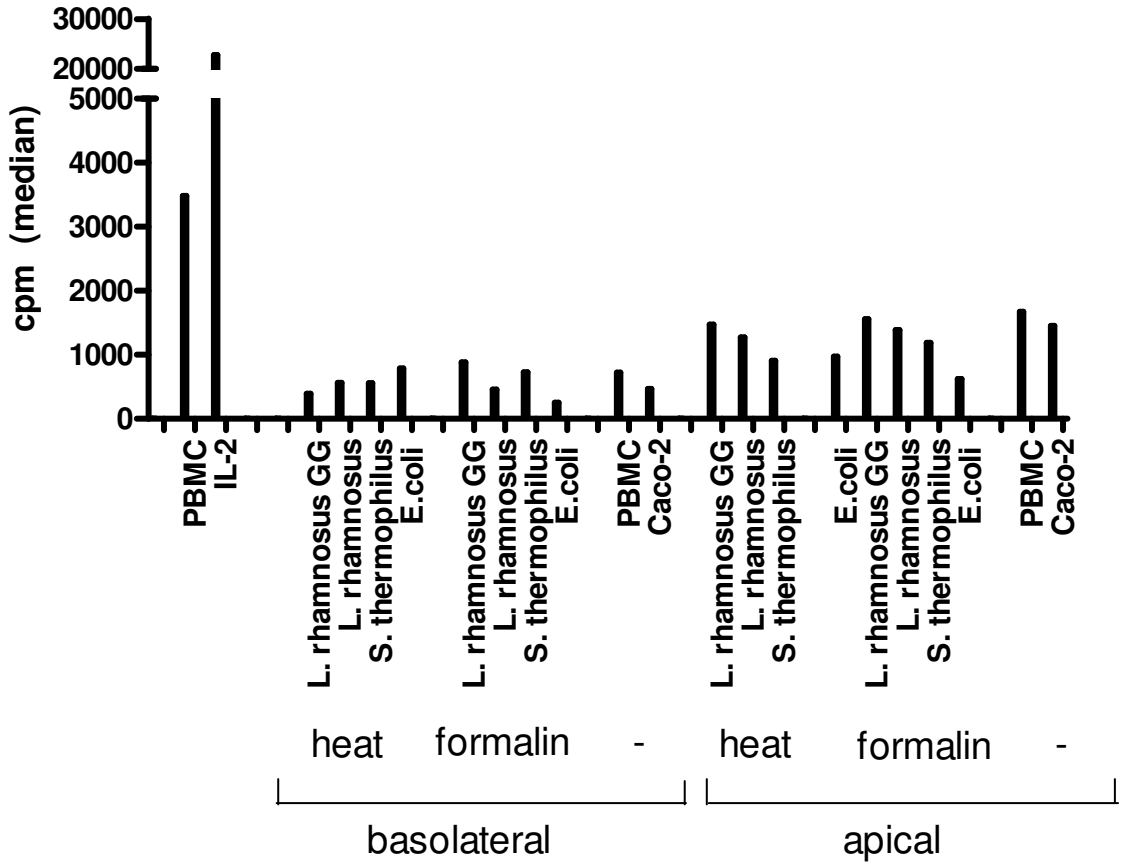


Figure 11. Caco-2 conditioned medium suppresses lymphoproliferation. Supernatants harvested from the basolateral and the apical sides of PBMC-Caco-2 cocultures after 40 hours +/- stimulation with heat- and formalin-killed bacterial strains were incubated with PBMC from the same donor. At day 3, lymphoproliferation was measured by 3H-Thymidine-uptake. PBMC cultured in non-conditioned medium and IL-2 (25IU/ml) served as controls. Results are expressed as median counts per minutes (CPM) of triplicate cultures of 5 individual donors.

7 DISCUSSION

7.1 The Caco-2-PBMC coculture model – characteristics and limitations of the experimental system

The Caco-2-PBMC coculture model has previously been used to mimic the complex interactions between the intestinal epithelium and immunocompetent cells, and IEC/leucocyte cross-talk upon challenge with apathogenic bacteria became evident with this model (*45*, *109*).

Following the concept of cellular cross-talk, the Caco-2-PBMC transwell system was chosen to analyze *in vitro* immune-modulatory features of *L. rhamnosus GG*, *L. rhamnosus*, *S. thermophilus*, and *E. coli*.

Caco-2 transwell cultures remained hypo-responsive to bacterial and allergen stimulation in the absence of the PBMC, confirming previous observations and supporting the concept of IEC/leucocyte interactions as a prerequisite in responses to antigenic stimuli (*45*, *109*). Like in previously published studies, a baseline IL-6, IL-8, and IL-10 production was detected in all cultures as soon as PBMC were present. This background cytokine production might on one hand be a characteristic feature of this intestinal epithelium *in vitro* model, reflecting the delicate interplay in the mucosal microenvironment to maintain gut homeostasis. On the other hand, it limits the investigation of cytokine pattern induced by probiotic strains. In particular, bacteria-induced IL-10 secretion was almost completely masked by background IL-10 synthesis, but IL-10 production is a central feature of probiotic strains (*21*, *27*, *47*, *95*, *101*). High IL-10 secretion by Caco-2-PBMC cocultures could however mirror the *in vivo* situation as IL-10 is thought to hold a key role in maintaining a tolerogenic milieu at the gut mucosal

surface (*12*, *47*, *75*). High IL-10 levels are contrariwise likely to have abrogated other cytokine levels in our experimental system.

In all Caco-2-PBMC experiments, cytokine levels varied greatly between individual PBMC donors. Consequently, calculating individual ratios of bacteria induced cytokine responses and Caco-2-PBMCcontrols seemed the only appropriate way to overcome this problem, although inter-individual differences remained wide. Using ratios might have also weakened statistical analysis, as cytokine induction compared to unstimulated co-cultures was often low, and to some extent limited comparison with literature.

The Caco-2-PBMC transwell system however is a simplified model that allows studying interactions of IEC, bacterial cells, and PBMC exclusively, but has its limitations. It does for instance not produce surface mucus, which represents an important barrier that prevents direct adherence of bacteria to the intestinal epithelium (*90*). *In vivo*, microbiota might not even directly adhere to IEC, raising the question by Corthésy et al. of whether IEC/leucocyte crosstalk can physiologically occur in the gut (*24*).

The transwell system also lacks DC, professional antigen-presenting cells that play a central role in polarizing T-cells towards the Th1, Th2, and T-regulatory phenotype. DCs reach through the epithelial cell layer with periscope-like protrusions to to sample non-invasive bacteria through the intestinal epithelium (*102*, *121*, *122*). Intestinal DCs are also involved in processing bacteria that are internalized via M-cells. It has been hypothesized that probiotics drive intestinal DC to induce and activate T-reg resulting in a constant suppressive environment (*20*, *125*).

Another contributor to gut homeostasis that is missing in the Caco-2-PBMC model is secretory IgA, the predominant immunoglobulin in defending invading pathogens at mucosal surfaces. Intestinal microbiotia are a constant stimulus of IgA production in the gut epithelium, consequently enforcing exclusion and elimination of pathogens (*58*, *67*, *84*).

The Caco-2-PBMC transwell model thus represents a reductionist model of IEC and leucocytes, but at present, there is no experimental model available considering all constituents of the complex micro-environment at the intestinal surface.

7.2 The Caco-2 barrier modulates cytokine responses upon stimulation with probiotic bacteria in Caco-2-PBMC cocultures

To further investigate the effects of IEC-leucocyte cross-talk on cytokine release induced by probiotic bacteria, we directly compared cytokine responses of Caco-2-PBMC- and direct PBMC-cultures stimulated with probiotic *L. rhamnosus GG*, *L. rhamnosus*, *S. thermophilus*, and non-fermenting *E. coli*.

In addition to previously described immune-modulating effects on PBMC, we show here that probiotic (*L. rhamnosus GG*, *L. rhamnosus*, *S. thermophilus*) and commensal bacteria (*E. coli*) induced distinct cytokine secretion by Caco-2-PBMC cocultures, suggesting strain-specific immunomodulatory capacities in the IEC/leucyte coculture system.

Screening basolateral supernatants of Caco-2-PBMC cocultures revealed that IL-6, IL-8, and IL-10 where the prevailing cytokines. These results are in accordance with a very similar experimental set-up by Parlesak et al. Upon stimulation of PBMC with nonpathogenic *E. coli* K12 for up to 62 hours across the Caco-2 monolayer, significant induction of IL-1-beta, IL-6, IL-8, IL-10, IFN-gamma, and TNF-alpha was observed. TNF-alpha peaked already after eight hours, which could explain why it was negative after 40 hours in our experiments, and IFN-gamma levels were generally very low. Consistent with the cited study, at a similar time point (32 hours), the cytokine being strongest induced over background by apical stimulation with all non-pathogenic strains was IL-6 (*109*).

The finding that IL-6 and IL-10 are the predominant cytokines in Caco-2-PBMC cocultures is of significance because either is required for IgA switch and -production by B-cells as well as maintaining tolerance. Both mechanisms are believed to orchestrate in securing gut homeostasis and protection from infectious agents and are assumed key in the immune-modulating action of probiotics and commensal microbiota (reviewed in (*24*)).

Although considering the numerous limitations of available *in vitro* systems, there is accumulating evidence that IEC play a crucial part in local immune responses. Here we show suppressive effects of the Caco-2 barrier on the production of the tested cytokines (IL-6, IL-8, IL-10) in comparison to direct PBMC culture and on lymphoproliferation.

IL-10 induction was significantly reduced when bacteria and PBMC were separated by confluent Caco-2. IL-6 and IL-8 induction was also attenuated in the presence of the Caco-2 monolayer, although differences did not reach significance

(Mann-Whitney-U test). However, absolute IL-6 levels in Caco-2-PBMC cocultures were reduced approximately by the factor 10, IL-8 secretion by the factor 40 compared to cultures were PBMC had direct contact o bacteria (absolute values not shown). Identically, Parlesak et al. reported a significant suppression of cytokine release when PBMC were challenged with nonpathogenic *E. coli* through the barrier of Caco-2 cells compared to direct PBMC-stimulation (IL-6, IL-8, IL-10, IL-1-beta, IL-12, TNF-alpha, IFN-gamma, and to a lesser extent TGF-beta).

Conditioned medium from bacteria-challenged Caco-2-PBMC cocultures also inhibitied lymphoproliferation. This effect has previously been described in the literature. Supernatants from IEC and Caco-2 cultures have previously been shown to attenuate T-cell (*19*, *117*, *118*), monocyte (*47*), and dendritic cell activation (*14*) through both soluble immune modulating factors and cell-cell interactions, conforming them to a hyporesponsive state similar to lamina propria T-cells (*117*, *118*).

However, from our experimental set-up, we cannot suggest the factors responsible for suppression of cytokine secretion and lymphoproliferation. We assent to the hypothesis introduced by Christ et al. that IEC secret soluble factor(s) that lead to a hyporesponsive milieu in *in vitro* cultures (*19*), because lymphoproliferation was reduced by conditioned medium recovered from Caco-2-PBMC cocultures. On the other hand, differential recognition of non-pathogenic bacteria and subsequent cytokine release actually required cell-cell contact of Caco-2 and PBMC. Only negligible cytokine secretion was detected in the absence of PBMC, and bacteria elicited considerably weaker cytokine responses when Caco-2 were grown on the upper side of filter inserts and PBMC were added to the lower compartment of the system hindering direct contact with Caco-2 (data not shown).

Probiotics have been studied for the prevention and treatment of a wide range of diseases in clinical trials, and there is strong evidence for their clinical efficacy. Immune-modulating properties of probiotic preparations are often characterized *in vitro*. It is tempting to select strains for therapeutic applications according to the cytokine pattern they induce in PBMC *in vitro*. We provide further evidence that in addition to effects on PBMC, probiotic (*L. rhamnosus GG*, *L. rhamnosus*, *S. thermophilus*), and commensal bacteria (*E. coli*) have immunomodulating capacities in IEC/leucocyte cultures. Yet our data also suggests that immunologic effects of non-pathogenic bacteria observed in *in vitro* PBMC-cultures should be extrapolated with caution. IEC-leucocyte-interactions and the consequential immuno-suppressive microenvironment might also be relevant *in vivo*. In addition, there are many other factors contributing to local immune reactions in the gut mucosa that cannot be simulated in *in vitro* models yet.

To characterize immune-modulating features of probiotics sufficiently, *in vitro* parameters should always be confirmed *in vivo*, e.g. in animal models or human interventional studies.

7.3 Heat- and Formalin-inactivation does not affect bacteria-induced cytokine responses in Caco-2-PBMC cocultures

In general, live specific probiotics are recommended for clinical use (*32*), but inactivated microorganisms may also be effective in certain conditions (*50*). However, data on *in vitro* immunologic effects of viable and non-viable probiotic bacteria is conflicting. Heat- and formaldehyde-treated strains have been shown to retain their immune modulating capacity in direct PBMC-cultures (*86*, *95*, *112*) as well as in IEC-models (*159*, *170*). On the other hand, Haller et al. found that heat-

killed *Lactobacilli* failed to induce TNF-alpha mRNA in Caco-2/leucocyte co-cultures (*46*). Heat- and gamma-irradiation was reported to attenuate the adhesive abilities of some probiotic strains, while increasing them in others (*105*). Epithelial adhesion and persistence in the gastro-intestinal tract is a commonly presumed prerequisite for probiotics, which has however been doubted lately (*24*).

Recently, Wong et al. demonstrated for the first time that challenge of Caco-2 cultures with heat-killed probiotic bacteria stimulated IL-6 and IL-8-production, whereas irradiated strains attenuated cytokine responses, indicating that the mode of inactivation itself is crucial as well and may explain opposing reports in literature (*164*).

To address the question of whether the mode of inactivation affects cytokine production induced by probiotic bacteria in Caco-2-PBMCtranswell cultures, we compared IL-6, IL-8, and IL-10 secretion as markers for immune-modulation by live, heat-, and formaldehyde-treated strains (*L. rhamnosus GG*, *L. rhamnosus*, *S. thermophilus*, and *E. coli*).

In our study, bacterial challenge enhanced IL-6, IL-8, and IL-10 production in the majority of experiments. Hence, in contrast to Wong et. al., we did not observe any consistent suppressive effects on Caco-2-PBMCcytokine responses subject to mode of inactivation of bacterial strains. Our findings are consistent with the cited study as far as heat-inactivated bacteria increased IL-6 and IL-8 synthesis too. However, heat- and formalin-treatment are related inactivation methods, which could explain the uniform immunologic response. Both lead to denaturation of proteins, whereas irradiation destructs the bacterial DNA without affecting protein components of the microorganism (*78*)**.**

Although it seemed that heat- and formalin-inactivated strains were less effective in inducing cytokine responses than viable mircroorganisms, differences were not

statistically significant when comparing IL-6, IL-8, and IL-10 induction by live, heat-, and formalin-inactivated bacteria directly (Mann-Whitney-U test). Similar findings of reduced but existent effects of inactivated probiotics on cytokine production have been reported in literature. Zhang et al. observed that heat-inactivated *L. rhamnosus GG* is less effective in inducing IL-8 in Caco-2 cells than live *L. rhamnosus GG* (*170*). Miettinen et al. demonstrated that glutaraledhyde-fixed lactic acid bacteria were slightly weaker stimulators of TNF-alpha, IL-6, and IL-10 in PBMC (*95*).

In summary, our results indicate that live, heat- and formalin-inactivated *L. rhamnosus GG*, *L. rhamnosus*, *S. thermophilus*, and *E. coli* induce strain-specific production of IL-6, IL-8, and IL-10 in Caco-2-PBMC cocultures. Consistent with literature, live bacteria appeared to be slightly more potent inducers of cytokine production than inactivated strains, but in direct comparison, differences were not statistically significant. No opposing trends in cytokine induction were observed with regard to inactivation method. We conclude that heat- and formalin-treatment of probiotic microorganisms are equivalent inactivation methods in terms of induction of IL-6, IL-8, and IL-10 production in Caco-2-PBMC cocultures. Nevertheless, since there are data suggesting that certain inactivation procedures invert immune-modulating effects of probiotic bacteria, it should be mandatory to give detailed information not only on the viability but also on the mode of inactivation when characterizing immunologic effects of probiotics *in vitro*.

7.4 Raji-induced M-cells in Caco-2 monolayers do not affect bacteria-induced cytokine production of Caco-2-PBMC cocultures

Beside IEC-leucocyte cross-talk, non-invasive bacteria can elicit local immune responses in the intestinal epithelium by crossing the intestinal barrier via M-cells, specialized antigen sampling cells in Peyer's Patches, and uptake by subepithelial dendritic cells (*79*, *136*).

In vitro, Caco-2 cells can be converted into cells expressing M-cell features by adding Raji cells, a human B-cell lymphoma cell line, to Caco-2 transwell cultures (*43*, *63*). With the Caco-2^{Raji} model, M-cell mediated transport of particles and microorganisms has been studied *in vitro* {Gullber, Leonoard, et al. 2000 243 /id(*25*, *43*, *88*, *128*, *132*).

We used the Caco-2^{Raji} –PBMC *in vitro* coculture model to investigate the contribution of M-cells to the concept of IEC-leucyte cross-talk, separate from their role as an antigen entry site in the intestinal epithelium. We hypothesized that the special characteristics of the M-cell surface known to promote adhesion of bacteria IEC (*74*) affected bacteria-IEC-leucocyte interaction and cytokine responses.

The use of filter insert membranes with a pore size of 0.4 µm according to the manufacturer prohibits penetration of whole microorganisms, therefore allowing to study interactions of the whole culture system (bacteria-Caco-2^{Raji} –PBMC) .

The presence of Raji-induced M-cell-like cells in Caco-2-PBMC cocultures did not significantly alter IL-6, IL-8, and IL-10 responses to the tested non-pathogenic

bacteria, suggesting that in this *in vitro* system, M-cell-like cells do not differ from enterocytes in probiotic bacteria-IEC-leucocyte responses.

This finding on one hand contributes to the general assumption that M-cells do not take part in defense mechanisms to antigens on the intestinal epithelial surface but primarily function as an antigen entry site for macromolecules and microorganisms (reviewed by (*74*)).

On the other hand, we cannot exclude that Caco-2 cells did not fully differentiate into M-cell-like cells. Raji-treated Caco-2 cultures showed a decrease in the activity of AP by 23-65% compared with Caco-2 monocultures. This is concordant with the reported down-regulation of enzyme activity in the *in vitro* M-cell model. We did not look for other M-cell markers like up-regulation of Sialyl Lewis A antigen, intercellular adhesion molecule-1 or vascular cell adhesion molecule (*43*). High background cytokine production of Caco-2-PBMC cocultures and great inter-individual variations in cytokine production might also have masked subtle effects.

Our data delineates that in a Caco-2^{Raji} *in vitro* model in which bacterial translocation is prevented by small pore size of membranes M-cell-like cells do not alter probiotic bacteria-Caco-2-PBMC interactions, and do not impact cytokine responses. These results support the assumption that the cardinal accomplishment of M-cells in responses to non-invasive bacteria seems to be acting as an antigen entry site, and directing microorganisms towards dendritic cells of the subepithelial dome region.

7.5 The Caco-2-PBMC coculture system is not applicable for measuring allergen-induced cytokine responses

The prevention and treatment of atopic diseases is a main field of interest for the application of probiotics (reviewed in (*106*)). According to the hygiene hypothesis, an explanatory model for the rise in allergic diseases in recent years, the lacking microbial stimuli of the immune system in industrialized countries lead to a Th2-skewed milieu favoring the development of type 1 allergies. Probiotic preparations are a promising therapeutic option, because modulation of the atopy-associated Th2 cytokine pattern is one of their attributive benefits. Lactobacilli and Bifidobacteria species have been demonstrated to re-balance PBMC Th2 cytokine profiles *in vitro* (*21*, *94*, *95*, *101*, *112*, *139*).

We aimed to investigate the capacity of *L. rhamnosus GG*, *L. rhamnosus*, *S. thermophilus*, and the control strain *E. coli* to revert an established allergen-specific cytokine response towards peanut allergen extract in the Caco-2-PBMC coculture model using PBMC of peanut allergic donors ($PBMC^A$).

In this set of experiments, cytokines were measured in ELISA, which had a higher sensitivity than the cytometric bead array, to detect subtle changes upon allergen stimulation. However, in contrast to allergen-challenge of $PBMC^A$ in the absence of Caco-2 monolayer, where peanut allergen extract significantly enhanced IL-5- and decreased IFN-gamma production (Wilcoxon matched-pairs signed rank test), allergen-specific cytokine induction by Caco-2-PBMC co-cultures failed to reach significance. As the barrier of Caco-2 cells diminished bacteria-induced cytokine responses in our experiments as well as in the literature, most likely, weaker effects in response to allergen-stimulation were completely abolished. Longer

incubation times might have boosted cytokine values, but at the same time increased leakages in the Caco-2 monolayer (*109*), and could therefore not be applied.

We also had to forbear from the approach to incubate allergen-pulsed $PBMC^A$ with conditioned supernatants from bacteria-challenged Caco-2-$PBMC^A$ cultures, as addition of conditioned supernatant from bacteria-challenged Caco-2-$PBMC^A$ cultures to PBMC of the same donor strongly suppressed lymphoproliferation, as discussed earlier. Suppressive effects could not be overcome by the polyclonal stimulants PHA either (data no shown).

We conclude that the Caco-2-PBMC coculture model as used in this study is not applicable for measuring peanut allergen-induced cytokine profiles. Hence immune modulatory properties of the probiotic strains on allergen-specific cytokine responses could not be tested in this experimental set-up.

7.6 Summary and Conclusion

Probiotics have been studied for the prevention and treatment of a wide range of diseases in clinical trials, and there is growing evidence for their clinical efficacy but not without controversy. Modulation of the host's immune system is one of the most commonly attributive effects of probiotic preparations, mainly characterized in PBMC *in vitro* cultures. Recently, IEC have been shown to participate in immune responses towards non-pathogenic bacteria. Considering IEC-leucocyte cross-talk, we here investigated immune-modulatory features of live, heat- and formalin-inactivated probiotic *L. rhamnosus GG*, *L. rhamnosus*, *S. thermophilus*, and non-fermenting, non-pathogenic *E. coli* in the Caco-2-PBMC transwell model.

We report that in addition to effects on PBMC reported in the literature, the tested bacterial strains have immunomodulating attributes in IEC-leucocyte cocultures, reflected by strain-specific patterns of IL-6, IL-8, and L-10. The Caco-2 barrier however had suppressive effects on cytokine production and lymphoproliferation. Investigating the impact of inactivation of microorganisms on cytokine responses, we found that live bacteria were slightly more potent inducers of cytokine production than non-viable strains, but heat- and formalin-treatment can be considered equivalent inactivation methods revealing comparable cytokine production. Introduction of M-cells into a Caco-2-PBMC model impermeable for microorganisms did not affect cytokine production, strengthening the general assumption that M-cells strictly act as antigen-sampling system in the intestinal epithelium. Caco-2 transwell cultures remained hypo-responsive to bacterial or allergen stimulation in the absence of PBMC, supporting the concept of IEC-leucocyte cross-talk in cytokine responses to antigenic stimuli in the gut epithelium.

The Caco-2-PBMC coculture system is a reductionist model of intestinal epithelial cells with numerous limitations, such as high background cytokine production of Caco-2-PBMC cocultures and great variations between PBMC-donors. The experimental system was not applicable to analyse modulatory features of probiotic strains on allergen-specific cytokine responses, because presumably subtle changes induced by allergens in PBMC cultures were masked in the presence of Caco-2 cells.

We conclude that the Caco-2-PBMC model is valuable for characterizing immunologic features of probiotic strains *in vitro*, but with limitations. Results must always be extrapolated deliberately. To accurately characterize immune-modulating properties of probiotics, studies directly comparing *in vitro* parameters

with clinical effects and *in vivo* immune responses towards probiotic strains are required.

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9 LIST of PUBLICATIONS

- Dehlink, E, Domig, KJ, Loibichler, C, Kampl, E, Eiwegger, T, Georgopoulos, A, Kneifel, W, Urbanek, R, Szépfalusi, Z. ***Heat- and formalin-inactivated probiotic bacteria induce comparable cytokine patterns in intestinal epithelial cell/leucocyte co-cultures.*** Accepted for publication, J Food Prot.
- Gruber, S, Dehlink, E, Eiwegger, T, Gut, S, Jaksch, P, Klepetko, W, Rumpold, H, Szépfalusi, Z. ***Immunoglobulin E-mediated allergies in lung-transplanted adults.*** Transplantation. 2007 Jul;84(2):275-9.
- Eiwegger, T, Rigby, N, Mondulet, L, Bernard, H, Krauth, MT, Boehm A, Dehlink, E, Valent, P, Wal, JM, Mills, EM, Szépfalusi, Z. ***Gastro-duodenal digestion products of the major peanut allergen Ara h 1 retain an allergenic potential***. Clin Exp Allergy. 2006 Oct;36(10):1281-8.
- Eiwegger, T, Dehlink, E, Schwindt, J, Pomberger, G, Reider, N, Frigo, E, Rokitansky, AM, Urbanek, R, Szépfalusi, Z. ***Early exposure to latex products mediates latex sensitization in spina bifida but not in other diseases with comparable latex exposure rates***. Clin Exp Allergy. 2006 Oct;36(10):1242-6.
- Szépfalusi, Z, Loibichler, C, Hanel-Dekan, S, Dehlink, E, Gerstmayr, M, Pichler, J, Eiwegger T, Horvart, R, Urbanek, R. ***Most of diaplacentally transferred allergen is retained in the placenta***. Clin Exp Allergy. 2006 Sep;36(9):1130-7.
- Dehlink, E, Gruber, S, Eiwegger, T, Gruber, D, Müller, T, Huber, WD, Klepetko, W, Rumpold, H, Urbanek, R, Szépfalusi, Z. ***Immunosuppressive therapy does not prevent the occurrence of IgE-mediated allergies in children and adolescents with organ transplants.*** Pediatrics. 2006 Sep;118(3):e764-70.
- Fritsch, MJ & Uxa, S, Horak, F, Putschoegl, B, Dehlink, E, Szépfalusi, Z, Frischer, T. ***Exhaled nitric oxide in the management of childhood asthma. A prospective 6-months study***. Pediatr Pulmonol. 2006 Sep;41(9):855-62.
- Kirchlechner, V, Dehlink, E, Szépfalusi, Z. **Cow's *Milk Allergy: Guidelines for the Diagnostic Evaluation.*** Klin Padiatr. 2006 Mar 15;
- Dehlink, E, Eiwegger, T, Gerstmayr, M, Kampl, E, Bohle, B, Chen, KW, Vrtala, S, Urbanek, R, Szépfalusi, Z. ***Absence of systemic immunologic changes during dose build-up phase and early maintenance period in effective specific sublingual immunotherapy in children.*** Clin Exp Allergy. 2006 Jan;36(1):32-9
- Eiwegger, T, Stahl, B, Schmitt, J, Boehm, G, Gerstmayr, M, Pichler, J, Dehlink, E, Urbanek, R, Szépfalusi, Z. ***Human milk--derived oligosaccharides and plant-derived oligosaccharides stimulate cytokine production of cord blood T-cells in vitro.*** Pediatr Res. 2004 Oct;56(4):536-40
- Dehlink, E, Prandstetter, C, Eiwegger, T, Putschoegl, B, Urbanek, R; Szépfalusi, Z. ***Increased prevalence of latex-sensitization among children with chronic renal failure.*** Allergy. 2004 Jul;59(7):734-8.

October 19th, 2007

10 CURRICULUM VITAE

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Date of Birth: June 25th , 1978
Place of Birth: Mödling, Austria
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EDUCATION

10/03-	Study of Medical Sciences/Doctor of Philosophy, PhD (Immunology) Doctoral thesis: *"***Immune-modulatory effects of lactic acid bacteria on human peripheral blood mononuclear cells through the barrier of intestinal epithelial cells (CaCo-2) in a trans-well system***",* Supervisors: Zsolt Szépfalusi, MD, Dept. of Pediatrics, Medical University of Vienna, Barbara Bohle, PhD, and Erika Jensen-Jarolim, MD, Centre of Physiology and Pathophysiology, Dept. of Pathophysiology, Medical University of Vienna
04/02	Graduation, MD
02/01-04/02	Doctoral thesis: *„***Immunological changes during sublingual immunotherapy in children***"* Supervisor: Zsolt Szépfalusi, MD, Dept. of Pediatrics, Medical University of Vienna
10/96-04/02	Medical School, University of Vienna
09/88-06/96	Secondary School Berndorf, Austria
06/96	General qualification for university entrance
09/84-06/88	Primary school Gainfarn, Austria

CURRENT POSITIONS

01/07-	Research Fellow, Children's Hospital Boston, Harvard Digestive Diseases Centre, GI Cell Biology Laboratory, Supervisor: Edda Fiebiger, PhD
10/02-	Resident in Pediatrics and Research Fellow, Dept. of Pediatrics, Medical University of Vienna

AUXILIARY ACTIVITIES

05-06/02	Clinical Clerkship/Elective, Dept. of General Medicine, Dept. of Cardiology, Monash University Clayton, Melbourne, Australia
99-00	Tutor and Research Fellow, Institute of Anatomy III, University of Vienna

DIPLOMAS

10/03	Asthma-behavior-trainer for pediatric and adolescent asthmatics according to the Guidelines of the German Consortium "Asthma-training in childhood and adolescence" (Asthma-academies Berchtesgaden/Salzburg, Austria and Munich/Gaissach, Germany)

SCHOLARSHIPS and AWARDS

01/07-	APART – Austrian Program of Advanced Research and Technology – Research Fellowship of the Austrian Academy of Sciences
09/06	Short Term Research Fellowship of the World Allergy Organization (WAO) at Children's Hospital Boston, Harvard Digestive Diseases Centre
06/06	Travel grant of the European Academy of Allergology and Clinical Immunology (EAACI)
10/05	Selection of the manuscript "*Increased prevalence of latex-sensitization among children with chronic renal failure*", Allergy 2004 59:734-738 for the book „Best Articles relevant to Pediatric Allergy and Immunology" by members of the Section on Allergology and Immunology of the American Academy of Pediatrics, Pediatrics 2005, 116, Supplements
06/05	Publication Prize 2004 of the Dept. of Pediatrics, Medical University of Vienna for the manuscript "*Increased prevalence of latex-sensitization among children with chronic renal failure*", Allergy 2004 59:734-738
06/05	Travel grant of the European Academy of Allergology and Clinical Immunology (EAACI)
03/03	Theodor-Körner-Grant for Promotion of Arts and Science, Austria
01/02	Excellence scholarship of the University of Vienna
01/00	Excellence scholarship of the University of Vienna

MEMBERSHIPS

2005-2006	Founding member and Board member of the Young Scientists' Association of the Medical University of Vienna (YSA)
2004-	Austrian Society of Pediatrics
2002-	Austrian Society of Allergy and Immunology (ÖGAI)
2002-	Austrian Society of Flowcytometry (OEGfZ)
2002-	Juniormember of the European Academy of Allergology and Clinical Immunology (EAACI)

Vienna, October 19th, 2007