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DIPLOMARBEIT

THE IMPACT OF PROBIOTIC BACTERICA ON GENE EXPRESSION OF INTERLEUKIN-8, INTERLEUKIN-17 AND CASP3 IN CACO-2 CELLS

Einfluss von probiotischen Bakterien auf die Genexpression von Interleukin-8, Interleukin-17 und CASP3 in Caco-2 Zellen

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List of Abbreveations

GI-tract	Gastrointestinal tract
IECs	Intestinal epithelial cells
LP	Lamina propria
FAE	Follicle-associated epithelium
M- cell	Micro fold- cell
APCs	Antigen-presenting cells
PP	Peyer's patches
DCs	Dendritic cells
NK- cell	Nature killer cell
GALT	Gut- associated lymphoid tissue
MALT	Mucosa-associated lymphoid tissue
MLN	Mesenteric lymph nodes
IEL	Intraepithelial lymphocytes
T_H	Helper T cell
T_R	Regulatory T cell
Ig	Immunglobulin
sIgA	Secretory IgA
IL	Interleukin
NFκ-B	Nuclear factor kappa B
TNF-α	Tumor necrosis factor alpha
IFN-γ	Gamma Interferon
TGF-β	Transforming growth factor
PRR	Pattern recognition receptor
TLR	Toll-like receptor
NOD	Nucleotide-binding oligomerization domain
NLR	NODs like receptor
PPAR	Peroxisome proliferators-activated receptor
PAMP	Pathogen-associated molecular pattern
UTR	Untranslated region

1. SCIENTIFIC BACKGROUND

1.1 Microbiota and probiotic bacteria

1.1.1 Microbiota

The human gut is colonized with about 10^{14} microorganism including bacteria, yeast and archea. The bacterial density varied within the gastrointestinal tract (GI tract). There is an increased augmentation of bacteria from the stomach to the distal colon. So the number of microbial cells is 10 time higher than the total number of all cells of the human body. About 500 to 1000 species inhabit the gastrointestinal tract. This microbes form a unique complex and dynamic ecosystem, also term as mircobiota (Artis 2008; Manson *et al.* 2008). Bacteria can have diverse effects to the host. Harmful bacteria such as *Clostridium* ssp., *Veillonella* ssp., *Proteus* ssp. and the family of *Enterobacteriaceae* induce pathogenicity or transform food components into harmful substances like ammonia, amines or hydrogen sulfide. Bacteria of the intermediate group including *Bacteroides* ssp., *Eubacterium* ssp. and anaerobic *Streptococci* ssp. have no pathogenic effects under normal condition. *Bifidobacterium* ssp. and *Lactobacillus* ssp. are beneficial bacteria suppressing harmful bacteria and have beneficial physiological effects to the host. The normal gut composition is termed microbiota (Gill and Prasad 2008).

1.1.1.1 Development of microbiota

The gut of a newborn is sterile and becomes colonized with different microorganism after birth. It needs 7 to 10 days until a baby develops a heterogeneous bacterial flora. Newborns feeding full-term with breast milk have a limited bacterial composition which is predominated of *Bifidobacteria* and also includes *Lactobacilli*, *Escherichia coli* and *Enterococci*. The microbiota of formula-fed babies are more complexer and includes species of *Bacteroides*, *Clostridia*, *Bifidobacteria*, *Lactobacilli*, gram-positive cocci and coliforme bacteria (Givens Bell 2007; Manson *et al.* 2008). During the first year of life the microbiota is characterized through a low diversity and high unstable bacterial composition. At the age of two years there are no more difference between the bacterial composition of breast-fed or formula-fed children (Bik 2009). The initial colonization is finished around three years of age. During this phase the human body

becomes imprinted to these microorganisms and tolerates them. This microbiological imprinting influence the immunological imprinting and thus the immune response (Artis 2008). The diversity and stabilization increases with age. But there a high differences between each segment of the GI tract (Bik 2009). The density of bacterial colonization increases from the stomach with $10^3 - 10^4$ cfu/g to the colon with 10^{10} to 10^{11} cfu/g. Also the composition alters within the GI tract (Gill and Prasad 2008). Following factors affect the establishment of bacteria within the GI tract: the rapid turnover of intestinal epithelium and their overlaying mucosa as well as mucin expression and the exposure to peristaltic activity, available nutrients, kind of transient bacteria, pH and secretion of gastric, pancreatic and bile acid. The composition of the adult microbiota is also influenced by kind of diet and the genetically background. The 500 to 1000 species in the gut are members of just 11 bacterial divisions. Eight of them are represented rare within the gut. About 93% of bacterial species are members of the division Cytophaga-Flavobacterium-Bacteroides, Firmicutes and Cytaphaga-Flavobacter-Bacteroides. The small intestine contains the genera *Bacteroides*, *Clostridia*, *Streptococci*, *Enterococci* and *γ-Proteobacteria*. The bacterial composition of colon includes species of *Bacteroides*, *Clostridia*, *Prevotella*, *Poryphyromonas*, *Eubacteria*, *Ruminococci*, *Streptococci*, *Bifidobacteria*, *Enterobacteria*, *Lactobacilli*, *Peptrostreptococcus* and *Fusobacteria (Manson et al. 2008)*.

1.1.1.2 Function of microbiota

The microbiota is a stable and well organized ecosystem playing an essential role for the human health. Some bacteria are capable to digest and synthesize nutrients which the human organism cannot digest or synthesize itself. For instance, *Firmicutes* sp. ferments human indigestible polysaccharide to short chain fatty acids such as butyrate using the host organism as energy source. The synthesis of vitamin K and vitamin B12 through *Bacteroides* and *Eubacterium* are essential for the human (Bik 2009). Furthermore, the commensal bacteria play a crucial role in the development of epithelium and regulation of immune response because they are able to avoid the colonization of potential pathogens through exclusion by competition for the same nutrients and attachment site. Microbiota also promotes angiogenesis within the epithelial cells and secretes anti-microbial peptide itself. As example for the immunomodulation function, germ-free mice's shown hypoplastic Peyer's patches, an

increased level of plasma cells, abnormal spleens and lymph nodes as well as underdeveloped intestinal villus capillaries. All this deficiencies can restore by colonization of one single Bacteroides species (Artis 2008; Bik 2009). Moreover, commensal bacteria are involved in the differentiation, recruitment and maintenance of mucosal-associated invariant T cells. The development of chronic inflammation is associated with changes in bacterial composition (Artis 2008).

1.1.2 Probiotic bacteria

FAO and WHO define probiotic bacteria as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO and WHO 2001).

The declared health benefits of probiotic bacteria include:

- Improvement of intestinal microbial balance
- Stabilization of gut mucosal barrier
- Characteristics of an immune adjuvant
- Prevention of infection and food allergies including abatement of intestinal bowel diseases symptoms and enhanced digestion of lactose in intolerant hosts
- Beneficial properties against respiratory tract infections
- Reduce the duration and frequency of viral diarrhea
- Reduction of serum cholesterol
- Anti-cancerogenic activity (Galdeano *et al.* 2007; Gill and Prasad 2008)

Conventional strains of lactic acid bacteria such as *Lactobacilli*, *Bifidobacteria*, *Streptococci* and *Lactococci* are used as probiotic bacteria. These bacteria are usual components of fermented milk products, cheese, fruit juice and sausages and used as single or mixed cultures of lived organisms for food preparation. To become a classification as a probiotic bacterium, the strain should have following properties:

- High cell viability even under conditions of low pH
- Be resistance against proteolytic enzymes, antimicrobial peptide, sIgA and bile acid

- Ability to persist in the intestinal tract even if the bacteria strain is not able to colonize within the gut
- Attachment to intestinal epithelial cells and interact or send signals to initiate an immune response (Galdeano *et al.* 2007).

To achieve a measurable effect a quantity of 10^8 to 10^9 cfu/day are required and the bacteria have to remain within the GI tract at 48- 74 hours. It could observe that a viability of *Lactobacillus delbruecki* ssp. *bulgaris* was not needed to initiate a positive immune response. But if live bacteria or non-viable bacteria are supplemented affect the time of resistance in the gut. Non-viable bacteria are cleared faster (Galdeano *et al.* 2007).

1.1.2.1 Probiotic bacteria affect the composition of microbiota

The treatment with *Bifidobacterium longum* led to a lower level of lecithinase-negative *Clostridia* in feces. *Bifidobacterium bifidum* B12 induced a higher number of resistant *Bifidobacteria* and a lower level of *Clostridia* in feces. After a supplementation for 7 days with *Lactobacillus acidophilus* LA2 an increased amount of resistant *Lactobacilli* and *Bifiodbacteria* was observed in feces samples. The combination of *L. acidophilus* and *B. bifidum* resulted in an increased number of resistant *Bifidobacteria* and a decreased level of coliforme species in feces after a consumption of 10 days. A relative long-term study with *Lactobacillus rhamnosus* DR20 has shown a change in *Lactobacilli* and *Enterococci* species in the feces of the most consumers. *Bifidobacterium animalis* subsp. *lactis* B19 was offered in 4 different concentrations from 10^8 to 10^{11} and it was found an increasing level of *B. lactis* B19 with higher dose. But the general bacterial composition was not altered. An elderly population was supplemented with *B. lactis* HN019 in 3 different concentrations (15×10^9, 10^9 and 6.5×10^7). Even at lowest dose an augmentation of resistant *Bifidobacteria*, a reduction of *Nitrobacteria* and a higher amount of *Lactobacilli* was observed. These few examples indicate that probiotic bacteria affect the composition of microbiota in a strain-dependent manner (Gill and Prasad 2008).

1.2 Immunoregulation in the gut

1.2.1 Introduction

The gut is not just the organ of digestion and absorption of nutrients. With a surface area of about 200 m² is the gastrointestinal tract (GI tract) the largest human surface where interactions with the exogenous environment take place. And is thus the most important site of entry for many pathogens. Mechanisms to protect the human body against invasion of pathogenic microorganism include physical barrier, anti-microbial components like anti-microbial peptides, mucin and trefoil peptides; specialized immune response as well as indigenous microbiota which prevent the establishment of pathogenic organisms through competitive exclusion. There are two kinds of immune responses: inflammatory or tolerance. The second task of the GI tract is the maintenance of the immune homeostasis through protecting the body from potentially pathogenic organisms and inducing tolerance against innocuous food, commensal microbes and self-antigens. Approximately 80% of the total human antibody production occurs in the gut and makes it to the most important physical and functional barrier of the immune system. A dysregulation of gut homeostasis lead to several inflammatory reactions such as bowel diseases. To cope with this task there are various mechanisms at the tissue, cellular and membrane level (Mason *et al.* 2008; Brandtzaeg 2009).

1.2.2 Morphology of GI-tract

To achieve a surface of about 200 m² an invagination/ projection at several levels is needed. The mucosa is formed into villi and crypt (figure 1). Villi are covered with intestinal epithelial cells which have additional microvilli. The tip of villus is bordered by filamentous brush border glycocalyx to optimize the uptake of nutrients and limits the invasion of bacteria or large molecules (Mason *et al.* 2008).

1.2.2.1 Mucus layer

The surface of epithelium is coated by a mucus layer which has visco-elastic properties and consists of intestinal glycoconjugates, mucin and glycocalyx. Mucin and glycoconjugates are important for the physical barrier function. Glycoconjugates are also required for the adhesion of pathogenic bacteria and toxins. There are also express a high amounts of sIgA on mucosal surface to bind to pathogens in the lumen and prevent their adhesion to epithelial cells. Thus the mucus layer protects the human organism by preventing invasion of microorganism to the underlying mucosa and may play a role in favoring colonization of the GI tract (Akarid *et al.* 2002; Velàzquez *et al.* 2005).

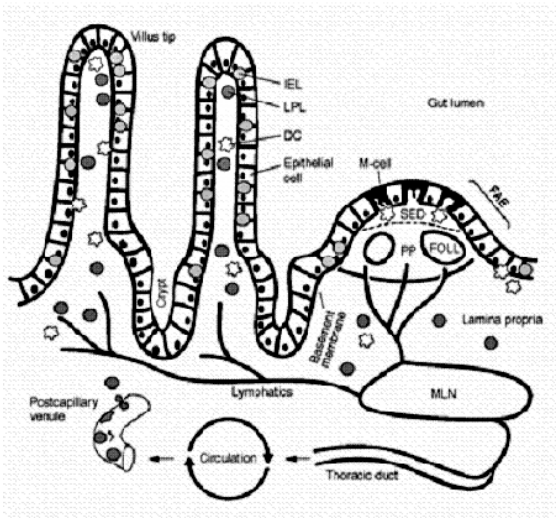

Figure 1: Overview of intestinal immune system (Lunds Universitet 2008)

1.2.2.2 Intestinal Epithelial cells

The intestinal epithelial cells (IECs), an array of mucosal lymphocytes and the mucosa-associated lymphoid tissue (MALT), are located between lumen (Dahan *et al.* 2009). The IECs contain enterocytes, colonocytes, globlet cells, enteroendocrine cells and paneth cells. Together with the mucus layer IECs compose the intestinal epithelium forming the physical barrier against pathogens and control the access to the mucosal compartments. Intestinal epithelial cells play a crucial role in the immune response in the gut because they are capable to secret immunostimulating signal molecules like cytokine, chemokine and eicosanoide (Mason *et al.* 2008). For instance, during a bacterial invasion IECs elicit a cascade of chemokines including IL-8, epithelial neutrophil-activating protein 78, monocyte chemotactic protein, macrophage inflammator protein 3α, and cytokines like IL-18, IL-7, IL-15, granulocyte-macrophage colony-stimulating factor, IL-6 and TGF-β. Thus IECs are also termed as non-professional antigen-presenting cells (APCs) (Dahan *et al.* 2009).

1.2.2.3 Follicle-associated epithelium

The follicle-associated epithelium (FAE) occurs segmental throughout the intestine (figure 1). Its development is regulated by underlying lymphoid cells (Velàzquez *et al.* 2005). FAE is a monolayer with a lower number of distinct brush border, less amounts of digestive enzymes and a higher level of immune cells (Mason *et al.* 2008). The function of FAE is the collection of antigens from luminal site. Therefore it is a part of Peyer's Patches or isolated lymphoid follicles (Velàzquez *et al.* 2005). Micro fold-cells (M-cells) are characteristic for FAE. They facilitate the transportation of antigens to the specialized antigen-presenting cells (APCs) within the subepithelial dome (SED) of Peyer's patches (Mason *et al.* 2008).

1.2.2.4 GALT

The tissue complex involving in the immune response within the GI tract is termed as gut-associated lymphoid tissue (GALT). The GALT is a subset of mucosa-associated lymphoid tissue (MALT). It is composed of intraepithelial lymphocytes, Peyer's patches (PP), lamina propria and appendix at the inductive sites of the gut. Soluble proteins and microbes are able to cross the epithelial barrier under the control of M-cells and are transmitted to Payer's patches. PP have large B cell follicles with intervention T cells in the subepithelial dome (SED) where antigens from the lumen are collected. Lamina propria is the effector site of GALT including professional immune cells such as macrophages, neutrophils, lymphocytes which produce a large number of pro- or anti-inflammatory cytokines and chemokines (Mason *et al.* 2008). The GALT is the largest lymphoid organ in the human body because it contains 70% of the total number of immune cells (Erickson and Hubbard 2009). The function of GALT is to limits and controls the immune response. Therefore it must be capable to distinguish harmless antigens presenting in food or commensal bacteria, from pathogenic microbes. That is difficult because pathogenic and symbiotic/ commensal microorganisms express the same molecular pattern. In general harmless microbes do not elicit an inflammatory immune response. Thus the recognition of microbial pattern is not crucial to indicate an immune response. Additionally signal of "danger" like molecules released from damaged or apoptotic cells as well as changes in membrane composition are necessary (Mason *et al.* 2008).

The mesenteric lymph nodes (MLNs) are the largest lymph nodes in the body mediating immune interaction between GALT and periphery. GALT and MLN are the primary tissue involving in the gut immunology (Mason *et al.* 2008).

1.2.3 Components of the Gut Immune Response

1.2.3.1 Introduction

The human immune system includes local immune tissues termed as mucosa-associated lymphoid tissue (MALT) and the systemic immune system taking place in the blood, spleen, liver and bone narrow. A subset of the MALT is the gut-associated lymphoid tissue (GALT). About 80% of total immune cells are contained within the GALT. There are two types of immune systems: the innate (non-specific) and the adaptive (specific) immune response. Both could be humeral or cellular mediated. The innate immune system includes cells like macrophages, neutrophils and NK-cells to kill the pathogenic organism. The adaptive system required the aid of antigen-presenting cells (APCs) or cytokines which are released in response to an infection or rather in response to pathogens. The actors of adaptive immune response are T and B lymphocytes. B lymphocytes induce a humoral response through specific antibody production. T lymphocytes lead to a cell-mediated response. Naïve T $CD4^+$ cells differentiate into helper T cells (T_H) after stimulation by antigen receptor of their surface. T_H1 activates macrophages and T_H2 induces a differentiation of B cells. Activated T CD8 cells kill pathogenic organism directly. B and T cells which are antigen-activated within the GALT migrate through the lymphatic system to the blood stream (Akarid *et al.* 2002).

1.2.3.2 Pattern recognition receptors

Intestinal epithelial cells are bordered with pattern recognition receptors (PRRs). PRRs like lectin, adhesins, family of nucleotide-binding oligomerization domain (NOD) receptors and Toll-like receptors (TLRs) are a part of the innate immune system. They activate an adaptive immune response. Their task is the recognition of the presence of infection as well as pathogenic organisms and the releasing of signaling molecules to elicit an adaptive response.

The most important PRR are Toll-like receptors. Intestinal epithelial cells express

TLRs variable, depend on type of cells and depend on cell compartment as well as the region within the GI tract. The major function is the protection of human body against a dysregulation of inflammation in the presence of commensal bacteria within epithelial cells (Dahan *et al.* 2009). There are different subset of TLRs with specific ligands binding to a various microbial and host components, also known as pathogen-associated molecular pattern (PAMP) (Mason *et al.* 2008). Characteristics of TLR ligands are 1) expressed only by microbes, but not by host cells, 2) invariant between microorganisms of a given class, 3) essential for the survival of microbes and 4) recognized by TLRs. Nowadays 13 TLR subset are known. For instance, TLR4 are stimulated by lipopolysaccharide (LPS) and endotoxin; TLR1, TLR2 and TLR6 binding specific to bacterial lipoproteins as well as zymosan; bacterial flagellins bind to TLR 5. TLR 9 is specialized to unmethylated CpG present in DNA (Erickson and Hubbard 2009). The dilemma is the discrimination between pathogenic microbes and beneficial organisms which is complicated by the fact that pathogenic as well as commensal species have the same PAMPs. Thus several other mechanisms like recognition of virulence factors or negative regulations at various stages of TLR signaling cascade, have to be also involved in the discrimination (Mason *et al.* 2008). When a ligand binds to a TLR it induces a signal transduction pathway often via NFκB leading to a pro-inflammation response (Dahan *et al.* 2009). A stimulation of TLR can results in an enhanced uptake of microorganisms by phagocytosis cells or killing of microbes by secretion of reactive oxygen and nitrogen intermediates. A recruitment of leukocytes to the site of infection are elicit because TLR induces the expression of E-selection as well as intracellular adhesions molecule-1 on cell surface. A chronic stimulation of TLR leads to IgA production, but this response does not result in an acute inflammation. The increased IgA secretion leads to a broadly increased protection against pathogenic bacteria at inductive site (Erickson and Hubbard 2009).

Dendritic cells (DC) have also various PRRs on their surface. Thus they have the ability to detect groups of microbes with PAMP by binding to PRRs on DCs surface which induces a downstream activation or inhibition of inflammatory pathways (Mason *et al.* 2008).

1.2.3.3 M-cells

Micro fold cells (M-cells) have short irregular microvilli (Erickson and Hubbard 2009) and facilitate the transport of macromolecules from the intestinal epithelial cells to the Peyer's patches (PP) where are professional antigen-presenting cells (APCs) like macrophages, dendritic cells as well as B and T cells. The transport of macromolecules is a very important step of mucosal immune response and thus a crucial point of pathophysiology. The transfer of information could be occurs indirectly through the expression of chemokines or directly by the transfer and present of APCs to lymphocytes. In response to pro-inflammatory signals M-cells up-regulate the production of MHC class I and MHC class II molecules (Dahan *et al.* 2009).

1.2.3.4 Peyer's patches

Peyer's patches (PP) are an aggregation of lymphoid follicles which are located along the antimesenteric side of the small intestinal mucosa and are a part of mucosa-associated lymphoid tissue. The epithelium associated site is composed of enterocytes and is also interspersed with M-cells. PP respons to antigens passing the intestinal epithelial barrier. They are subdivided into follicular and parafollicular area as well as follicle-associated epithelium. The germinal center of PP contains proliferating B lymphocytes, follicular dendritic cells (DCs) and macrophages. The dome area above the follicle comprises B cells, DCs, macrophages and T cells. The activation of B lymphocytes leads to the production of IgA and IgM (Erickson and Hubbard 2009).
Lymphocytes activating in PP are transmitted to mesenteric lymph nodes and from there return to lamina propria and other secretory tissues through thoratic dut and blood vascular system (Erickson and Hubbard 2009).

1.2.3.5 Dendritic cells

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) presenting antigens to T cells in the lymphoid tissue and NK cells. Subsets of specialized DCs was found on the inductive as well as effector site within GI mucosa. They are the major gatekeeper to interact with the innate and adaptive immune response within the GI tract. All subsets of DCs express the signal molecules CD11c and major

histocompatibility complex (MHC) class II. But they are different in the release of CD8a and CD11b. Myeloid DCs $CD11c^+$ $CD11b^-$ are located in the subepithelial dome of Peyer's patches (PP) and plasmacytoid CDs are located in the interfollicular region of PP. An inflammatory or tolerated immune response is influenced by released signaling molecules from DCs and T cells. For instance, $CD11^+$ subsets in PP expressing low level of Interleukin (IL) -12 and a high amount of IL-10 playing a crucial role in tolerated response. In contrast $CD8^+$ $CD11b^-$ has an increased expression of IL-12 and a reduced expression of IL-10 leading to an inflammatory response. Further the location of DCs is crucial of induced immune response. As an example, the activation of DCs of PP by the ligand RANK leads to an induction of polarized T cells to produce IL-10, an anti-inflammatory cytokine. But spleenic DCs would induce an IL-12 production resulting in a pro-inflammatory response. That indicates DCs proximity to intestinal epithelial cells (IEC) induce tolerance when there is a steady-state, absence of inflammatory signals and/ or infection in IECs (Mason *et al.* 2008). DCs also stimulate $CD4^+$ T cells inducing the development of immunoglobulin (Ig)A- committed B cells in lymphoid follicle. These B cells migrate from PP to mesenteric lymph nodes (MLN) via lymphatic channels. Further they migrate back to intestinal lamina propria (LP) through thoratic duct and blood vascular system. Arrived at the effector site B cells and plasmablasts differentiate into IgA producing plasma cells under the influence of IL-5 and IL-6 which are secreted by T_H2 cells. Several forms of IgA bind to polymeric Ig-receptors leading to secreatory IgA (sIgA). SIgA is not associates with an inflammatory response. They are resistant against proteolysis and mediate immune exclusion of foreign antigens by preventing binding to IECs as well as penetration of microorganisms (Erickson and Hubbard 2009).

1.2.3.6 T cells

T cells are a part of the adaptive immune system which is activated by antigen-presenting cells. For instance, helper T cells (T_H) are activated after a presentation of antigens in context with major histocompatibility complex (MHC). The subset T_H1 is involved in an inflammatory response and stimulation of cytotoxines in T cells. Whereas T_H2 producs cytokines which activate specific B cells. And T_H3 has a suppressive affect to effetor cells by releasing the inhibitor cytokine transforming

growth factorβ (TGFβ) (Erickson and Hubbard 2009). Regulatory T cells (T_R), also known as suppressor T cells, are involved in the regulation of tolerance. In the GALT there are antigen-nonspecific and antigen-specific T_Rs. Natural T_Rs express CD25, CTLA-4, GITR and Foxp3. Subsets of $CD4^+$ $CD25^+$ are important for the maintenance of homeostasis within the human body by recognition of self-antigen and preventing an inflammatory response to chronic stimulation. Adaptive T_Rs subsets like T_H3 and T_R1 cells, both are $CD4^+$ T cells, are required for intestinal immunity. T_H3 is activated after an induction of oral tolerance and are capable to influence the expression of IgA. T_R1 cells produce IL- 10 leading to an anti-inflammatory response (Mason *et al.* 2008).

1.2.3.7 B cells and antibody production

80% of all plasma B cells are located in the GI tract. B cells produce specific antibodies. For the production a stimulation of helper T cells are partly needed. The response to the commensal intestinal microbiota is in general independ of T cells induction. A theory of this observation is it requires a more broadly spectrum of IgA to protect the body against microbiota, whereas a production of IgA to neutralize bacterial toxins requires a high specific affinity which needs the induction by T cells (Mason *et al.* 2008). Precursor B cells of Peyer's patches migrate to the gut mucosa where they ensconce into their follicular site. After a successfully antigen-dependent activation they move away to mesenteric lymph nodes and from there to lamina propria where they mature to antibody-producing plasma cells (Velàzquez *et al.* 2005). The major immunoglobulin (Ig) isotype on the mucosal surface is secretory IgA (sIgA) having anti-inflammatory properties and protects the mucosal surface from pathogenic invasion. SIgA is also required to maintain the gut homeostasis and tolerance. An IgA deficiency is associated with an increased risk of gastrointestinal infection in human (Mason *et al.* 2008). About 3 g of dimeric sIgA are translocated to the gut every day. For that reason the gut is the largest antibody producing organ in the human organism (Brandtzaeg 2009). But IgA also plays a necessary role in the regulation of gut microbiota. For instance, in human an IgA deficiency is associated with an higher amount of *Escherichia coli* strains with pro-inflammatory properties (Mason *et al.* 2008).

1.2.3.8 Phagocytes

Phagocytes are non-specific immune cells recognizing pathogens and destroy or eliminate them by phagocytosis. Phagocytosis is a term of enzymatic digestion of pathogenic organism within host cells. Furthermore, phagocytes secret cytokines to warn other cells in the organism as well as attract other immune cells to the site of invasion. They also secrete anti-microbial peptides and reactive oxygen species (ROS) to kill microorganisms outside the own cell. Phagocytes are necessary for the discrimination of "self" or "non-self" cells (Danilova 2006).

1.2.3.9 Macrophages

Macrophages play a crucial role as antigen-presenting cells which release factors attacking other immune cells and pro-inflammatory mediators like IL-1, IL-6 and TNFα after ungulfing and phagocytosis of pathogens. When macrophages ingest apoptotic cells they express anti-inflammatory factors like TGF and IL-10 (Danilova 2006).

1.2.3.10 NK cells

Nature killer cells (NK cells) have the task to screen cells for the presence of specific molecules like MHC class I molecules expressing only by host cells. A high secretion of MHC class I molecules protects cells from NK cells attack. During cancer or viral infection the expression of MHC class I molecules is often decreased. Thus NK cells are able to recognize and remove pathogens, tumors and virus-infected cells. Target cells are killed by secreted granules containing membrane-distinguishing peptide, perforine, serine protease and ganzyme. But a presence of MHC class I and other molecules is not restricted to host cells because some cytotoxic cells are also capable to release this kind of molecules (Danilova 2006).

1.3 Cross-talk between intestinal epithelium and the microbiom

Intestinal epithelial cells interact and response to environmental signals by secretion of chemokines and cytokines to activate an innate and/ or adaptive immune response. Whereas the nature of response is depend on the kind and location of stimulation. For instance, the presences of commensal bacteria lead to high amounts of IgA without the use of adaptive immune system. But the production of sIgA against specific pathogens requires the interaction of T and B cells in Peyer's patches or antigen-presenting cells within lymphoid tissues (Galdeano *et al.* 2007).

1.3.1 Epithelial cell recognition of commensal bacteria by TLRs and NLRs

Intestinal epithelial cells (IECs) express pattern recognition receptors such as Toll-like receptors (TLRs) and NODs-like receptors (NLRs) on apical or basolateral surface. TLRs recognize different kinds of bacterial molecules (like describe above). Under steady-state condition there are only a slight or no expression of TLR2 and TLR4 on apical surface leading to a diminished recognition of bacterial lipopolysaccharid (LPS). It was observed that other TLRs and NLRs localizing in subcellular regions may facilitate the discrimination of commensal or pathogenic bacteria. For instance, TLR5 recognizing bacterial flagellin is exclusively expressed on basolateral surface of IECs. TLR3, TLR7, TLR8 and TLR9 are expressed in intracellular endosomal organelles and some NLRs are found in the cytoplasm of IECs. Thus TLRs and NLRs on basolateral surface recognize only pathogenic (or commensal) organism overcoming the epithelial cell barrier. Ligation to these receptors elicit an innate immune response including initiate expression of pro-inflammatory cytokines and chemokines as well as anti-microbial peptides (Artis 2008). Commensal LPSs and peptidoglycans help to maintain the gut homeostasis and protect the mucosa from cell damages through inducing the expression of protective cytokines such as IL-6, TGF-β, KC-1 and diverse heat shock proteins (Dinalo and Relman 2009). Usually a TLR ligation induces the NF-κB pathway or nitrogen-activated protein kinase (MAPK) leading to an inflammatory response, but do not cause an inflammatory diseases after a stimulation of commensal bacteria. This tolerance against commensal is caused by varied anti-inflammatory/ inhibitor

mechanisms- one of them is described below. This cross-talk is crucial to maintain the epithelial integrity. Dsyregulation of the commensal- TLRs interaction can results in inflammatory diseases (Kelly *et al.* 2005; Clark and Coopersmith 2007; Artis 2008).

The NF-κB pathway is the most important pro-inflammatory signaling cascade of the innate immune response because NF-κB regulates and controls the gene expression of several inflammatory mediators like cytokines. During an absence of initiate signals NF-κB is bound to the inhibitor ligand IκB in the cytoplasm. Stimulating signals result in an IκB phosphorylation by IκB-kinase. A following ubiquitin-dependent degradation split NFκ-B from IκB. The activated NF-κB migrate from the cytoplasm to the nucleus where it promotes the transcription of some target genes involving in inflammatory response (Artis 2008; Won-Jae 2008).

Pathogen-associated molecular pattern like LPS and peptidoglycan elicit a NF-κB-dependent inflammatory response but it does not lead to an inflammation. Because there are many regulatory mechanisms including bacterial-mediated regulation like the promotion of nuclear-cytoplasmic retribution of NF-κB through peroxisome proliferation-activated receptor-γ pathway (Won-Jae 2008). For instance, the neddylation of cullin-1 by Ubc12 is needed for a correct arrangement of enzymatically active SCF complex. A commensal-bacteria-mediated production of reactive oxygen species (ROS) results in an inactivation of Ubc12. Therefore the neddylation of cullin-1 by Ubc12 is not possible and cullin-1 associated and neddylation-dissocated protein-1 (CAND 1) is capable to bind to the deneddylated form of cullin-1 which leads to an inactive SCF complex. Under inflammatory conditions the formation of NEDD8-UBC12 thioester complex activates the SCF complex. The activated SCF complex is involved in the ubiquitin-dependent degradation of IκB leading to activated NF-κB which promotes the transcription of inflammatory genes. This bacterial-regulated IκB stabilization through ROS is an example for coexist of commensal bacteria within the GI tract without causing inflammation. Whereas some commensal bacteria have a higher potential as ROS-inducer than other bacteria. A dysregulation of NF-κB pathway is associated with the pathogenesis of chronic inflammatory bowel diseases (Won-Jae 2008).

1.3.2 Epithelial integrity

The epithelium has a function as physical barrier between human organism and exogenous environment. A critical parameter of barrier function is the permeability of the epithelium. Tight junctions are involved in the regulation of epithelial permeability. They connect epithelial cells with each other and form a physical as well functional barrier against paracellular invasion of pathogenic substance from the intestinal lumen. Cytokines are crucial modulators of the expression and the localization of tight junctions (Clark and Coopersmith 2007). For instance, pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-4 and IL-13 are associated with an increased permeability. Whereas cytokine IL-10 preventes the pro-inflammatory-induced augmentation of permeability. A leakage of the tight junction barrier as well as intestinal barrier can leads to an increased intestinal permeability and in the following to a penetration of luminal antigens into the epithelium which cause an inflammatory response (Clark and Coopersmith 2007; Hörmannsperger and Haller 2010). Such as TNF-α reduces the expression of ZO-1 protein and changes the protein localization. A reduction of ZO-1 protein triggers the expression of inducible nitric oxide synthase (iNOS) leading to an enhanced synthesis of nitric oxide (NO) within the intestinal epithelial cells. NOs play an essential role in homeostasis and development of diseases. Endogenous NO is needed to maintain the mucosal permeability. A disorder in NO production is dangerous for intestinal integrity (Clark and Coopersmith 2007).

Furthermore, apoptosis, the programmed cell death, is an important regulatory mechanism which is also influenced by commensal bacteria. Apoptosis is initiated by receptor-mediated pathway or by mitochondrial-mediated pathway. Apoptotic cells release several signaling molecules influencing the surrounding cells. In general, a decreased level of apoptotic cells within the GI tract is associated with the secretion of anti-inflammatory cytokines and the suppression of pro-inflammatory cytokines (Clark and Coopersmith 2007).

Permeability and apoptosis of intestinal epithelial cells interact with each other. For instance, in rats with an increased permeability were observed a higher level of apoptotic cells (Clark and Coopersmith 2007). A reduction of intestinal barrier function induced by bacterial stimulation leads to a dysregulation of inflammatory signals and augmented apoptosis level (Hörmannsperger and Haller 2010).

1.4 How probiotic bacteria influence the immune response within the GI tract

Probiotic bacteria affect the host in different ways. They influence the composition of intestinal microbiota, the intestinal barrier function and modulate the innate as well as the adaptive immune system. Whereas different probiotic strains and their products induce a variance of different protective mechanisms (Delcenserie *et al.* 2008; Hörmannsperger and Haller 2010). Clinical relevance has *Escherichia coli* Nissle 1917 and the probiotic mixture VSL#3 containing following 8 bacteria species: *Lactobacillus acidophilus*, *Lactobacillus bulgarius*, *Lactobacillus casein*, *Lactobacillus plantarum*, *Streptococcus thermophilus*, *Bifidobacterium breve*, *Bifidobacterium infantis* and *Bifidobacterium longum* (Hörmannsperger and Haller 2010). Probiotic bacteria have to interact with the epithelial cells and/ or intestinal immune cells to promote an immune response. For instance, bacterial flagellin binds to TLRs and induces an immune response. But probiotic organism also interact with M-cells of Peyer's patches and may be is also collected by dendritic cells (Hörmannsperger and Haller 2010).

1.4.1 Probiotic bacteria and epithelial integrity

An increased apoptosis within intestinal epithelial cells (IECs) is often associated with the penetration of pathogenic microorganisms. In ulcerative colitis patients were observed a higher level of apoptotic epithelial cells and a reduced intestinal barrier function. In gnotobiotic mice, *Escherichia coli* Nissle up-regulated the expression of tight junction protein ZO-1. In vitro *E. coli* Nissle prevented the penetration of pathogenic bacteria such as *Salmonella dublin*, *Yersina entercolitca*, *Shigella flexuri*, *Legionella pneumophilla* and *Listeria monocytogenes* through secretion of soluble factors. That indicates an anti-apoptotic effect of *E. coli* Nissle. Such an anti-apoptotic effect of probiotic proteins is dependent on simultaneous stimulation of heat shock protein (HSP) production in IECs. For instance, pro-inflammatory cytokines TNFα and IFN-γ suppress the expression of cytoprotective HSP like hsp25 und hsp70 which augment the number of apoptotic cell within epithelium (Hörmannsperger and Haller 2010). *Lactobacillus rhamnosus* GG releases the proteins p40 and p75 blocking the TNFα-induced pro-apoptotic signal pathway and trigger the Akt-dependent survival

pathway in IECs (Fang *et al.* 2007).

1.4.2 Probiotic bacteria and immunomodulation function

The immunomodulation function of probiotic bacteria to the intestinal epithelial cells (IECs) are depend on bacteria species and strain as well as bacterial immune-derived stimulation (Delcenserie *et al.* 2008; Hörmannsperger and Haller 2010). Like describe above, IECs are capable to detect components of microbiota by expression of TLRs or cytolic NLRs and through MHC class II detection and co-stimulatory proteins. A continuing activation of NF-κB by increased expression of chemokine IL-8 or RANTES and IP-10 are crucial signal molecules in the development of chronic inflammation within the GI tract. Because a dysregulated, steady synthesis and expression of these chemokines lead to an persistent recruitment and activation of effector cells such as T cells, neutrophils and macrophages in the mucosal tissue. A pro-inflammatory response is required to fight against potential pathogenic organisms. In healthy humans this response is terminated after a successful elimination of infection. For instance, *Lactobacillus plantarum* plays a role in the modulation of NF-κB- dependent pathway in duodenal mucosa. And soluble factors of *E. coli* Nissle block the TNFα-mediated expression of IL-8 in IECs. It was also observed that VSL#3-derived DNA decreased the expression of IL-8 in IECs. This effect was initiated by probiotic bacteria which reduce the protosomal activity in IECs leading to suppressed degradation of IκB, the inhibitor of NF-κB, in IECs. A mix of soluble probiotic proteins were able to inhibit a *Salmonella-dublin*-induced activation of NF-κB. *Lactobacillus casein*, extracted from VSL#3, shown a specifically inhibition of IP-10 expression in IECs resulting in a decreased recruitment of activated T lymphocytes. The priming of naive T cells into effector or regulatory T cells is depends on cytokines milieu and depends on co-stimulatory signals from antigen-presenting cells. Probiotic bacteria are capable to effect pro-inflammatory cytokines expression; inducing effector T cells development as well as anti-inflammatory cytokines mediated development of regulatory T cells. As an example, VSL#3 promotes an anti-inflammatory response by induction of mucosal $CD4^+CD25^+$ and $CD4^+$ LAP positive regulatory T cells. VSL#3 also increased the transcription of regulatory Foxp3. In pouchitis patients VSL#3 suppressed the expression of pro-

inflammatory IL-1β. And in patients with ulcerative colitis a treatment with the bacteria mixture VSL#3 reduced the expression of IL-8 as well as IFN-γ and IL-1β in dendritic cells or T cells (Hörmannsperger and Haller 2010).

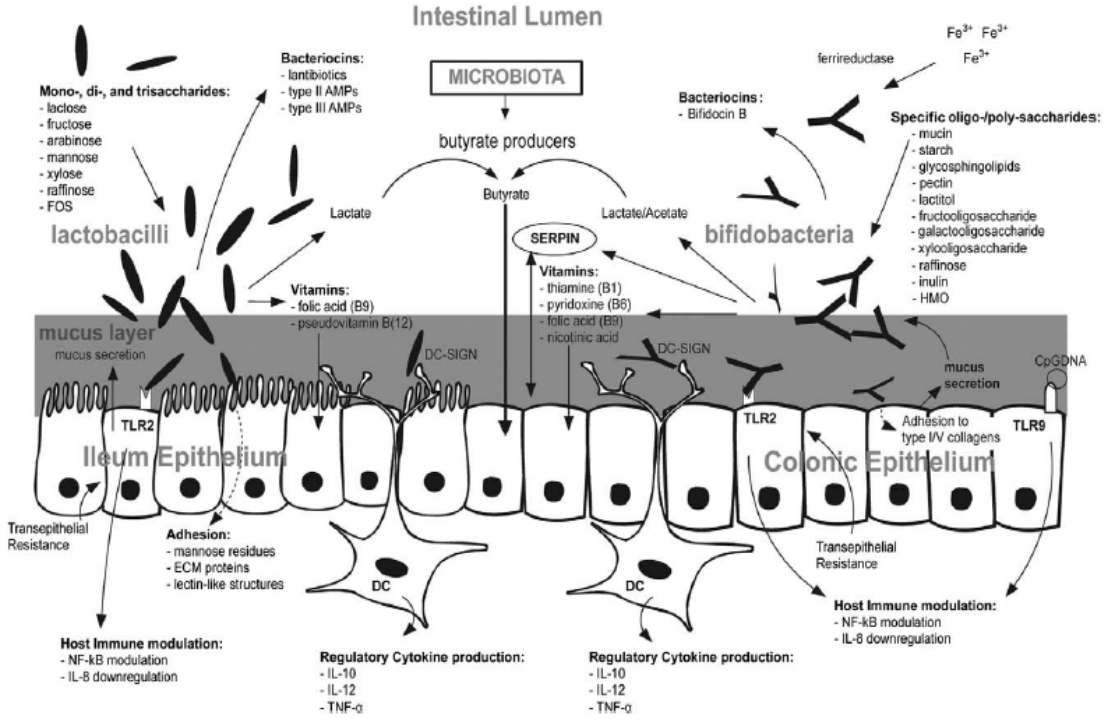

Figure 2: Overview of immunomodulated effect of Lactobacilli and Bifidobacteria (Boesten and de Vos 2008)

IL-1β induces an IL-6 expression and production. *Lactobacillus paracasein* ssp. *paracasein* F19 and *Lactobacillus plantarum* 2362 (10^8 cells/ml) did not affect the IL-6 production in Caco-2 cells, but the combination of IL-1β with one of these viable bacteria or heat-inactivated or sonicated bacteria led to an increase of IL-6 concentration by 6-fold in case of *L. paracasein* F19 and by 2-fold in case of *L. planatrum* 2362 compared to IL-1β stimulation alone. That was also reflected in an augmentation of IL-6 mRNA level in a time-dependent manner. The commensal vaginal bacteria *Lactobacillus jensinii* as well as the non-pathogenic *E. coli* DH5α in combination with IL-1β showed a slight rise of IL-6 production. These results indicate that probiotic bacteria or their cell wall components in combination with IL-1β are able to improve the IL-6 production in Caco-2 cells. Whereas this affect is strain-dependent. This beneficial effect could be relevant for clinical treatment of bowel inflammation. The combination of TNF-α and *L. paracasein* F19 did not led in a

measurable IL-6 production which indicate *L. paracasein* have no influence on IL-6 production via TNF-α pathway (Reilly *et al.* 2007). Vinderola et al used small intestinal epithelial cells (SIEC) of mousses to study the effect of probiotic *Lactobacillus casein* CRL431 and *Lactobacillus helveticus* R389 as well as non-pathogenic *E. coli* MM245 to the IL-6 production. All bacteria showed a reduction of IL-6 concentration at a concentration of 10^8cells/ml and an augmentation, especially a 2-fold increase after *E. coli* stimuli, at a concentration of 10^6 and 10^7bacteria/ml. After a treatment of non-viable bacteria the IL-6 production was greater as with viable-treated SIEC. An ex-vivo test with this mouse model and an oral admission of probiotic bacteria found *L. casein* increased the IL-6 concentration by about 8-fold after two days and about 2-fold after day 5 and 7. *L. helveticus* led to a rise of IL-6 by 8-fold after the second day and an increase by 3-fold after day 5 and 6-fold after day 7. This study shown epithelial cells in natural context have a higher capacity to influence the IL-6 production (Vinderola *et al.* 2005).

Further, *Lactobacillus plantarum* reduced the LPS (from *Salmonella enterica serovar typhimurin*)- induced IL-23 expression and protein secretion, and thus shown also anti-inflammatory properties (Paolillo *et al.* 2009).

1.5 The Interleukin 8

1.5.1 The role of IL-8 in the immune system

Interleukin 8 (IL-8) is a member of CXC-chemokines, also term as chemotractic cytokine. A wide variety of human cells such as endothelial cells, fibroblasts from different tissues, several types of epithelial cells and tumor cells are capable to secrete IL-8 in response to various pathogenic microbes and pro-inflammatory cytokines such as tumor necrosis factor (TNF) and IL-1. The IL-8 expression is regulated at the transcriptional level by the nuclear factor (NF)-κB. IL-8 and other CXC-chemokines are capable to recruit neutrophils (Baggiolini *et al.* 1995; Remick 2005; O'Hara *et al.* 2006). As a neutrophil chemoattractans, IL-8 induces chemotaxis leading to the migration of neutrophils to the site of inflammation and results in some shape changes including transient augmentation of intracellular Ca-ion, secretion of granule contents, up-regulation of adhesions proteins, formation of bioactive lipids and respiratory burst (Baggiolini *et al.* 1995). Because IL-8 plays a crucial role in the regulation of inflammatory response and its relative longevity at site of acute inflammation, it is used as mediator of inflammation (Baggiolini *et al.* 1995; Remick 2005).

1.5.2 Influence of bacteria to IL-8 expression and protein level

During the last years several studies researched the affect of pathogenic and commensal bacteria, some of them are (potential) probiotic bacteria, to the IL-8 expression as well as IL-8 secretion. A study with three different human intestinal cell lines (Caco-2, HT-29 and HCT 116) shown *Enterococcus feacalis* (EC1, EC3, EC15, EC16) down-regulate the IL-8 production in all cell lines. In Caco-2 cells *Lactobacillus delbrueckki bulgaris* D1, *Lactobacillus rhamnosus* GG, *Lactobacillus paracasein* LP33, *Lactobacillus brevis* T6 and *Streptococcus thermophilus* NCIMB10387 also inhibited the IL-8 secretion. But the IL-8 mRNA level was increased by 10^7cfu/ml of *E. feacalis* (EC1, EC3, EC15, and EC16), *L. rhamnosus* GG and *L. bulgaris* D1. And there were not observed any alternation in IL-8 mRNA expression with bacteria by 10^8 cfu/ml (Wang *et al.* 2008). These data of IL-8 expression are not consistent with IL-8 protein concentration indicating there have to be involved post-transcriptional or post-

translational mechanisms.

Caco-2 cells, pre-treated with live or UV-inactivated *L. rhamnosus* GG, shown a decreased IL-8 production by 66% or 59% after stimulation with flagellin of *Pseudomonas aeruginosa* compare to the flagellin stimulation alone which led to a 17-fold increase of IL-8 production. Live *L. rhamnosus* GG alone induced an IL-8 expression by 2-fold. Whereas UV-inactivated *L. rhamnosus* GG alone had no influence to IL-8 expression (Lopez *et al.* 2008). A further experiment studied the impact of *Bifidobacterium animals* MB5 and *L. rhamnosus* GG to an enterotoxigenic *E. coli* K88 (ETEC)-induced pro-inflammatory response. ETEC alone induced a strong up-regulation of IL-8 expression as well as IL-1β and TNF-α and a down-regulation of TGF-α expression. Both bacteria, *L. rhamnousus* GG and *B. animalis* MB5, reversed the ETEC-induced effect to the expression of IL-8, IL-1β and TNF-α to the level of untreated cells and up-regulated the TGF-α expression to the basal level. Like describe above the cytokines IL-1β and TNF-α are positive associated with IL-8 expression. It is known that TGF-α inhibits the IL-1β expression and also influence the IL-8 expression. Thus *B. animals* and *L. rhamnosus* GG have an anti-inflammatory effect to an ETEC-induce inflammatory (Roselli *et al.* 2006). In Caco-2 cells pre-treated with supernatant of *Lactobacillus casein* subsp. *casein* 2756, *Lactobacillus carvatus* 2775 and *L. platarum* 2142 were observed a reduction of *Salmonella enteritidis*-mediated IL-8 production by 3- to 5-fold compare with the rise of *S. enteritidis* alone (Nemeth *et al.* 2006). Pathogenic bacteria including *Vibrio cholera* O1 and *V. cholera* O139, enterohaemorrhagic *Escherichia coli* (EHEC), *Salmonella typhimurin* and *Shigella flexneri* induced a IL-8 production in HT29 and T84 cell lines. The probiotic bacteria *Lactobacillus rhamnosus* GG and *Lactobacillus plantarum* alone did not affect the IL-8 secretion, but pre- or co- cultured with the pathogenic bacteria resulted in a suppressed IL-8 secretion caused by *V. cholera*, *S. typhimurin* and EHEC. The both lactobacilli did not influence the Shigella-induced IL-8 production (Nandakumar *et al.* 2009). Similar effects were shown for *Bifidobacterium infantis* and *Lactobacillus salivarius* which reduced the IL-8 secretion in a dose-dependent manner in untreated HT-29 cells and also inhibited the *S. typhimurin*-induced IL-8 secretion by 23.5% or 31%. Further, *B. infantis* inhibited the IL-8 secretion caused by flagellin of *S. typhimurium*, but not *L. salivarius* (O'Hara *et al.* 2006). In contrast, Vizoso Pinto et al

observed in their experiment that HT29 cells pre-treated with *L. plantarum* BFE 1685 or *L. rhamnosus* GG and infected with S. *typhimurium* led to an augmented IL-8 concentration by about 2.5-fold (Vizoso Pinto *et al.* 2009). Further, an inhibitory effect on IL-8 production caused by *Listeria monocytogenes* was observed in C2Bbe1 cell culture which was pre-treated with *Lactobacillus salivarius* UCC118 or *Lactobacillus acidophilus* NCDO 1748, *Lactobacillus casein* NCDO 1205, *Lactobacillus lactis* NZ9000 and *Bifidobacterium breve* UCC2003, *B. infantis* CCUF36569 as well as *Bifidobacterium longum* JCM 7050 (Corr *et al.* 2007).

For instance, IL-8 production is mediated by TNF-α. In Caco-2 cells with and without antibiotics were shown that *L. rhamnosus* GG inhibited the TNF-α up-regulation at a concentration of 10^6 and 10^8 cfu/ml. *L. rhamnosus* GG at a concentration of 10^{10} cfu/ml increased explicit the IL-8 production in a TNF-α-dependent manner, equal if in media with our without antibiotics. Heat-killed *L. rhamnosus* GG also blocked the TNF-α-induced IL-8 up-regulation at a concentration of 10^6 and 10^8 cfu/ml, but a concentration of 10^{10} cfu/ml led to a slight rise of IL-8 production without TNF-α (Zhang *et al.* 2005). Jea Sung Ko et al observed the same inhibitory effect of TNF-α-induced IL-8 secretion for *Lactobacillus plantarium* ATCC 8014 at a concentration of 10^7 cfu/ml (Jae *et al.* 2007). TNF-α pre-treated HT-29 cells shown a higher IL-8 concentration by 2.5- to 3-fold after addition of *Lactobacillus plantarum* BFE 1685 and *Lactobacillus rhamnosus* GG (Vizoso Pinto *et al.* 2009). Contrary to this finding, McCracken et al observed indeed an increase of IL-8 mRNA level by 6.6- fold in HT-29 cells pre-treated with TNF-α and incubated with *L. plantarum* 299v compare to HT-29 cells treated with TNF-α alone. But this higher IL-8 gene expression did not result in an augmentation of IL-8 protein concentration. In contrast, TNF-α pre-treaded HT-29 cells shown a reduction of IL-8 level after addition with *L. plantarum* 299vb (McCracken *et al.* 2002). A dose-dependent inhibition of TNF-α- induced IL-8 production was also shown for *Bifidobacterium longum* Bar33 and *Lactobacillus acidophilus* Bar13 as well as a mixtures of them (Candela *et al.* 2008).

IL-1β induces a lower IL-8 production compare to TNF-α. In Caco-2 cells the combination of IL-1β with *Lactobacillus paracasein* F19 did not affect the IL-8

concentration (Reilly *et al.* 2007). HT-29 cells, pre-treaded with IL-1β, shown a reduced IL-8 level after incubation with *Bifidobacterium longum* Bar33 and *Lactobacillus acidophilus* Bar13 as well as a mixtures of them (Candela *et al.* 2008). The lipopolysaccahride (LPS) from *E. coli* O55:B5 (100 ng/ml) blocked the IL-1β as well as TNF-α induced IL-8 secretion, but did not affect the IL-6 concentration in Caco-2 cells (Savidge *et al.* 2006).

These data show probiotic bacteria at a moderate level have a beneficial influence on IL-8 production mediated by pro-inflammatory cytokines and/or chemokines as well as pathogens. But this effect seems to be species- and strain-specific.

1.6 The Interleukin 17

1.6.1 The role of IL-17 in the immune system

The distinctive cytokine Interleukin (IL-) 17 is major released by T_H17 cells. But also nature killer cells and nature killer T cells are able to express it. The differentiation of naïve T cells into T_H17 cells is mediated by IL-6 and TGF-β- dependent expression of the transcription factor ROR-γt. IL-23 regulates the expansion as well as survival of T_H17 cells. Interleukin 17 occurs relative at the end of an inflammatory cascade (Miossec *et al.* 2009). Nowadays six subsets from IL-17A to IL-17F are known, whereas IL-17E is also termed as IL-25. The IL-17 family members are expressed in different cells of tissues or organs. The most important subsets are IL-17A, also called as IL-17, and IL-17F which are quite similar to each other (Kolls and Lindén 2004; Gaffen 2009). The primary target cell type are neutrophils which induce an inflammatory response through expression of IL-1β, TNF-α, IL-6 and some chemokines involving in neutrophil recruitment such as IL-8 (Maloy and Kullberg 2008; Miossec *et al.* 2009). Further target cells include endothelial cells, fibroblasts, osteoblasts and chondrocytes. In addition to its function as pro-inflammatory cytokine it is also involved in the control of extracellular pathogens, inducing matrix destruction and has synergy effects with TNF-α and IL-1β. An overproduction of IL-17 is associated with chronic inflammation and serve immunopathologic conditions (Miossec *et al.* 2009).

1.6.2 The affect of bacteria on IL-17 production

An experiment with germ free (GF) mice and conventionally reared (CNV) mice showed an increased IL-17 gene expression by 2-fold in the large intestine of GF mice compare with them of CNV mice. Further, in the large intestine the IL-17 mRNA level was 3-fold higher in antibiotic treated CNV mice then in CNV mice. There was no significant alternation in the small intestine. Zaph et al also found GF mice treated with IL-25 have a lower IL-17 mRNA level in the large intestine compare with GF mice. And the commensal-dependent expression of IL-25 reduced the IL-23 concentration and the frequency of T_H17 cells in the large intestine. That suggests commensal bacteria have an inhibitor effect on IL-17 expression in the large intestine (Zaph *et al.* 2008). The IL-17 production in the lamina propria of small intestine in BALB/c mice was not affect by supplementation of *Lactobacillus acidophilus* A9, *L. acidophilus* 1462, *Lactobacillus casein* CRL 431 and *Escherichia coli* 129 after two days as well as after day 5 and 7. Only *E. coli* 13-7 induced an IL-17 production in a time-dependent manner. Dogi et al assume this observation is associated with an augmentation of IFNγ and IL-12 which have a suppressive affect on IL-17 expression (Dogi *et al.* 2008).

It was shown that IL-27 inhibits the IL-17 production. Murine splenocytes treated with TGF-β plus IL-6 led to a drastic increase of IL-17 production and a lower IL-27 concentration. The addition of heat-killed *Bifidobacterium infanits* to these murine splenocytes reduced the TGF-β and IL-6 induced IL-17 production and increased the IL-27 secretion. Whereas heat-killed *Bifidobacterium bifidum* led to a higher IL17 concentration and heat-killed *Lactobacillus acidophilus* and *Lactobacillus bulgaris* induced a slight IL-17 production and a reduction of IL-27. That indicates bacteria modulate the IL-17 production in a species-dependent manner. In this experiment IL-17 level is negative correlated with IL-27 level in TGF-β and IL-6 treated cell. Further, in colon organ culture heat-killed *B. bifidum*, *B. infantis* and *Bifidobacterium cantenulatum* decreased the dextran sodium sulfate-induced IL-17 production without changes in IL-27 production (Tanabe *et al.* 2008). An increase of IL-17 production after addition with *B. bifidum* was also observed in human monocyte-derived dendritic cells. Whereas this effect seems to be strain-specific (López *et al.* 2010).

1.7 The CASP *3* gene

1.7.1 Caspase-3 and apoptosis

Caspase-3 is a member of caspases, a class of proteolytic enzymes which are essential for the apoptosis. Apoptosis is an active and energy-dependent programmed cell death occurring in presence or absence of caspase activation. Mostly the cell death is caspase-mediated and thus executioner caspase such as CASP3, CASP6 and CASP7 leading directly to apoptosis and can used as markers for cell death. There are two apoptotic signaling pathways. The extrinsic pathway, also named as death-receptor pathway, is activated through ligation to death receptors like tumor necrosis factor receptor (TNFR) and Fas. This stimulation leads to a recruitment of pro-caspase 8 and activation (cleavage) of caspase 3. The intrinsic pathway or mitochondrial/ER pathway is induced through cytokines like IL-1 and IL-6, chemokines and growth factors. In this pathway bcl-2 family members and disintegration of the mitochondrion also induce caspase 3 activation. The homeostasis of apoptosis is important for intestinal epithelial development and their function (Perl *et al.* 2005).

1.7.2 How bacteria influence caspase-3 activation

In dextran sodium sulfate (DSS)-induced colitis mice a treatment with *Bacillus polyfermenticus* led to a lower number of apoptotic cells in colonic mucosa. Human colonic epithelial cells which were treated with apoptosis-inducing factor FasL or *Clostridium difficile*toxin A, shown in presence of *B. polyfermenticus*-conditioned medium a strong reduction of FasL. *C. difficile*toxin A induced PARP cleavage and an inhibition of caspase-3 cleavage inducing by *C. difficile*toxin A. That indicates soluble factors from *B. polyfermenticus* are able to suppress apoptosis in colonic mucosa as well as colonic epithelial cells (Eunok *et al.* 2009).

In an experiment with Caco-2 cells which were co-cultured with *Atopobium minutum*, *Lactobacillus rhamnosus* GG, *Bifidobacterium lactis* Bb12, non-pahtogenic *Escherichia coli* K-12 strains and pathogenic *E. coli* (EPEC and VTEC), was observed a translocation of BAX and BAK, both Bcl-2 family members, into mitochondrion for all bacteria without an augmentation of BAX or BAK concentration. Whereas this translocation was 2- to 3-fold higher after the treatment with pathogenic *E. coli*. Further all

bacteria, with the exception of *E. coli* K-12 strains, showed an activation of caspase-9 and a reduction of pro-caspase 9 leading to proteolytic cleavage of caspase-3. Thus pathogenic *E. coli*, the used probiotic bacteria and *A. minutum* have an apoptosis-inducing effect in colon cancer Caco-2 cells (Altonsy *et al.* 2010). A treatment of Caco-2 cells with *Lactobacillus rhamnosus* GG, *L. rhamnosus* Lc705, *Propionibacterium freundenreichii* subsp. *shermanii*, *Bifidobacterium breve* Bb99 and pathogenic *Helibacter pylori* resulted only in the case of *P. shermanii* in an increased caspase-3 activity. In bacteria pre-treated Caco-2 cells infecting with *H. pylori* were observed a reduced caspase-3 activity under the control level for the pre-treatment with *L. rhamnosus* GG, *L. rhamnosus* Lc705 and the combination of all four bacteria after 24 h suggesting a preventive role in caspase-3 activity in *H. pylori* infected Caco-2 cells (Myllyluoma *et al.* 2008). An anti-apoptotic effect for *L. rhamnosus* GG was also shown in an experiment with i*n vitro* intestinal epithelial cells and *in ex- vivo* with cells of the murine gut of two week-old mices feeding oral with viable *L. rhamnosus* GG. In these experiments could decreased a chemical-induced caspase-3 activation and the number of epithelial apoptotic cells (Lin *et al.* 2008). Further, the pro-apoptotic effect of the genus *Propionibacteria* was observed in a study with *P. acidipropionici* CNRZ80, *P. freundenreichii* subsp. *freudenreichii* ITG18 and *P. freudenreichii* subsp. *shermanii* SI41 where it could be shown that the short-chain fatty acids (SCFAs) propionate and acetate producing by these bacteria, inducing the cleavage of procaspase-3 as well as the activation of caspase 3 in HT-29 and Caco-2 cells. The *Propionibacteria* were even able to kill the both human colon cancer cell lines. Whereas the extent of cytotoxic effect is depend on the produced SCFAs amount (Jan *et al.* 2002).

1.8 Characterization of bacteria

1.8.1 Lactic acid bacteria

Lactic acid bacteria (LAB) are inhabitants of the human and animal gastrointestinal tract. Some species are used for the production of dairy or other fermented foods or as probiotic food. The most three important genera are *Lactobacillus*, *Bifidobacteria* and *Streptococcus*. Further common LAB contain species of *Enterococcus* and *Lactococcus*. LAB are gram positive, non-spore forming and usually catalase negative bacteria growing under microacophilic or strictly anaerobic atmosphere. They are capable to ferment carbohydrates often to the end product lactic acid. Other properties contain resistance to low pH and/ or bile, tolerate different concentration of NaCl and are often resistance against antibiotics. Furthermore, LAB are capable to grow under conditions of different nutrient media and defined temperatures (Klein *et al.* 1998).

1.8.1.1 Lactobacillus acidophilus NCFM™

Lactobacillus acidophilus NCFM™ is a common probiotic strain using in conventional foods like milk, yoghurt, toddler formula and juices, as well as in dietary supplements in the United States of America since the mid-1970s. This strain has an excellent stability within milk and fermented dairy products insolated with a density of about 10^7cfu/ml. Toxonomical *Lactobacillus acidophilus* NCFM™ is a strain of type A1 *L. acidophilus*, a gram-positive and catalase-negative homofermenter that ferments carbohydrates to 34% $_{D-}$ and 66% $_{L-}$ lactic acid. From the three chosen probiotic bacteria, *L. acidophilus* produced the highest amount of lactic acid and lower explicit the pH in its fermented products. Like all probiotic strains *L. acidophilus* NCFM™ is able to tolerate low pH condition and is capable to adhere to intestinal epithelium. In studies with Caco-2 cells, an enhanced attachment was observed in the presence of calcium ions. Further, this probiotic strain survives the gastrointestinal transit in healthy as well as sick humans. The properties to produce anti-microbial compounds such as lactic and acetate acid, hydrogen peroxide, diacetyl, bacterial or bacteriostatic peptides and proteins, are necessary for competitive exclusion of pathogens including *Staphylococcus aureus*, *Salmonella typhimurin*, enterpathogenic *Escherichia coli* and *Clostridium perfringens*. Other beneficial attributes are reducing the incidence of

pediatric diarrhea, decreasing the concentration of toxic amines in blood of dialysis patients with small bowel bacterial overgrowth and decreasing the cholesterol level. An adequate daily intake of *L. acidophilus* NCFM™ may facilitates the lactose digestion in lactose-intolerant individuals (Sanders and Klaenhammer 2001). A prophylaxis supplementation to children between 3 and 5 years of age with *L. acidophilus* NCFM™ alone or in combination with *Bifidobacterium animalis* subsp. *lactis* Bi-07 during the six winter months reduced explicit the incidence of fever, cough and rhinorrhea as well as the duration of illness and the use of antibiotics. There are certain strains of probiotic bacteria being a safe and effective way to prevent cold and influenza-like symptoms in children. Whereas *L. acidophilus* NCFM™ alone was effective, but in combination with *Bifidobacterium animals* subsp. l*actis* Bi-07 was shown a boarder protective effect (Leyer *et al.* 2009). Moreover the consumption of *L. acidophilus* NCFM™ in combination with the prebiotic lactitol led in elderly persons who take NSAID, to an increased stool frequency without side-effects and to a higher level of faecal *L. acidophilus* NCFM™ and *Bifidobacteria*. This measured level is similar to reported *Bifidobacteria* concentration of healthy young adults. And the concentration of PGE and spermidine were also increased. These data indicate that the consumption of *L. acidophilus* NCFM™ with lactitol enhance mucosal function and alter the microbiota composition (Ouwehand *et al.* 2008). In a study with isolated intestinal epithelial cells from a mouse fetuses *L. acidophilus* NCFM™ resulted in a slower but higher production of cytokines and chemokines than *E. coli* Nissle. *E. coli* Nissle induced a up-regulation of IL-6, IL-10 and TNF after 2 h and shown a down-regulation of IL-10 after 4 h. Whereas *L. acidophilus* NCFM™ achieved a similar expression level of IL-6 and TNF after 4 h and shown the highest protein concentration after 18 h. Interestingly, *E. coli* Nissle stimulated the transcription of TLR-2, a receptor to detect gram-positive bacteria, and inhibited the transcription of TLR-4 which is a LPS receptor. Thus *E. coli* may induced a *L. acidophilus* NCFM™-mediated slower but potentiated up-regulation through an increase level of TLR-2 (Zeuthen *et al.* 2010).

L. acidophilus NCFM™ also influence the cytokines expression in dendritic cells (DC) in a dose-dependent manner. The anti-inflammatory cytokines IL-10 and IL-6 were

higher expressed at an increasing concentration of *L. acidophilus* NCFM™ (at a ratio 1:100 DC/bacteria cells). Whereas IL-12p70 expression was increased at lower bacteria concentration suggesting a beneficial effect of *L. acidophilus* NCFM™ because a higher amount of DCs express more IL-10 and IL-6 which is associated with the capability to modulate the T cells maturation forward T_{h17} or $_{reg}T$ cells (Konstantinov *et al.* 2008).

1.8.1.2 Bifidobacterium animalis subsp. lactis Bt420

Bifidobacterium animalis subsp. *lactis* Bt420 (*B. lactis* Bt420) is a gram-positive, non-motile and anaerobic bacterium which produces short chain fatty acids like acetate and lactate but do not produce butyrate and propionate. Further, B. lactis strains synthesize folic acid (Nurmi *et al.* 2005) and show an anti-microbial activity against *Staphylococcus aureus* and *E. coli*. Its probiotic properties are associated also with the capability to adheres to human mucus in subjects of different age groups (Klein *et al.* 2008). The consumption of the combination of *L. acidophilus* 74-2 (2.8×10^9 cfu/day) and *B. lactis* 420 (9.0×10^8 cfu/ day) led to an increased proportion of both bacteria in fecal samples after the intervention period of 5 weeks. That indicates a transient colonization of these bacteria. In addition a rose level of granulocytes and monocytes with an higher phagocytic activity was observed during the probiotic intervention period suggesting the combination of this both bacteria strains are able to affect the unspecific cellular immune response. The supplementation also resulted in a lower serum concentration of triacylglyceroles but did not affect the serum cholesterol (Klein *et al.* 2008). A similar study with a probiotic drink containing *Lactobacillus paracasein* Lpc-32, *L. acidophilus* 74-2 and *B. lactis* 420 compared the influence of probiotic between healthy adults and atopic dermatitis (AD) patients. In addition to the results as described above, in healthy persons an increase of $CD57^+$ and a decrease of $CD3^+$ $CD4^+$ were observed after the consumption of this probiotic drink. These results indicate an immune stimulating effect of the probiotic drink which may leads to a lower incidence and duration of infection among healthy individuals. AD patients showed a reduction of SCORAD by 15.5% and lower level of $CD4^+$ and $CD54^+$, but no alternation among major lymphocytes and nature killer cells after probiotic supplementation. The beneficial effect on therapy of AD patients is only shown for mild and moderate AD. This findings further suggest probiotic foods affect the

peripheral immune parameters depend on immune status of an individual (Roessler (nee Klein) *et al.* 2007).

Caco-2 cells stimulated with *B. lactis* 420 supernatant showed a higher transepithelial electrical resistance (TEER). In contrast supernatant of pathogenic *Escherichia coli* O157:H7 (EHEC) led to a strong decrease of TERR indicating a damage of tight junction. Interestingly, in Caco-2 cells pretreated with *B. lactis* 420 supernatant, the EHEC-induced tight junction damage could prevented (Putaala *et al.* 2008). Furthermore, the addition of *B. lactis* 420 to Caco-2 cells led to a strong increased expression of Cox-1 playing amongst others a crucial role in the maintenance of gastrointestinal integrity and has a cytoprotectiv function within the GI tract. And it led to a reduction of Cox-2 expression whereas a high expression of Cox-2 gene is associated with inflammatory and cancerous diseases. The stimulation with *L. acidophilus*, *E. coli* and *Salmonella enteritis* shown no influence on Cox-1/ Cox-2 ration (Nurmi *et al.* 2005). Interestingly, the metastatic gastric adenocarcinoma cell line NCI-N87 treated with bacteria *B. lactis* 420 and *L. acidophilus* 74-2 did not affect the Cox-1 or Cox-2 expression, but the cell- free supernatant of *L. acidophilus* 74-2 induced a Cox-1 expression (Mahkonen *et al.* 2008). Thus potentially anti-inflammatory and anti-carcinogenic properties relating to Cox-1/ Cox-2 ratio seem to be bacteria specific as well as depend on absence or presence of colon cancer.

1.8.1.3 Streptococcus salviarius subsp. thermophilus ST21

The non-pathogenic bacteria *Streptococcus salivarius* subsp. *thermophilus (St. thermophilus)* is a gram-positive, non-motile, thermophile cocci with an optimal growth temperature of 45°C. These bacteria are facultative anaerobe and are capable to generating energy in form as adenosine triphosphate (ATP) under oxygen use or through fermentation without oxygen. Further, *St. thermophilus* is oxidase negative, chytochrom negative as well as catalaze negative. Its property to produce lactic acid is commercial used as starter culture in the production of milk, cheese and other dairy products (GenomeProject; MicrobeWiki 2006)

St. thermophilus STR21 as starter culture is used in combination with other probiotic bacteria. A study with 162 children of the age between 9 months and 10 years who had a high index of exposure to environmental microorganism, showed that there

were no significant differences in the rate of immunoglobulin and isoagglutinin acquisition among children supplemented for 4 months with low-fat milk fermented by *St. thermophilus* or low-fat milk fermented by *St. thermophilus* and *Lactobacillus casein* which was further added with *Lactobacillus acidophilus*, oligofructose and inulin after the fermentation process (Pérez *et al.* 2009). In hospital patients with an average age of 74 years, the supplementation twice a day with a probiotic drink containing *L. casein*, *Lactobacillus bulgaris* and *St. thermophilus* led to a reduced incidence of antibiotic or *Clostridium difficile* associated diarrhea (Hickson *et al.* 2007). The mixture of *St. thermophilus*, *L. acidophilus* and *Bifidobacterium animalis* subsp. *lactis* (YO-MIX™ Y109 FRO 1000) led to a strong TNF-α gene expression and to a moderate reduction of IL-6 mRNA level in the colon of Wistar rats suggesting a pro-inflammatory reaction. In Wistar rats with Trinitroenzenesulfonic acid (TNBS)-induced colitis were observed a strong augmentation of TNF-α and IL-6 which could explicit reduced through the supplementation of the probiotic mix (Amit-Romach *et al.* 2010).

A study in human peripheral blood mononuclear cells (PMBC) researched the affect of *St. thermophilus* THS alone or in combination with *Bifidobacterium longum* 1/10, *Bifidobacterium breve* Bb99 and *Bifidobacterium animalis* subsp. *lactis* Bb12 or non-pathogenic *Escherichia coli* DH5α to the production of TNFα, IL-12, IFN-γ and IL-10. The cytokine production was dose- as well as time-dependent. *St. thermophilus* THS alone induced a strong TNF-α procution, a sign of inflammatory response. In combination with *B. longum* 1/10 was observed a higher TNF-α production, whereas together with *B. breve* Bb99 and *B. lactis* Bb12 did not alter this level. Other *St. thermophilus* THS alone increased explicit the IL-12 as well as IFN-γ concentration, whereas the combination with the three other probiotic bacteria resulted in a suppression of *St. thermuphilus* THS-induced IL-12 and IFN-γ production. No significant influence of IL-10 concentration was found for *St. thermophilus* THS alone. But the combination of *B. longum* 1/10 and *B. breve* Bb99 led to a slight augmentation of IL-10. *St. thermophilus* THS in combination with *E. coli* DH5α showed a higher TNF-α and IL-10 level compare with *E. coli* DH5α-induced stimulation. No alternation was observed for this combination on IL-12 and IFN-γ production (Kekkonen *et al.* 2008). In contrast, in 6 weeks of age old BALB/c mice feeding with

St. thermophilus CRL 412 led to a strong augmentation of IFN-γ and IL-4 concentration after day 2, 5 and 7. No affect was observed of the production of TNF-α, IL-12, IL-2 and IL-10 at all three times. Moreover, *St. thermophilus* CRL 412 did not impair the Th1/Th2 balance and did not change the Bcl2 protein level (Perdigón *et al.* 2002). Cell debris of *Streptococcus thermopilus* MB455 did not affect the IL-8 production in HT-29/19A cells, but extract fraction of this strain increased the IL-8 level at a concentration of 10^7 to 10^{10} cfu/ml (Lammers *et al.* 2002).
These data suggest the immunoregulated effect of *St. thermophilus* is strain-dependent as well as is affected by the presence of other bacteria and further depend on the researched model.

1.8.2 Control bacteria

1.8.2.1 Eubacterium biforme^T

Eubacterium biforme^T is an obligatory anaerobic, gram-positive and non-motile bacterium occurring in rods and coccoid from in pairs or chains. The optimal growth temperature is 37°C, but some strains are able to grow at 45°C. *E. biforme*^{T} ferments PY-glucose-Tween 80 into DL-lactic acid and butyric acid as well as into moderate amounts of ethanol and occasionally succinice, pyruvic and acetic acid. The fermentation of PY-pyruvate-Tween 80 results in butyrate as well as lactate and acetate production. *E. biforme*^{T} is a normal inhabit of the human intestinal tract (v. Holdeman and Moore 1974).

1.8.2.2 Escherichia coli^{TC} XL blue

Escherichia coli is a gram negative rod-shaped bacterium which is able to grow under facultative anaerobic conditions at an optimal temperature of about 37°C. Under anaerobic atmosphere *E. coli* used mixed-acid fermentation with the production of lactate, succinate and acetate. The harmless strains of *E. coli* inhabit the lower intestine in humans as well as animals. As a part of the normal microbiota *E. coli* has the function to promote the host against the establishment of pathogenic bacteria. E. coli is also able to produce vitamin K2 (MicrobeWiki 2008). The *E. coli* strain XL blue is characterized through its nalidixic acid and tetracycline resistant (OpenWetWare).

An experiment comparing the effect of gram-positive and gram-negative bacteria in non-polarized Caco-2 cells, showed *E. coli* strains led to a strong increase of IL-8 concentration in a strain-dependent manner. *E. coli* Nissle resulted in a lower augmentation than the *E. coli* strains F 18, BJ4, MG1655 and UTI. Interestingly, live *E. coli* strains have a stronger affect than UV- killed *E. coli* strains. Moreover, *E. coli* strains led to a higher rise of IL-8 production as *Bifidobacterium* ssp. or *Lactobacilus* ssp. A similar effect was observed for the TGF-β1 production. And only the gram-negative *E. coli* strains induced a semimaturation of dendritic cells which underlied Caco-2 cell layer in a TLR-4-dependent manner (Zeuthen *et al.* 2010).

The non-pathogenic *E. coli* strain K12 reduced the concentration of IL-8, IL-6, TNF-α, IL-1β after apical stimulation of Caco-2 cells in the basolateral presence of leucocytes compared to the direct treatment of bacteria on leucocytes. The apical addition of bacteria to the Caco-2 cells especially augmented the IL-8 and IL-6 concentration, but the measured concentrations were far below those observing after apical bacteria stimulation of Caco-2 cell layer in the basolateral presence of leucocytes. The IL-8 mRNA expression was strong increased in Caco-2 cells by 15.4-fold and in leucocytes by 10.4- fold which did not attended by the observed relation of rose IL-8 production (Parlesak *et al.* 2004). The augmentation of IL-8 expression in Caco-2 cells after stimulation with non-pathogenic *E. coli* seems to be dose-dependent and can significantly suppressed through the presence of *Lactobacillus rhamnosus* GG and *Lactobacillus casein* at a concentration of 10^{10} to 10^{11}cfu/ml. Butyrate at a concentration of 10 mM and 20 mM was also capable to inhibit the *E. coli*-induced IL-8 expression. The LPS from *E. coli* did not affect the IL-8 mRNA level and *E. coli* flagellin led to a slight increase (Shinji *et al.* 2009).

2 OBJECTIVES

A long-term altered expression of chemokines such as IL-8 or cytokines such as IL-17 can be a marker for all activations but can also cause an over-reaction of the human immune system and can lead to chronic inflammatory bowel diseases including Crohn's diseases and colitis ulcerosa or diverse immunopathologycal disorders (Remick 2005; Miossec *et al.* 2009). It is well know that the kind of diet and special foods or nutrients have an effect on appearance and concentration of inflammatory mediators and may affect the symptoms of diseases on a beneficial but also on a negative way. As such special food probiotic bacteria are discussed.
In many cell culture or animal studies were demonstrated that IL-8 gene expression and IL-8 protein release are mediated by a variety of commensal and pathogenic bacteria (Hörmannsperger and Haller 2010). During the last years the role of IL-17 get more and more clear and it seems that IL-17 plays also a crucial role in the inflammatory regulation (Miossec *et al.* 2009).

The aim of this diploma thesis was to investigate the influence of probiotic bacteria on the inflammatory mediator IL-8 and IL-17 as well as their affect on caspase-3 mediated apoptosis. IL-8 ELISA and gene expression of IL-8, IL-17 and CASP3 was analyzed in Caco-2 cells after the treatment with probiotic bacteria and commensal gut bacteria. The Caco-2 cell line was chosen as a model for the human gut because of its similar properties to mature enterocytes (Sambuy *et al.* 2005).
This study is the first step in the work group of Uni-Doz. Dr. Alexander G. Haslberger to establish Caco-2 cell experiments with bacteria and present the basic for future studies with the focus on the epigenetic mechanism behind the effects of probiotic bacteria.

3 MATERIALS AND METHODS

3.1 Cell line and culture conditions

For this experiment I used the human colon carcinoma cell line CaCo-2 culturing in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum, 4 mM glutamine, and 100 units each of streptomycin and penicillin, at 37°C in a humidified atmosphere with 5% CO_2 in air. After a growth period of about 14 days there was added 10 ng/ml Interleukin(IL)-1β to the half part of cells. After that cells with and without IL-1β were treated with 0.5 ml lactic acid bacteria such as *Lactobacilus acidophilus* NCFM™, *Bifidobacterium animalis* subsp. *lactis* Bt420 and *Streptococcus salivarius* subsp. *thermophilus* ST21 which as grew on medium, as well as supernatant of this three bacteria. I also added lyophilized probiotic bacteria at a concentration of 5 x 10^8 cfu/ml cell medium to the Caco-2 cells without IL-1β pre-treatment. The pepetting scheme is listed below. Further, I added 0.5 ml supernatant of *Escherichia coli*^{TC} XL blue and of the human gut bacteria *Eubacterium biforme*^{T} to the Caco-2 cells. The incubation time averaged 48 hours. For using IL-8 ELISA culture supernatant fluid was stored in aliquots. Cells were washed twice with ice-cold PBS before the DNA and mRNA were isolated.

	L. acidophilus NCFM™	B. animalis subsp. lactis Bt420	St. salivarius subsp. thermophilus ST2
concentration of lyophilisate	150 billion cfu/g	170 billion cfu/g	200 billion cfu/g
stock solution	0.1 g lyophilisate in 0.2 ml cell medium → 7.5 x 10^{10} cfu/ml	0.1 g lyophilisate in 0.2 ml cell medium → 8.5 x 10^{10} cfu/ml	0.1 g lyophilisate in 0.2 ml cell medium → 1x 10^{11} cfu/ml
volume bacterial suspension/ well to get 5 x 10^8 cfu/ml	13 µl	12 µl	10 µl

Table 1: pipetting scheme for lyophilized probiotic bacteria to get a final concentration at 5 x 10^8 cfu/ml

3.2 Enzyme-linked immunosorbet assay (ELISA)

To get an overview about the IL-8 concentration after stimulation the IL-8 Eli-pair kit from Diaclone was implemented with the cell media.

3.2.1 Principle of the method

The enzyme-linked immunosorbant assay (ELISA) is a method to measure a single protein in each sample. For the IL-8 measurement I used a double antibody sandwich ELISA. Thus a 96 microtiter plate is covered with IL-8 antibody attaching to the bottom of a well and provides antibody capture. In a second step IL-8 standard dilutions and samples are added to the plate. During the incubation the IL-8 proteins ligate to the antibodies on the bottom of the well. After incubation detection antibody which is conjugate to an enzyme, is added to each well. By dint of an UV plate reader the different intensities of the color are detected. The standard dilutions allow the quantification of IL-8 in the samples (Leng *et al.* 2008).

3.2.2 Procedure

For this experiment the IL-8 Eli-pair kit from Diaclone was used.

Coating

- Per plate 50 µl of the capture antibody was diluted in 10 ml of coating buffer.
- 100 µl of the solution was pipetted into each well of the plate.
- The plate was sealed and incubated overnight at 4°C.
- After incubation the wells were discharged and washed twice with 400 µl of wash buffer.
- 250 µl of saturation buffer was added to the wells and was incubated for 2.5 hours at room temperature.
- In the last step the saturation buffer was discarded and the plate had to dry one day at room temperature.

Sample application

Using the standard stock solution and the standard diluent buffer to receive a standard curve with the following scheme:

standard	concentration in pg/ml	volume standard	volume buffer
1 (stock solution)	2000		
2	1000	210 µl standard 1	210 µl
3	500	210 µl standard 2	210 µl
4	250	210 µl standard 3	210 µl
5	125	210 µl standard 4	210 µl
6	62.5	210 µl standard 5	210 µl

Table 2: IL-8 standard concentration and pipetting schema

- 100 µl of each standard and each sample were applied to the wells in duplicate whereas two wells contained only standard diluent buffer as blank.
- The plate was incubated for two hours at room temperature.
- The detection antibody solution was made out of 100 µl biotinylated detection anti-IL-8 and 5 ml of biotinylated antibody diluent buffer.
- 50 µl of the prepared solution was distributed to each well and was incubated for one hour at room temperature.
- The plate was emptied and washed three times with 400 µl of wash buffer.

Color development

- 5 µl of horseradish peroxidase-streptavidin (HRP-streptavidin) was diluted in 500 µl of HRP- streptavidin diluent buffer and 150 µl of this solution was also diluted in 10 ml of the same buffer.
- 100 µl of the HRP-streptavidin was dispensed to each well and incubated for 20 minutes at room temperature.
- For color development 100 µl of 3,3' 5,5'- Teramethylbenzidine was added to each well and incubated for eight minutes to achieve a color development.
- The color reaction was stopped by adding 100 µl of 1 molar sulfuric acid to the wells.
- The absorbance was measured immediately at 450 nm.

Analysis

The standard curve was drawn in Microsoft Office Excel 2000 and the sample concentrations were calculated with the determined formula.

3.3 Polymerase Chain Reaction (PCR) and real-time PCR analysis

Polymerase chain reaction (PCR) allows the detection of nucleic acid sequences in a complex sample through amplification in a cycle process. During the amplification a large number of identical copies are generated which can be analyzed. In contrast to the end-point PCR measurement, the real- time PCR analysis detects the specific DNA sequences simultaneous to amplification. This detection is provided by monitoring of fluorescence dyes or probes which show the amount of formed products during the amplification.

For the PCR amplification following components are required:

- Desoxynucleotide triphosphats (dNTPs) of the four DNA-bases adenine, guanine, cytosine and thymine as building blocks for the new synthesized DNA fragments.
- DNA-Polymerase, an enzyme building DNA out of dNTPs.
- Magnesium-chloride as a co-factor of the DNA-polymerase.
- Primers, short oligonucleoitides of about 15 to 40 base pairs length flanking the DNA sequence or promotor region of the amplified gene with the function as starter signal for DNA-polymerase. It is important to design a primer which binds stable and specific to the target sequence and not to any other sequences.
- Fluorescent reporter is required for detection and quantification of the formed DNA (Kubista *et al.* 2006).

3.3.1 PCR- reaction

The PCR- reaction is performed by a temperature cycling dividing into three steps: denaturation, annealing and elongation. A normal PCR analysis includes 30 to 40 cycles.

Denaturation

At the first step the double helical DNA is denaturized (melted) at a high temperature between 90°C and 95°C. The used temperature and duration are depending on the length of target DNA as well as on the instrument and used reaction components.

Annealing

In the second step the primer binds to the target DNA whereas the annealing temperature depends on the used primer and its melting temperature. In general, the annealing temperature is between 50°C and 70°C.

Elongation

The Elongation takes place at a temperature about 72°C because this is the optimal temperature for the polymerase binding to the primer and start the DNA strands forming (Kubista *et al.* 2006).

3.3.2 Real-time PCR detection system

For the detection of amplified DNA during the cycles a fluorescent reporter is needed which binds to the target DNA product. Used reporters are fluorescence dyes like SYBR Green I, or probes such as Taqman. They show no fluorescence when they are free, but become a brightly fluorescence after binding to DNA. The fluorescence signal relates with the amount of formed double stranded products. During the initial cycles the signal is low and do not differ from the background signals. After achieving the threshold the signal increase exponentially till the saturated level is reached. At this point the reaction products stagnate cause of a running out of some crucial reaction components (Kubista *et al.* 2006).

3.4 Gene expression

3.4.1 Principle of the method

Measurement the gene expression means the measurement of messenger RNA (mRNA), a product of DNA transcription which is directly proportional to the gene activity. But before using the PCR the mRNA have to be converted into more stable complementary DNA (cDNA) by reverse transcription. Three primering strategies base on anchored-oligo (dT) primer, random hexamer primer and sequence specific primer are used for reverse transcription.

- Anchored-oligo(dT) primer which consists only thymine and binds to the

poly-A tail of mRNAs.

- Random hexamer primer is a small primer of six base pairs length and binds randomly to all mRNAs existing within the sample.
- Sequence specific primer binds only to specific genes sequences or part of their sequences (Kubista *et al.* 2006).

3.4.2 Procedure

3.4.2.1 Genomic mRNA isolation

For the mRNA extraction the NucleoSpin RNA II from Macherey-Nagel was used and followed the instruction applied.

3.4.2.2 Reverse transcription

To convert the mRNA into cDNA the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics) was used.

For each sample agents were mixed as following:

transcriptor reverse transcriptase reaction buffer	4.0 µl
deoxynucleotide mix	2.0 µl
protector RNase inhibitor	0.5 µl
anchored-oligo(dT) primer	1.0 µl
transcriptor reverse transcriptase	0.5 µl
water	6.0 µl
RNA	6.0 µl
total volume	20.0 µl

Table 3: reaction mix for reverse transcriptase

- The mixes were set at 50°C for 60 minutes for transcription.
- The samples were heated up to 85°C for 5 minutes to inactivate the transcriptase.

3.4.2.3 Real-time PCR

The primers, sample composition, and cycling conditions for the IL-8, IL-17 and CASP3 gene expression detection are listed in the tables below.

Primer	Sequence	annealing temperature	concentration
IL-8 sense	ATG ACT TCC AAG CTG GCC GTG GCT	66 °C	1.5 pmol/µl
IL-8 antisense	TCT CAG CCC TCT TCA AAA ACT TCT C	66°C	1.5 pmol/µl
IL-17 sense	ACT TCC ACC GCA ATG AGG AC	67°C	2.5 pmol/ µl
Il-17 antisense	GTG GAC AAT CGG GGT GAC AC	67°C	2.5 pmol/ µl
CASP3 sense	AGG GGA TCG TTG TAG AAG TCT	67°C	5.0 pmol/ µl
CASP3 antisense	GCC AAG AAT AAT AAC CAG GTG CT	67°C	5.0 pmol/ µl

Table 4: Primer sequence, annealing temperature and primer concentration for the gene expression

Sample composition:

	IL-8	IL-17	CASP3
SensiMix	5.0 µl	5.0 µl	5.0 µl
water	2.6 µl	2.8 µl	2.8 µl
primer	1.0 µl	1.0 µl	1.0 µl
Sybr Green	0.2 µl	0.2 µl	0.2 µl
4 mM MgCl2	0.2µl	---	---
total volume	10 µl		

Table 5: reaction mix for real-time PCR

PCR cycling conditions:

	IL-8		IL-17		CASP3	
polymerase activation	10 min at 95°C					
cycling	40 times		45 times		40 times	
denaturation	30 sec	95°C	30 sec	95°C	30 sec	95°C
annealing	40 sec	66°C	40 sec	67°C	40 sec	67°C
elongation	30 sec	72°C	30 sec	72°C	30 sec	72°C
melt curve	0,5°C every 5 sec from 50°C to 90°C					

Table 6: PCR cycling condition for gene expression

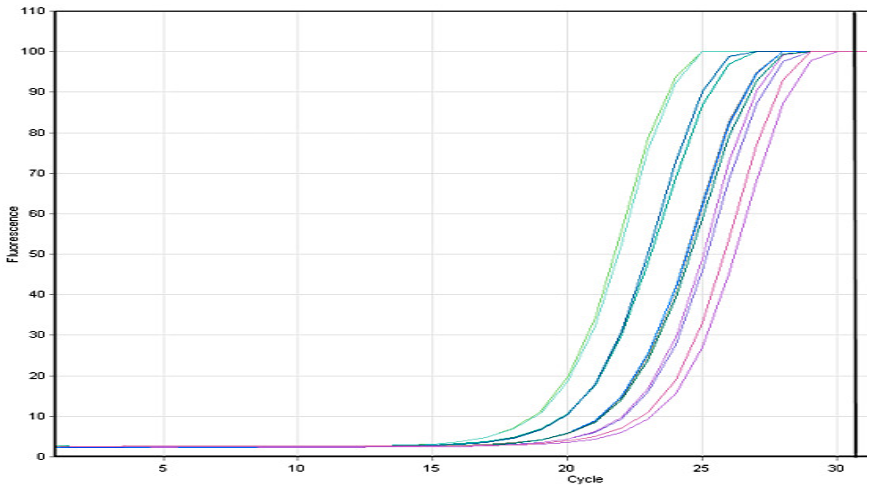

Figure 3: Real-time PCR curve of gene expression reaction

3.5 Data and Statistical analysis

The results of the real-time PCR were analyzed with the software Rotor-Gene Version 6.1 belonging to the Corbett Rotor Gene 3000 real time cycler. Hypothetic concentrations were calculated by the software and the amplification efficiency was adjusted between individual samples. The gene expression of IL-8, IL-17 and CASP3 are normalized to our reference gene Glyceraldehyde-3-phosp (GAPDH). Average and the standard deviations were calculated with Microsoft Office Excel 2000. A two tails t-test was used to determine the significance of the results which also was performed in Microsoft Office Excel 2000. Results were regarded as significantly different at $P < 0.05$.

4 RESULTS

4.1 IL-8 ELISA

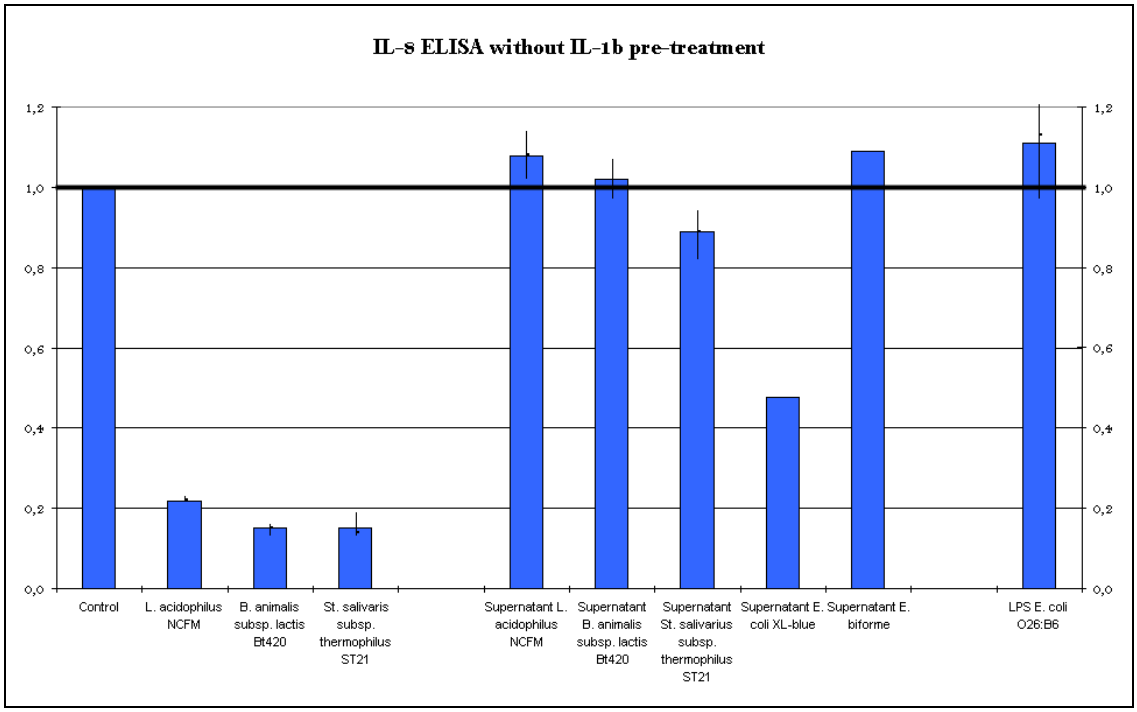

Figure 4: IL-8 concentration in cell medium of Caco-2 cells after treatment with bacteria und bacterial supernatant as well as LPS from E. coli O26:B6

A strong reduction of IL-8 concentration was observed after treatment with the bacteria *Lactobacillus acidophilus* NCFM™ by 0.22 ± 0.01 (P< 0.0001), *Bifidobacterium animalis* subsp. *lactis* Bt420 by 0.15 ± 0.01 (P< 0.0001) and *Streptococcus salivarius* subsp. *thermophilus* ST21 by 0.15 ± 0.03 (P< 0.001). Further, the supernatants of *Escherichia coli*^{TC} XL blue and *St. thermophilus* ST21 also inhibited the IL-8 production by 0.48 and 0.89 ± 0.06 (P< 0.05). No significant affect had the supernatants of *L. acidophilus* NCFM™ (1.08 ± 0.05), *B. lactis* Bt420 (1.01 ± 0.04) and *Eubacterium biforme*^{T} (1.09). The addition of LPS from *E. coli* O26:B6 to Caco-2 cells did not influence the IL-8 level (1.11 ± 0.13) (figure 4).

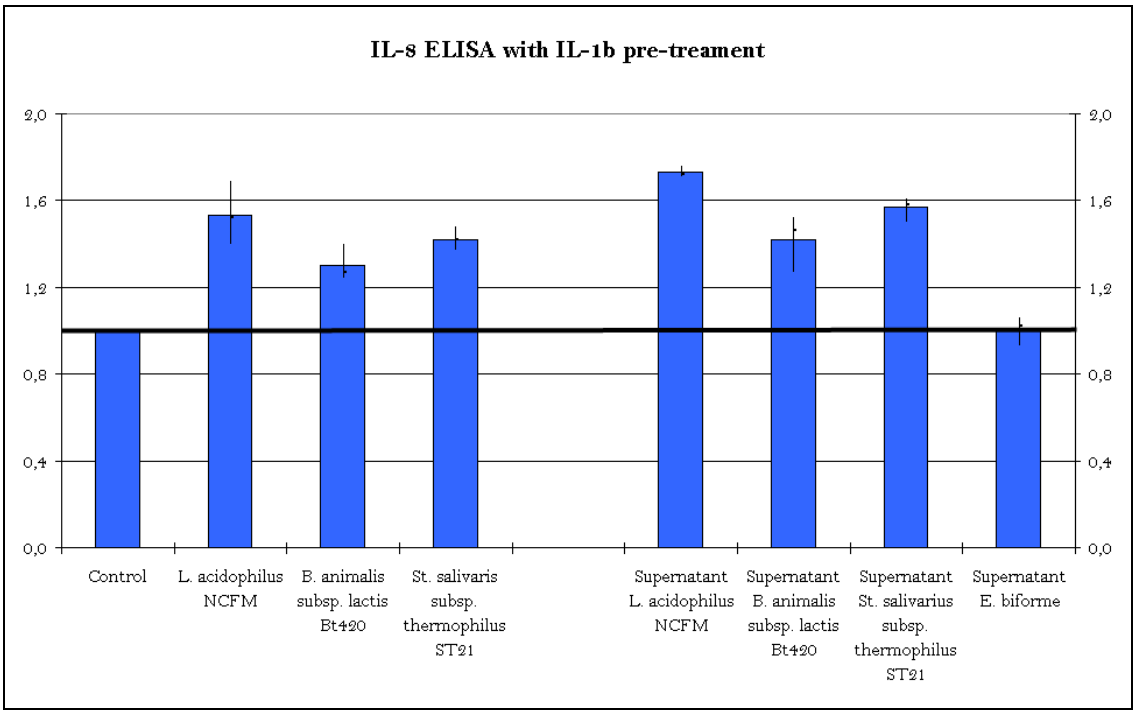

Figure 5: IL-8 production in IL-1β pre-treated Caco-2 cells after stimulation with bacteria and bacterial supernatant

Figure 5 shows the results of IL-8 ELISA in IL-1β pre-treated Caco-2 cells. All three bacteria and their supernatants slight increased significant the IL-8 concentration after IL-1β pre-treatment. Numerical an augmentation was shown for bacteria *L. acidophilus* NCFM™ by 1.53-fold ± 0.12 ($P < 0.01$), *B. lactis* Bt420 by 1.30-fold ± 0.07 ($P < 0.001$) and *St. thermophilus* ST21 by 1.42-fold ± 0.05 ($P < 0.001$). The supernatants of *L. acidophilus* NCFM™, *B. lactis* Bt420 and *St. thermophilus* ST21 induced an IL-8 production by 1.73-fold ± 0.02 ($P < 0.0001$), 1.42-fold ± 0.11 ($P < 0.01$) and 1.57-fold ± 0.05 ($P < 0.0001$). No significant effect was observed for the supernatant of *E. biforme*^{T} (1.01 ± 0.05).

4.2 IL-8 gene expression

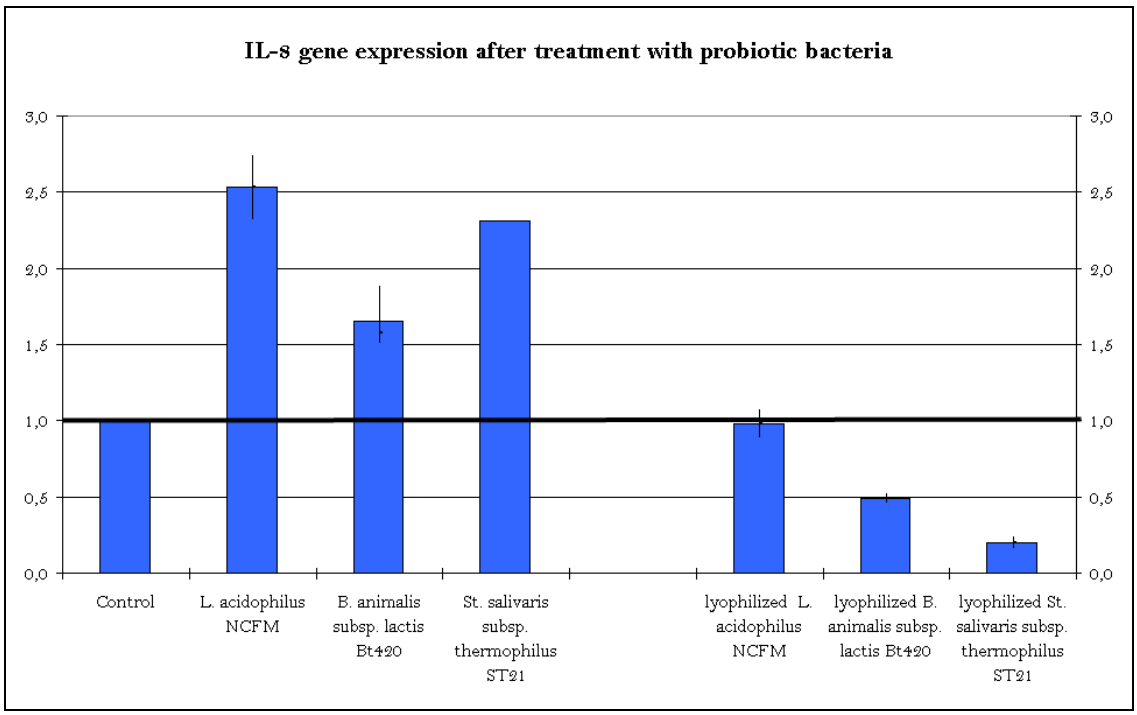

Figure 6: IL-8 gene expression in Caco-2 cells after stimulation with probiotic bacteria and lyophilisate of probiotic bacteria

The results for the IL-8 gene expression after treatment with probiotic bacteria and the lyophilisate of them are listed in figure 6. All bacteria grown in medium led to an increased IL-8 gene expression. The strongest affect was observed for *L. acidophilus* NCFM™ by 2.53-fold ± 0.24 ($P < 0.001$), followed by *St. thermophilus* ST21 with a rise of 2.31-fold and *B. lactis* Bt420 with 1.65 ± 0.18 ($P < 0.001$). In contrast the lyophilized bacteria *St. thermophilus* ST21 reduced the IL-8 mRNA level by 0.20-fold ± 0.04 ($P < 0.0001$) and *B. lactis* Bt420 by 0.49-fold ± 0.03 ($P < 0.0001$). Lyophilized *L. acidophilus* NCFM™ did not alter the IL-8 gene expression (0.98 ± 0.11).

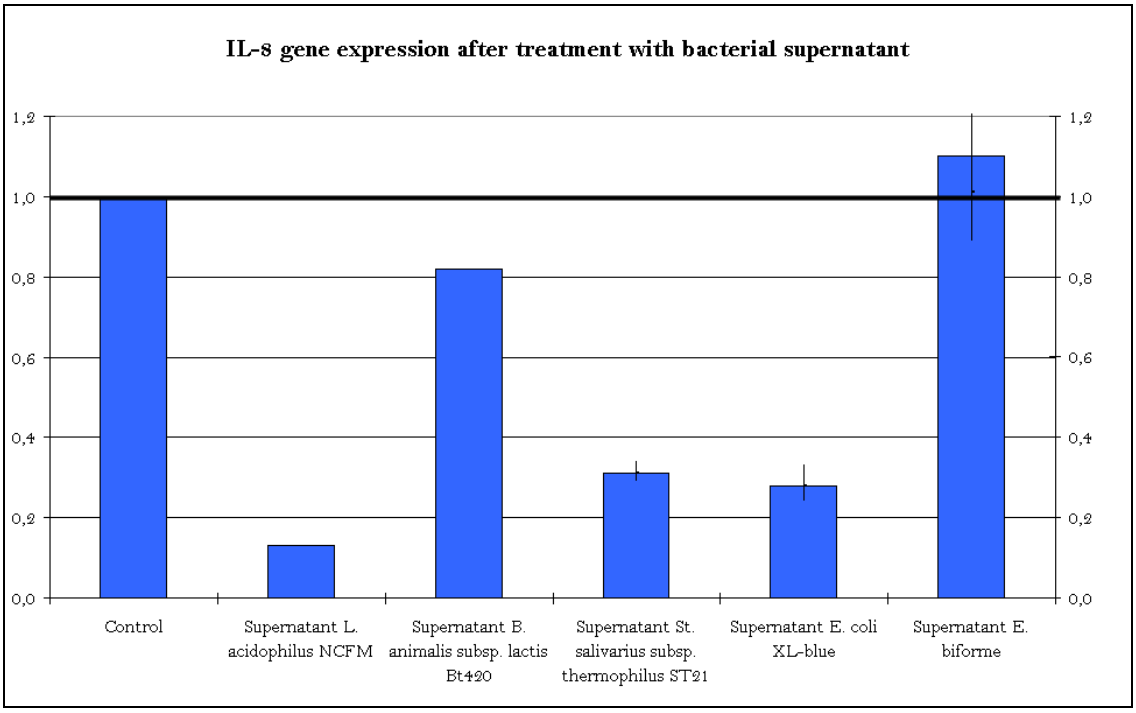

Figure 7: IL-8 expression in Caco-2 cells after stimulation with bacterial supernatant

In contrast to the treatment of bacteria, the stimulation with the supernatants of the bacteria resulted in a down-regulation of IL-8 gene expression in case of *L. acidophilus* NCFM™ by 0.13-fold, *B. lactis* Bt420 by 0.82-fold, *St. thermophilus* ST21 by 0.31-fold ± 0.03 ($P < 0.0001$) and *E. coli* XL blue by 0.28-fold ± 0.05 ($P < 0.0001$). The supernatant of *E. biforme*^{T} had no significant affect on IL-8 mRNA level (1.10 ± 0.18).

4.3 IL-17 gene expression

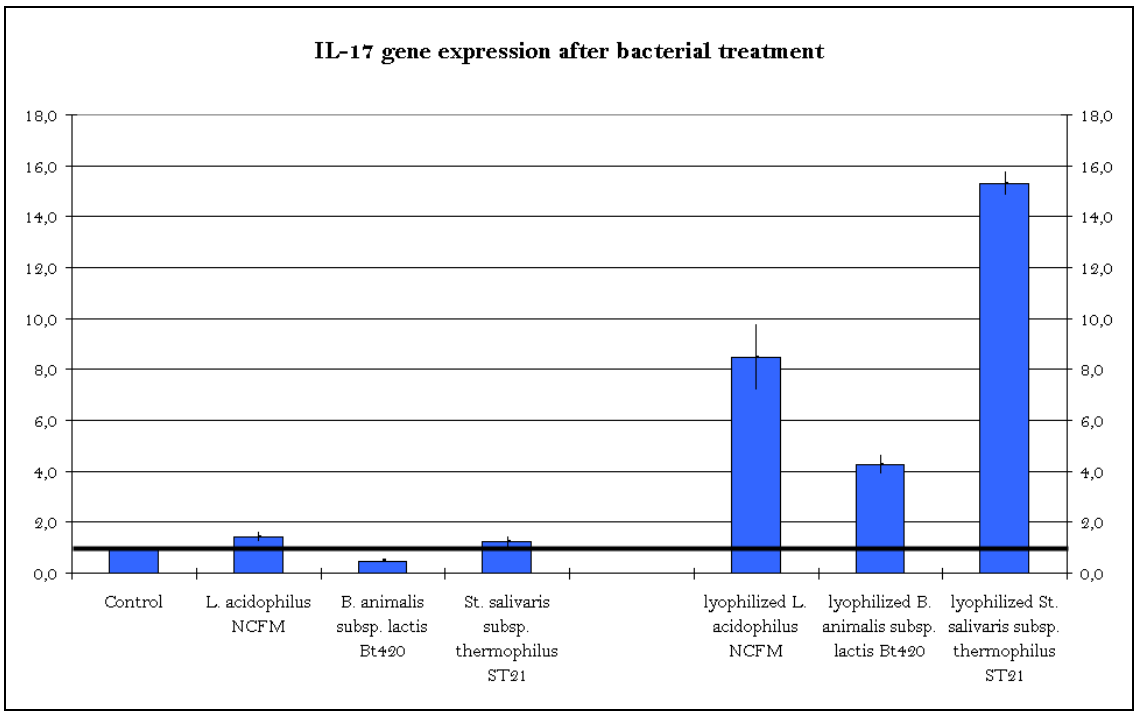

Figure 8: IL-17 gene expression in Caco-2 cells after stimulation with probiotic bacteria and lyophilized probiotic bacteria

A slow significant augmentation of IL-17 gene expression was seen for *L. acidophilus* NCFM™ by 1.42-fold ± 0.20 ($P < 0.01$). To a reduction of IL-17 mRNA level led *B. lactis* Bt420 by 0.48-fold ± 0.02 ($P < 0.01$). No significant influence had the stimulation with *St. thermophilus* ST21 (1.23 ± 0.22). All lyophilized probiotic bacteria induced a strong up-regulation of IL-17 gene expression. The strongest effect was observed for the lyophilisate of *St. thermophilus* St21 by 15.30-fold ± 0.51, followed by lyophilized *L. acidophilus* NCFM™ (8.47 ± 1.48) and lyophilized *B. lactis* Bt420 (4.27 ± 0.42).

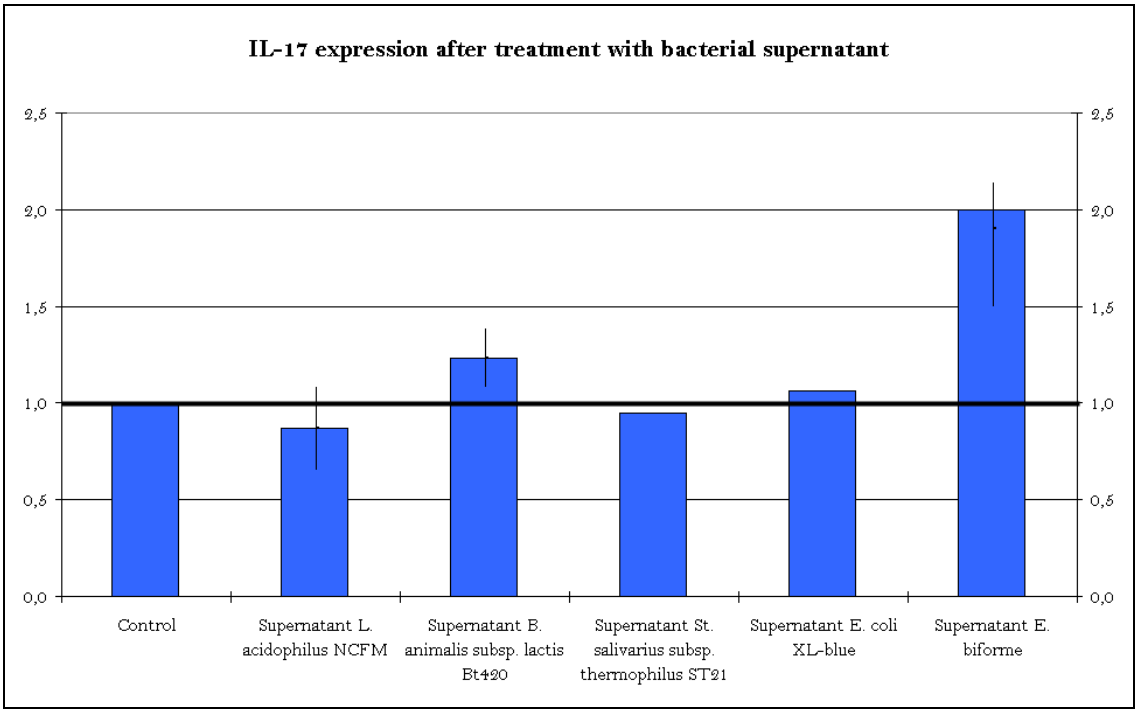

Figure 9: IL-17 gene expression in Caco-2 cells after stimulation with bacterial supernatant

Only the supernatant of *E. biforme*^{T} led to a significant augmentation of IL-17 gene expression by 2.00-fold ± 0.13 ($P < 0.0001$). The other supernatants did not alter the IL-17 gene expression (*L. acidophilus* NCFM™ by 0.87± 0.25, *B. lactis* Bt420 by 1.23 ± 0.17, *St. thermophilus* ST21 by 0.95 and *E. coli* XL blue by 1.06).

4.4 CASP3 gene expression

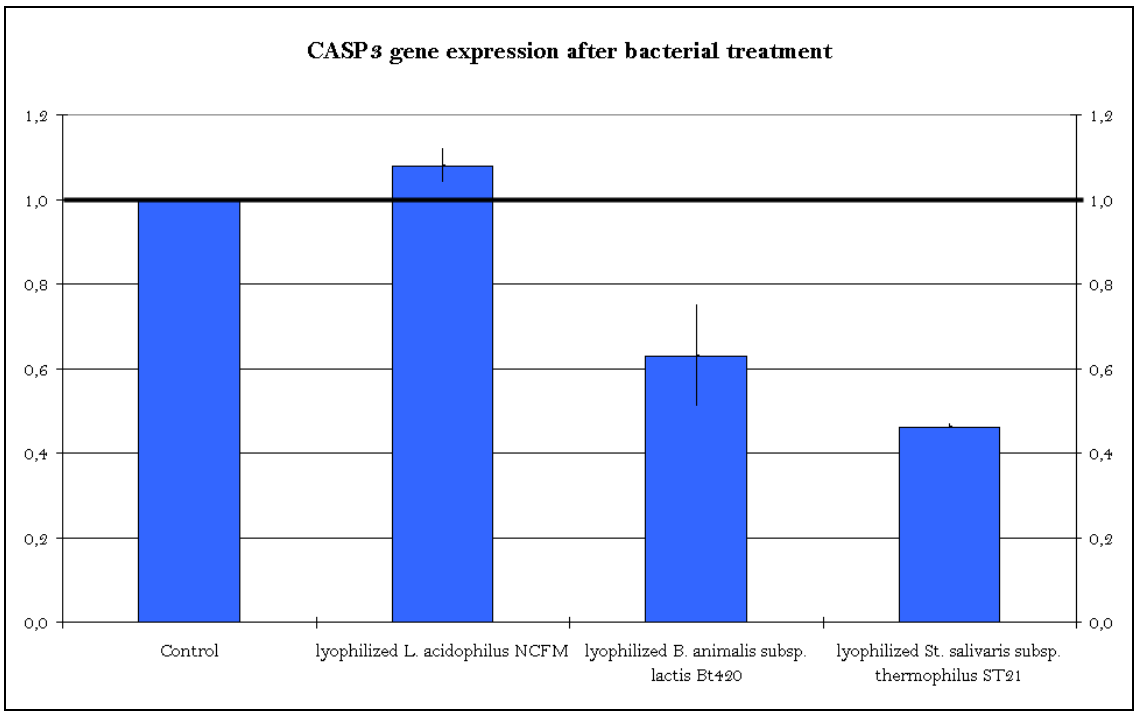

Figure 10: CASP3 gene expression in Caco-2 cells after stimulation with lyophilized probiotic bacteria

The lyophilized probiotic bacteria *St. thermophilus* ST21 inhibited the CASP3 gene expression by 0.46 ± 0.01 ($P < 0.001$) and *B. lactis* Bt420 led to an Il-17 mRNA level reduction by 0.63 ± 0.14 ($P < 0.01$). No significant alternation was observed for the treatment with the lyophilisate of *L. acidophilus* NCFM™ (1.08 ± 0.05).

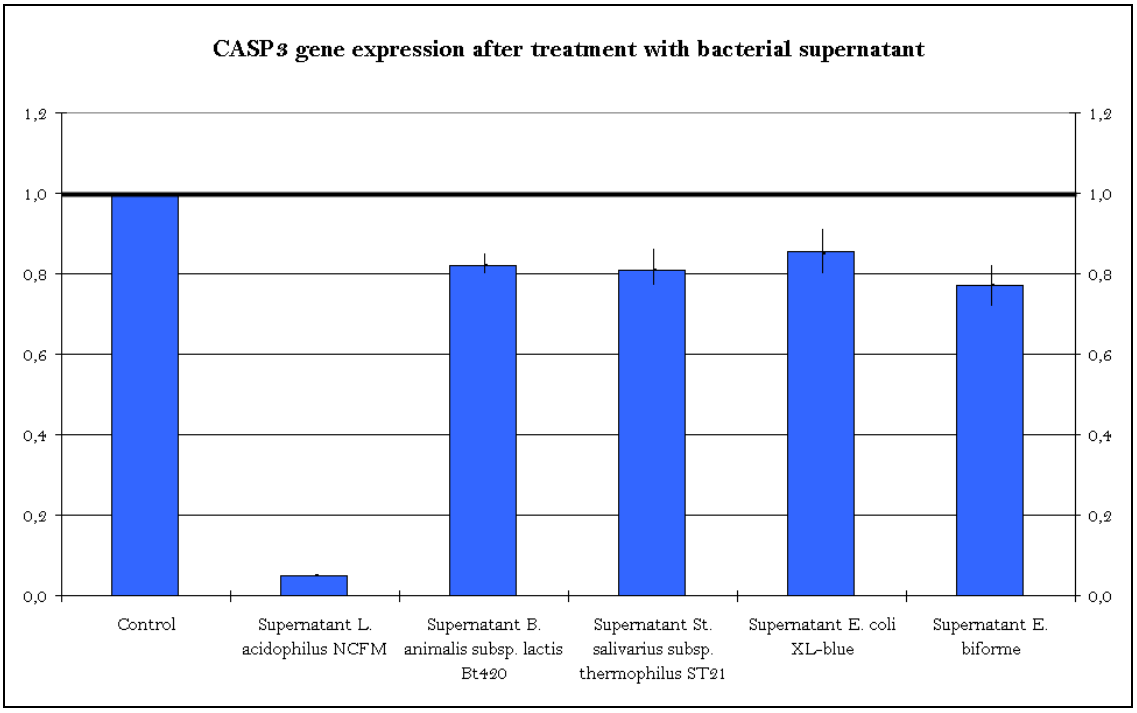

Figure 11: IL-17 gene expression in Caco-2 cells after treatment with supernatant of bacteria

An explicit reduction of CASP3 gene expression was showed for the treatment with supernatant of *L. acidophilus* NCFM™ by 0.05-fold ± 0.01 ($P < 0.0001$). A slight, but significant decrease was resulted after the stimulation with the supernatants of *B. lactis* Bt420 by 0.82-fold ± 0.03 ($P < 0.0001$), *St. thermophilus* ST21 by 0.81-fold ± 0.07 ($P < 0.001$), *E. coli* XL blue by 0.85-fold ± 0.08 ($P < 0.01$) and *E. biforme*^{T} by 0.77-fold ± 0.08 ($P < 0.001$).

5 DISCUSSION

5.1 Probiotic bacteria

5.1.1 Lactobacillus acidophilus NCFM™

The *Lactobacillus acidophilus* strain NCFM™ is well known and is used in dairy products and other fermented products since about 30 years. Its beneficial effects on humans health includes exclusion of pathogenic bacteria, reduction of the incidence of pediatric diarrhea, lower the concentration of toxic amines in blood of dialysis patients with small bowel bacterial overgrowth and decrease of cholesterol level. An adequate daily intake of *L. acidophilus* NCFM™ may facilitates the lactose digestion in lactose-intolerant individuals (Sanders and Klaenhammer 2001). In children at the age of 3 to 5 years *L. acidophilus* NCFM™ alone or in combination with *Bifidobacterium animalis* subsp. *lactis* Bi-07 reduce explicit the incidence of fever, cough and rhinorrhea as well as the duration of illness and the use of antibiotics during the six winter months. In few studies it could be shown that *L. acidophilus* NCFM™ induces a production of pro- and anti-inflammatory cytokines such as IL-6 and IL-8 or IL-10 in the intestinal epithelial cells as well as dendritic cells (Konstantinov *et al.* 2008; Zeuthen *et al.* 2010).
Many data suggest a protective role of probiotics in case of bacteria-induced infection. It could be shown that a pre-treatment with (potential) probiotic bacteria inhibit a pro-inflammatory response (Nemeth *et al.* 2006; Roselli *et al.* 2006; Lopez *et al.* 2008; Nandakumar *et al.* 2009).
I have found similar results in my experiments. As inflammatory marker used the chemotractic cytokine Interleukin(IL)-8 which is released in response to microbial stimulation and in the presence of pro-inflammatory cytokines such as tumor necrosis factor (TNF) and IL-1. This chemokine is a good inflammatory marker because IL-8 occurs in the early state of an inflammation and cause of its longevity at site of acute inflammation. Further, IL-8 plays a crucial role in the neutrophli recruitment and regulation of inflammatory response in general (Baggiolini *et al.* 1995; Remick 2005).
The addition of *L. acidophilus* NCFM™ grown in medium to Caco-2 cells led to a strong reduction of the pro-inflammatory cheomokine IL-8 by 0.22-fold ± 0.01 ($P < 0.0001$). Whereas the pre-stimulation with the pro-inflammatory cytokine IL-1β

followed by *L. acidophilus* NCFM™ apply resulted in a slight increase of IL-8 production by 1.53-fold ± 0.12 ($P < 0.01$) compared to the IL-1β-induced IL-8 production. That indicates *L. acidophilus* NCFM™ has no anti-inflammatory effect after an induced pro-inflammatory reaction. This finding is contrary to the published results of Candela who could observe a suppressive effect of *L. acidophilus* in case of IL-1β-induced IL-8 production (Candela *et al.* 2008). Perhaps the modulation of IL-1β-induced IL-8 secretion is strain-specific. Interestingly, the IL-8 gene expression was up-regulated by 2.53-fold ± 0.24 ($P < 0.001$). Therewith the IL-8 gene activity is in contrast to the measured IL-8 protein concentration suggesting *L. acidophilus* NCFM™ itself induces a pro-inflammatory response at transcriptional level, but there might be also other regulatory mechanisms which blocked the pro-inflammatory cascade within Caco-2 cells. The work group of Wang et al have observed the same phenomenon for the bacteria species *Enterococcus feacalis* (EC1, EC3, EC15, EC16), *Lactobacillus rhamnosus* GG and *Lactobacillus bulgaris* D1 in Caco-2 cells (Wang *et al.* 2008). In TNF-α pre-treated HT-29 cell line was also found an increased IL-8 mRNA level, but a lower extracellular IL-8 concentration after addition of *Lactobacillus plantarum* 299v (McCracken *et al.* 2002).

There are two regulation mechanisms which could explain this observation - either a post-transcriptional or a post-translational regulatory mechanism. The post-transcriptional regulons coordinate the mRNA stability and the translation of mRNA into proteins. The most post-transcriptional control mechanisms interact with the 3' untranslated region (UTR) of mRNA to block the protein synthesis because UTRs encoding often proteins which are able to induce mRNA degradation and/ or translation inhibition. Such mechanisms targeting either the mRNA translation initiation process or lead to a selectively inhibition of the translation of capped mRNA as well as to a reduction of recruited 60S ribosomal subunits and other RNA-independent factors. For instance, Interferon-γ-activated inhibitor of translation (GAIT) complex binds to GAIT elements which are located in 3' UTRs of a selected mRNA group encoding inflammatory mediators. After binding to GAIT elements, the GAIT complex influences components of the translation initiation pathway to block the protein synthesis. Other examples are the transcriptional co-activator SRC3 increasing the activity of several transcription factors involving in the inflammatory

response, and diverse zinc proteins or miRNA. Interestingly, mediators involving in mRNA degradation or inhibition of mRNA translation, are capable to repress rapidly the protein expression despite an on-going gene transcription (Anderson 2010). In addition to the post-transcriptional mechanisms, the post-translation regulation is also a crucial step in the secretion or storage as well as degradation of produced proteins. The synthesized chemokines contain a single peptide that mediates the chemokines transport into the secretory apparatus of the cell. Chemokines of the membrane-bound chemokines family are released after the proteloytic cleavage by proteases of ADAM family. The release of chemokines is limited by diverse complex interactions with extracellular as well as cell surface components. But chemokines can be also stored within the cells to mediate a more rapidly secretion on a further cell activation. It seems that IL-8 storage has the function as endothelial cell memory on inflammatory stimulation (Comerforda and Nibbs 2005).

Hörmannsperger et al have demonstrated in an experiment that *L. casein* did not impair the TNF-induced interferon-inducible protein (IP-) 10 promotor activity and gene expression of IP-10 mRNA, a pro-inflammatory T cell chemokine, but it blocked the IP-10 protein secretion as well as IP-10 mediated T cell transmigration. The anti-inflammatory effect was caused by a heat-labile *L. casein* cell surface protein which did not alter the IP-10 protein production, but it led to a lower level of intracellular IP-protein stability blocking the IP-10 secretion. The blocking of IP-10 secretion through brefeldin A resulted in a degradation of IP-10 instead of intracellular accumulation. Moreover, *L. casein*-induced ubiquitination of IP-10 regulated the degradation of chemokines without affect to the proteins for proteasomal and lysosomal degradation. Further, the *L. casein* surface protein had properties as 3-methyladenine (3-MA) leading to an inhibition of vesicular trafficking of IP-10 and its intracellular degradation. This affect was not observed for IL-6. That suggests probiotic bacteria are able to mediate post-transcriptional or post-translational regulation mechanisms in intestinal epithelial cells and have so an anti-inflammatory effect (Hörmannsperger *et al.* 2009).

In contrast to the bacteria grown in medium, the lyophilized *L. acidophilus* NCFM™ did not alter the IL-8 gene expression (0.98 ± 0.11). The reason for this observation could be different medium growth conditions and different metabolism as well as metabolites. These findings suggest that the IL-8 response in Caco-2 cells depends on

the condition in which bacteria are applied.

The supernatant of *L. acidophilus* NCFM™ showed a strong reduction of IL-8 mRNA by 0.13-fold without a significant alternation of IL-8 protein concentration suggesting cell wall components of *L. acidophilus* NCFM™ induced another inflammatory response than its metabolites. The discrepancy between IL-8 mRNA level and secreted IL-8 also indicates the involvement of post-transcriptional or post-translation mechanisms.

As the second inflammatory indicator I used the pro-inflammatory cytokine Interleukin (IL)-17. It is released major by T_h17 cells and occurs in the late state of inflammatory response. The target cell type of IL-17 is neutrophil. IL-17 secretion is associated with chronic inflammation and serve immunopathologic conditions (Zaph *et al.* 2008). As far as I know there are no studies which have focused on IL-17 gene expression in enterocytes. *L. acidophilus* NCFM™ induced a slight augmentation of IL-17 gene expression by 1.42-fold ± 0.20 ($P< 0.01$) and lyophilisate of *L. acidophilus* NCFM™ increased it even by 8.47 ± 1.48. Whereas the supernatant of *L. acidophilus* NCFM™ did not alter significant the IL-17 mRNA level (0.87± 0.25) suggesting the metabolites of *L. acidophilus* NCFM™ did not induce a response in enterocytes. In contrast to that the presence of the bacteria activates an inflammatory response at the transcriptional level. My data indicates that *L. acidophilus* NCFM™ grown in medium as well as lyophilized *L. acidophilus* NCFM™ induce an augmentation of IL-17 gene expression, but I'm not able to describe the scale of this effect because in non-treated Caco-2 cells the IL-17 gene expression was to small (a gently inclined of cycle curve compare to the others) to use it as a "good" control value. Further, I did not measure the IL-17 protein concentration thus I am not able to say if this increase mRNA level is also reflected in the IL-17 extracellular protein level or if even IL-17 is released by enterocytes such as Caco-2 cells. But in murine splenocytes treated with TGF-β plus IL-6 were shown a drastic increase of IL-17 production and a lower IL-27 concentration. The addition of heat-killed *Lactobacillus acidophilus* and *Lactobacillus bulgaris* induced a slight IL-17 production and a reduction of IL-27 (Tanabe *et al.* 2008) indicating *L. acidophilus* induced a IL-17 production which attendant with a higher IL-17 mRNA level. It is published that commensal bacteria have a suppressive effect of IL-17 concentration in larger intestine, but no influence of *Lactobacillus*

species was found in lamina propria of small intestine (Dogi *et al.* 2008). Thus the immunomodulation properties of *L. acidophilus* in connection with IL-17 seem to be depends on location of stimulation.

To see if the used probiotic bacteria have a cytotoxic effect to the Caco-2 cells, I analyzed the CASP3 gene expression. Caspase3, a proteolytic enzyme, is a member of the executioner caspases leading directly to apoptosis, the programmed cell death. Because caspase 3 is activated through both, the extrinsic as well as intrinsic pathway, it is a good marker for induced cell death. The regulation of apoptosis is essential for the maintenance of cell integrity and intestinal epithelial cell function (Perl *et al.* 2005). The lyophilisate of *L. acidophilus* NCFM™ did not change the CASP3 gene activity (1.08 ± 0.05). I found only an explicit reduction of CASP3 gene expression in the case of supernatant of *L. acidophilus* NCFM™ by 0.05-fold ± 0.01 ($P < 0.0001$) indicating metabolites of *L. acidophilus* NCFM™ are capable to induce an anti-apoptotic effect in Caco-2 cells. In the case of *Lactobacillus rhamnosus* GG was observed a reduction of caspase-3 activity in *Heliobacter pylori*-infected Caco-2 cells (Myllyluoma *et al.* 2008) and a decrease of a chemical-induced caspase-3 cleavage as well as the number of epithelial apoptotic cells in the murine gut of two week-old mice (Lin *et al.* 2008). This data suggests the effect to caspase-3 activity seems to be strain-dependent and is may induced by metabolites of the bacteria.

5.1.2 Bifidobacterium animalis subsp. lactis Bt420

Bifidobacterium animalis subsp. *lactis* Bt420 (*B. lactis* Bt420) has some beneficial effects for the human. On the one hand its metabolic function including producing of short chain fatty acids like acetate and lactate as well as the synthesis of folic acid (Nurmi *et al.* 2005) and on the other hand its anti-microbial activity against *Staphylococcus aureus* and *E. coli* (Klein *et al.* 2008).The consumption of *B. lactis* 420 in combination with other probiotic bacteria is associated with beneficial stimulation effect of cellular immune system (Klein *et al.* 2008) leading to a lower incidence and duration of infection among healthy individuals (Roessler (nee Klein) *et al.* 2007), and to a reduction of serum level of triacylglyceroles (Klein *et al.* 2008).

In my Caco-2 experiment the treatment with *B. lactis* Bt420 resulted in a drop of IL-8

concentration by 0.15-fold ± 0.01 (P< 0.0001) attendant with a suppression of inflammatory response like describes in human studies. Stimulation with IL-1β followed by addition of *B. lactis* Bt420 showed a small, but significant increase by 1.30-fold ± 0.07 (P< 0.001) compare with Caco-2 cells treated with IL-1β alone. This observation suggests that a supplementation with *B. lactis* Bt420 during an infection does not have anti-inflammatory effects. Like observed for *L. acidophilus* NCFM™, *B. lactis* Bt420 also led to an up-regulation of IL-8 mRNA expression by 1.65 ± 0.18 (P< 0.001) which is contrary to the measured reduced IL-8 protein level. The reason for this observation could be post-transcriptional or post-translational mechanisms which are described in detail above by *L. acidophilus* NCFM™. Because *B. lactis* Bt420 led to a slighter increased IL-8mRNA expression as well as IL-8 production after IL-1β pre-treatment, it could be suggest that *B. lactis* Bt420 induced a slighter pro-inflammatory response compare to *L. acidophilus* NCFM™. Interestingly, the lyophilisate of *B. lactis* Bt420 inhibited the IL-8 expression by 0.49 ± 0.03 (P< 0.0001). Thus there is evidence that the condition in which the bacteria are applied or the medium growth conditions are crucial for the induced inflammatory response in Caco-2 cells.

The supernatant of *B. lactis* Bt420 showed no significant alternation of IL-8 concentration (1.01 ± 0.04) in Caco-2 cells, but an increased IL-8 expression level by 1.42-fold ± 0.11 (P< 0.01) in IL-1β pre-treated Caco-2 cells in addition to *B. lactis* Bt420. The similar increase of IL-8 production in IL-1β pre-treated Caco-2 cells after stimulation with the bacterium or its supernatant indicates that supplementation with live bacteria or their supernatant have the same effect in infected cells, but the differences of IL-8 secretion in "healthy" Caco-2 cells suggest whole bacteria with its cell components are required for the protective effect of *B. lactis* Bt420. In contrast to the bacterium *B. lactis* 420, its supernatant induced a slight reduction of IL-8 mRNA by 0.82-fold which does not alter the IL-8 protein concentration suggesting post-transcriptional and/ or post-translational regulations are involved in the immune response.

Further, *B. lactis* Bt420 is the only of my analyzed probiotic bacteria leading to a reduction of IL-17 gene expression by 0.48-fold ± 0.02 (P< 0.01). In contrast the lyophilized *B. lactis* Bt420 induced an IL-17 expression by 4.47 ± 0.42. That result

indicates a protective effect of *B. lactis* Bt420 in case of chronic inflammation or immunopathologic disorders which are mediated by IL-17. But I did not measure the IL-17 extracellular protein concentration, so it is unclear if the altered gene activity is also reflected in the protein level. Or if IL-17 is generally released by Caco-2 cells because as far as I know IL-17 secretion was only found in T_h17 or nature killer cells (Zaph *et al.* 2008; Gaffen 2009) as well as in murine splenocytes (Tanabe *et al.* 2008) and monocytes-derived dendritic cells (López *et al.* 2010). In an experiment with TGF-β plus IL-6 treated murine splenocytes was observed a drastic increase of IL-17 production and a lower IL-27 concentration. The addition of heat-killed *Bifidobacterium infanits* to the murine splenocytes reduced the TGF-β- and IL-6-induced IL-17 production and increased the IL-27 secretion. Whereas heat-killed *Bifidobacterium bifidum* led to a higher IL-17 concentration (Tanabe *et al.* 2008). This finding indicates that the affect to IL-17 production seems to be species specific. In case of *B. lactis* Bt420 it may be an IL-17 inhibitory effect. Because its supernatant did not significantly impair the IL-17 gene expression (1.23 ± 0.17) in Caco-2 cells suggesting the effect has to be mediated by bacterial cell surface components.

The CASP3 gene expression was slighter inhibited through the supernatant of *B. lactis* Bt420 by 0.82 ± 0.03 ($P< 0.001$) and has thus not a cytotoxic effect. The stimulation with lyophilized *B. lactis* Bt420 also reduced the CASP3 mRNA-level by 0.62 ± 0.14 ($P< 0.01$). In the case of *B. lactis* Bt420 I have expected an increased CASP3 gene expression because for *Propionibacteria* species were found a cleavage of caspase-3 activity in Caco-2 cells caused of the production of propionate and acetate (Jan G. 2002). *B. lactis* strain also produce acetate (Nurmi *et al.* 2005). And for *B. lactis* strain Bb12 an apoptosis-inducing effect in Caco-2 cells was observed (Altonsy *et al.* 2010). But maybe *B. lactis* Bt420 produce acetate in to small concentration that this production does not affect the CASP3 gene activation. And it also produces lactate which may inhibit the cytotoxic effect of acetate.

5.1.3 Streptococcus salivarius subsp. thermophilus ST21

Streptococcus salivarius subsp. *thermophilus* (*St. thermophilus*) is common used as starter culture in combination with other probiotic bacteria, whereas the immunomodulated effect is strong depends on used *St. thermophilus* strain as well as the combinated probiotics and the kind of observed model. As an example, in children no significant differences were observed in the rate of immunoglobulin and isoagglutinin acquisition among children supplemented for 4 months with low-fat milk fermented by *St. thermophilus* or low-fat milk fermented by *St. thermophilus* and *Lactobacillus casein* which was further added with *Lactobacillus acidophilus*, oligofructose and inulin after fermentation process (Pérez *et al.* 2009). But in elderly hospital patients the supplementation with a probiotic drink containing *L. casein*, *Lactobacillus bulgaris* and *St. thermophilus* twice a day led to reduced incidence of antibiotic or *Clostridium difficile* associated diarrhea (Hickson *et al.* 2007). And in Wistar rats feeding with a mixture of *St. thermophilus*, *Lactobacillus acidophilus* and *Bifidobacterium animalis* subsp. *lactis* (YO-MIX™ Y109 FRO 1000) were found a strong gene expression of the pro-inflammatory TNF-α and a moderate reduction of the IL-6 mRNA level, an anti-inflammatory act cytokine, in the colon. In Wistar rats with Trinitroenzenesulfonic acid (TNBS)-induced colitis were observed a strong augmentation of TNF-α and IL-6 which could explicit reduced through the supplementation of the probiotic mix (Amit-Romach *et al.* 2010).

For the *St. thermophilus* strain ST21 I could observe a decrease IL-8 concentration by 0.15-fold ± 0.03 ($P < 0.001$) in Caco-2 cells and a slight increase of IL-8 production by 1.42-fold ± 0.05 ($P < 0.001$) in IL-1β pre-treated Caco-2 cells. That was also found for the other two probiotic bacteria which indicate a preventive anti-inflammatory effect of the used lactic acid bacteria, but sadly no protective effect after an induced pro-inflammatory response.

Interestingly, like the other two probiotic bacteria *St. thermophilus* ST21 induced an up-regulation of IL-8 gene expression by 2.31-fold which is contrary to the measured IL-8 protein level. To explain this phenomenon also in the case of *St. thremophilus* ST21 post-transcriptional and/or post-translational mediated regulations have to be involved like described above by *L. acidophilus* NCFM™. The lyophilized *St. thermophilus* ST21 blocked the IL-8 gene expression by 0.20 ± 0.04 ($P < 0.0001$)

indicating the affect to IL-8 gene expression is mediated by conditions in which the bacteria are grew and also on its metabolism and its metabolites.

The supernatant of *St. thermophilus* ST21 is the only one of the lactic acid bacteria supernatants leading to a lower concentration of IL-8 by 0.89-fold ± 0.06 ($P < 0.05$) in Caco-2 cells. An increase by 1.57-fold ± 0.05 ($P < 0.0001$) was measured in IL-1β pre-treated Caco-2 cells in addition to *St. thermophilus* ST21 supernatant suggesting a small preventive anti-inflammatory effect of *St. thermophilus* ST21 supernatant, but without a protection effect in Caco-2 cells with an induced inflammatory cascade. The IL-8 mRNA expression was explicit blocked by 0.31-fold ± 0.03 ($P < 0.0001$) after stimulation with supernatant of *St. thermophiuls* and is reflected in a slight reduced IL-8 secretion. The IL-17 gene expression was not affected by the treatment with *St. thermophilus* ST21 and its supernatant (1.23 ± 0.22 and 1.23 ± 0.17). Whereas lyophilized *St. thermophilus* ST21 induced a strong augmentation by 15.30 ± 0.51. This observation indicates that the affect of *St. thermophilus* St21 on IL-17 response is modulated by the condition in which the bacteria are applied and thus by different metabolites.

A slight suppression of CASP3 expression was observed for the supernatant of *St. thermophilus* ST21 by 0.81-fold ± 0.07 ($P < 0.001$) which is conform with the finding that *St. thermophilus* CRL 412 did not changed the Bcl2 family level (Perdigón *et al.* 2002) which are involved in the activation of apoptosis cascade. So *St. thermophilus* ST21 only induced an IL-8 response and seems to be tolerates by Caco-2 cells. The lyophilisate of *St. thermophilus* St21 inhibited the CASP3 activity by 0.46 ± 0.01 ($P < 0.001$) suggesting *St. thermophilus* ST21 and/ or its cell components lead to a stronger reduction of CASP-3 gene expression and thus stimulate the cell growth.

5.2 Control bacteria

5.2.1 Eubacterium biforme ^{T}

We used the supernatant of the common gut bacteria *Eubacterium biforme*^{T} as a reference to compare the effect of probiotic bacteria to a commensal bacterium.

Like the supernatants of *L. acidophilus* NCFM™ and *B. lactis* Bt420, the supernatant of *E. biforme*^{T} also did not affect the IL-8 concentration in Caco-2 cells (1.09). Further, the supernatant of *E. biforme*^{T} was the only one which did not alter the IL-8 production (1.01 ± 0.05) in IL-1β pre-treated Caco-2 cells indicating commensal bacteria did not mediate am immune response even in the case of inflammatory response. The observation in IL-8 production are conform to the measured IL-8 gene expression which is not significantly changed after stimulation with *E. biforme*^{T} supernatant (1.10 ± 0.18). Intestinally, only the supernatant of *E. biforme*^{T} led to a strong augmentation of IL-17 gene expression by 2.00-fold ± 0.13 ($P< 0.0001$), but I do not know if the increased IL-17 mRNA level convert also to protein level because I did not analyzed the extracellular IL-17 concentration. Whatever, this finding is quite interesting because the commensal bacterium *E. biforme*^{T} did not alter neither IL-8 gene expression nor IL-8 protein concentration in normal as well as IL-1β pre-treated Caco-2 cells. Thus any metabolites of *E. biforme*^{T} are capable to induce a chronic inflammatory reaction through IL-17 gene expression. Perhaps the metabolite butyrate which is major produced by *E. biforme*^{T}, is the reason for this observation because butyrate alone induced an augmentation of IL-17 expression by 3.72- fold ± 1.23 in Caco-2 cells (Annex 3).

A similar inhibitor effect was found for the supernatant of *E. biforme*^{T} to the CASP3 gene expression by 0.77-fold ± 0.08 ($P< 0.001$) compare to the supernatants of *B. lactis* Bt420, *St. thermophilus* ST21 and *E. coli* XL blue which is a sign that probiotic or commensal bacteria have no harmful effects on cell integrity and its function. A main reason for this finding could be that this bacteria major produce butyrate and lactate which do not induce a caspase activation in contrast to propionate and acetate (Jan *et al.* 2002). In contrast to the published results I could found that the addition of butyrate induced to a rise of CASP-3 mRNA level by 1.88 ± 0.07. But I only analyzed

the gene activity so I am not able to describe if there is an effect to the protein level.

5.2.2 Escherichia coli^{TC} XL blue

The harmless species of the gram-negative bacteria *Escherichia coli* are common inhabitants in the human gastrointestinal tract. In Caco-2 cells *E. coli* strains led to a strong augmentation of IL-8 concentration. Whereas the strain *E. coli* Nissle induced to a lower rise than the *E. coli* strains F 18, BJ4, MG1655 and UTI. Further, *E. coli* strains led to a higher rise of IL-8 production as *Bifidobacteria* ssp. or *Lactobacilli* ssp. A similar effect was observed for the TGF-β production (Zeuthen *et al.* 2008). The non-pathogenic *E. coli* strain K12 reduced the concentration of IL-8, IL-6, TNF-α, IL-1β after apical stimulation of Caco-2 cells in the basolateral presence of leucocytes compared to the direct treatment of bacteria on leucocytes. The apical addition of bacteria to the Caco-2 cell especially augmented the IL-8 and IL-6 concentration, but the measured concentration were far below those observing after apical bacteria stimulation of Caco-2 cell layer in the basolateral presence of leucocytes. The IL-8 mRNA expression was strong increased in Caco-2 cells by 15.4- fold and in leucocytes by 10.4- fold (Parlesak *et al.* 2004). The augmentation of IL-8 expression in Caco-2 cells after stimulation with non-pathogenic *E. coli* is dose-dependent and can significantly suppressed by *Lactobacillus rhamnosus* GG and *Lactobacillus casein* at a concentration of 10^{10} to 10^{11} cfu/ml. Butyrate at a concentration of 10 mM and 20 mM was also capable to inhibit the *E. coli*-induced IL-8 expression (Shinji *et al.* 2009).
In contrast to the published results of *E. coli*, I found a drop of IL-8 concentration by 0.48-fold in Caco-2 cells after stimulation with supernatant of *E. coli* XL blue indicating supernatants of *E. coli* may have another affect on immune regulation than bacteria of *E. coli*. Unfortunately I could only use the supernatant of *E. coli* and not the bacteria itself for this experiment. Thus I am not able to compare the affect of *E. coli* XL blue bacteria or its supernatant to IL-8 production in Caco-2 cells. But supernatant of *E. coli* XL blue also reduced the IL-8 gene expression by 0.28-fold ± 0.05 ($P < 0.0001$) suggesting *E. coli* XL blue induce an alternation of gene expression which is reflected by extracellular IL-8 concentration. And the addition of LPS from *E. coli* O26:B6 to Caco-2 cells did not influence the IL-8 protein level (1.11 ± 0.13).

That is conform with the observation that LPS from *E. coli* did not affect the IL-8 mRNA level (Shinji *et al.* 2009). Also the gene expression of IL-17 was not influenced by supernatant of *E. coli* XL blue (1.06). The treatment with *E. coli* XL blue led to a slight suppression of CASP3 expression by 0.85-fold ± 0.08 ($P < 0.05$). For the non-pathogen *E. coli* Nissle was observed an anti-apoptotic effect (Perl *et al.* 2005), whereas the pathogenic *E. coli* O157:H7 (EHEC) led to cell damages (Putaala *et al.* 2008) indicating a tolerance of Caco-2 cells against non-pathogenic *E. coli*.

6 SUMMERY/ ZUSAMMENFASSUNG

To analyze how probiotic bacteria influence mediators of inflammatory response in intestinal cells I treated Caco-2 cells with the probiotic bacteria *Lactobacillus acidophilus* NCFM™, *Bifidobacterium animalis subsp. lactis* Bt420 und *Streptococcus salivarius* subsp. *thermophilus* ST21 as well as their supernatants and lyophilisates. Some of the Caco-2 cells were pre-treated with the pro-inflammatory cytokine IL-1β. The impact on gene expression of Interleukin-8 (IL-8), Interleukin-17 (IL-17) and CASP3 was measured by Real-time RCR and was calibrated to GAPDH Expression. Furthermore, the influence of IL-8 production was analyzed by IL-8 ELISA. As control the Caco-2 cells were also treated with the supernatants of *Eubacterium biforme*^{T} und *Escherichia coli* XL blue.

The stimulation of Caco-2 cells with the probiotic bacteria induced an up-regulation of IL-8 gene expression. In case of *Lactobacillus acidophilus* NCFM™ was measured an augmentation by 2.53-fold ± 0.24 ($P< 0.001$). *Bifidobacterium animalis* subsp. *lactis* Bt420 and *Streptococcus salivarius* subsp. *thermophilus* ST21 induced an increase of IL-8 mRNA level by 1.65-fold ± 0.18 ($P< 0.001$) and 2.31-fold. These results are in contrast to the strong decreased IL-8 protein concentration. For instance, *L. acidophilus* NCFM™ inhibited the IL-8 production by 0.22 ± 0.01 ($P< 0.0001$). *B. lactis* Bt420 led to a reduction of IL-8 concentration by 0.15 ± 0.01 ($P< 0.0001$) like *St. thermophilus* ST21 (0.15 ± 0.03, $P< 0.001$). That observation suggests that these three bacteria might induce also post-transcriptional and/or post-translational mechanisms which suppressed the pro-inflammatory response in Caco-2 cells. But the anti-inflammatory effect was only observed in Caco-2 cells which were not pre-treated with the pro-inflammatory cytokine IL-1β. In Caco-2 cells where an inflammation was induced through IL-1β pretreatment, an increased IL-8 production was measured compare to the IL-1β stimulation. Interestingly, lyophilized *B. lactis* Bt420 and *St. thermophilus* ST21 decreased the IL-8 gene expression by 0.49-fold ± 0.03 ($P< 0.0001$) and by 0.20-fold ± 0.04 ($P< 0.0001$) which indicates that IL-8 response in Caco-2 cells is mediated by different metabolites and bacterial growth conditions.

The supernatants of *L. acidophilus* NCFM™ and *B. lactis* Bt420 as well as the supernatant of the commensal bacteria *Eubacterium biforme*^{T} did not affect the IL-8 production. But only the supernatants of *B. lactis* Bt420 and *E. biforme*^{T} also did not alter the IL-8 gene expression. Interestingly, the supernatant of *L. acidophilus* NCFM™ inhibited the IL-8 gene expression, but that is not reflected in a reduced IL-8 production. The treatment with *St. thermophilus* ST21 supernatant resulted in a decrease of IL-8 concentration (0.89 ± 0.06; $P< 0.05$) which is also shown in a lower IL-8 mRNA level (0.31 ± 0.03; $P< 0.0001$). Only the supernatant of the control bacteria *Escherichia coli* XL blue induced an explicit reduction of IL-8 production by 0.48-fold. For the LPS from *E. coli* O26:B6 did not alter the IL-8 production. Like the bacteria, all supernatants, with exception of the supernatant of *E. biforme*^{T}, led to a small rise of IL-8 in IL-1β pre-treated Caco-2 cells. The finding that lactic acid bacteria have an anti-inflammatory effect, but not their supernatant, indicate that bacterial cell surface components induced this effect. This hypothesis is proved through the observation that butyrate, a fermentation product of these bacteria, did not alter the IL-8 mRNA expression (1.37 ± 0.37) as well as IL-8 concentration (1.07 ± 0.22) in Caco-2 cells (Annex1 and Annex 3).

A suppression of IL-17 gene expression was found in the case of *B. lactis* Bt420 by 0.48-fold ± 0.02 ($P< 0.01$). Interestingly, the lyophilized *B. lactis* Bt420 induced an IL-17 up-regulation by 4.27 ± 0.42. In contrast to *B. lactis* Bt420, *L. acidophilus* NCFM™ increased the IL-17 gene expression by 1.42-fold ± 0.20 ($P< 0.01$). No significant change was found for the treatment with *St. thermophilus* ST21. Also the addition lyophilisates of *L. acidophilus* NCFM™ and *St. thermophilus* St21 to Caco-2 cells led to an increased IL-17 mRNA-level by 8.47-fold ± 1.48 and 15.30-fold ± 0.51. These data suggest that *B. lactis* Bt420 have a broader anti-inflammatory effect to Caco-2 cells than *L. acidophilus* NCFM™ and *St. thermophilus* ST21. No effect was observed for the supernatants, with exception the supernatant of *E. biforme*^{T}, leading to an augmentation of IL-17 expression.

Furthermore, only the supernatant of *L. acidophilus* NCFM™ (0.05 ± 0.01; $P< 0.0001$) and the lyophilized bacteria *B. lactis* Bt420 (0.63 ± 0.14; $P< 0.01$) and *St. thermophilus* St21 (0.46 ± 0.01; $P< 0.0001$) induced an explicit anti-apoptosis effect in Caco-2 cells

which is perhaps correlated with the observed inhibition of IL-8 gene expression.
In conclusion probiotic bacteria such as *L. acidophilus* NCFM™, *B. lactis* Bt420 and *St. thermophilus* ST21 showed a preventive anti-inflammatory effect.

Um den Einfluss von probiotischen Bakterien auf Entzündungsmediatoren in Darmzellen zu bestimmen, wurden Caco-2 Zellen mit und ohne IL-1β Vorbehandlung mit den probiotischen Bakterien *Lactobacillus acidophilus* NCFM™, *Bifidobacterium animalis subsp. lactis* Bt420 und *Streptococcus salivarius* subsp. *thermophilus* ST21 sowie deren Überstände und Lyophilisats der Bakterien behandelt. Und deren Auswirkung auf die Genexpression von Interleukin-8 (IL-8), Interleukin-17 (IL-17) und CASP3 mit Hilfe der Real-time PCR bestimmt. Außerdem wurde der Einfluss der Bakterien auf die IL-8 Produktion mittels ELISA gemessen. Zum Vergleich wurden die Caco-2 Zellen mit den Überstand von *Eubacterium biforme*^{T} und *Escherichia coli* XL blue behandelt.

Die Behandlung von Caco-2 Zellen mit den probiotischen Bakterien *Lactobacillus acidophilus* NCFM™, *Bifidobacterium animalis* subsp. *lactis* Bt420 und *Streptococcus salivarius* subsp. *thermophilus* ST21 führte zu einem Anstieg der IL-8 Genexpression. Wobei die Zugabe von *Lactobacillus acidophilus* NCFM™ einen Anstieg um das 2,53-fache ± 0,24 ($P< 0,001$) zur Folge hatte. *Bifidobacterium animalis* subsp. *lactis* Bt420 und *Streptococcus salivarius* subsp. *thermophilus* ST21 induzierten einen erhöhten IL-8 mRNA Spiegel um das 1,65-fache ± 0.18 ($P< 0,001$) beziehungsweise um das 2,31-fache. Diese Ergebnisse stehen im Widerspruch zu den verringerten IL-8 Produktionen. Zum Beispiel hemmte *L. acidophilus* NCFM™ die IL-8 Produktion um 0,22 ± 0,01 ($P< 0,001$). Genau wie *St. thermophilus* ST21 (0,15 ± 0,03; $P< 0,001$) führte *B. lactis* Bt420 zu einer Reduktion der IL-8 Konzentration um das 0,15-fache ± 0,01 ($P< 0,0001$). Diese Beobachtung deutet darauf hin, dass die verwendeten probiotischen Bakterien zudem einen post-transkriptionalen und/oder post-translationalen Regelmechanismus in Gang setzen können, der die eingeleitete Entzündungsreaktion in den Caco-2 Zellen unterdrückt. Dieser entzündungshemmende Effekt war aber nur in den Caco-2 Zellen zu beobachten, die vorher nicht mit den entzündungsfördernden Cytokin IL-1β behandelt wurden. In den Caco-2 Zellen mit einer IL-1β Vorbehandlung konnte ein Anstieg der IL-8 Konzentrationen gemessen werden. Interessanterweise bewirkte die Stimulierung der Caco-2 Zellen mit lyophilisierten *B. lactis* Bt420 und *St. thermophilus* St21 eine

Hemmung der IL-8 Genexpression um das 0,49-fache ± 0,03 (P< 0,0001) und um das 0.20-fache ± 0,04 (P< 0,0001), was darauf schließen lässt, dass in Caco-2 Zellen die IL-8 Antwort abhängig ist von den unterschiedlichen Stoffwechselprodukten sowie von den Bedingungen unter denen die Bakterien gezüchtet werden.

Für die Überstande von *L. acidophilus* NCFM™ und *B. lactis* Bt420 sowie dem Überstand vom Darmbakterium *Eubacterium biforme*^{T} konnten keine signifikanten Änderungen in Bezug auf die IL-8 Produktion nach gewiesen werden. Wobei lediglich nur für die Überstande von *B. lactis* Bt420 und *E. biforme*^{T} auch keine Veränderung der IL-8 Genexpression beobachtet werden konnte. Dagegen hemmte der Überstand von *L. acidophilus* NCFM™ die IL-8 Genexpression, was sich aber nicht in der IL-8 Konzentration wieder spiegelt. Diese Beobachtung weist darauf hin, dass zwar die Stoffwechselprodukte von *L. acidophilus* NCFM™ im Überstand potentiell die IL-8 Genexpression hemmen, aber es gleichzeitig post-transkriptionale und/ oder post-translationale Mechanismen aktivieren muss, die diesen Effekt wieder aufheben. Der Überstand von *St. thermophilus* ST21 induzierte eine kleine Verringerung der IL-8 Konzentration um das 0,89-fache ± 0,06 (P< 0.05), die sich auch in der verminderten IL-8 Geneaktivität (0,31 ± 0,03; P< 0,0001) wieder spiegelt. Nur der Überstand des von *Escherichia coli* XL blue, der als Kontrolle benutzt wurde, führte zu einer deutlichen Reduzierung der IL-8 Produktion um das 0,48-fache. Für den LPS von *E. coli* O26:B6 konnte keine Veränderung gemessen werden.

Wie bei den Bakterien, konnte auch für die Überstande, mit Ausnahme des Überstandes von *E. biforme*^{T}, ein geringer Anstieg von IL-8 in IL-1β vorbehandelten Caco-2 Zellen beobachtet werden. Die Beobachtung, dass Milchsäurebakterien, nicht aber ihre Überstände, einen entzündungshemmenden Effekt haben, deutete darauf hin, dass Bestandteile der Zelloberflächen für diesen Effekt verantwortlich sein müssten. Diese Hypothese wird auch von der Tatsache unterstützt, dass Butyrat, ein Fermentationsprodukt der Bakterien, keinen Einfluss auf die IL-8 Genexpression und IL-8 Konzentration in Caco-2 Zellen hatte (siehe Annex1 und Annex 3).

Ein hemmender Einfluss auf die IL-17 Genexpression konnte nur für das Bakterium *B. lactis* Bt420 (0,48 ± 0,02; P< 0,01) beobachtet werden. Interessanterweise induzierte das lyophilisierte Bakterium *B. lactis* Bt420 eine Stimulierung der IL-17 Genexpression um das 4,27-fache ± 0,42. Im Gegensatz zu *B. lactis* Bt420 erhöhte *L.*

acidophilus NCFM™ die IL-17 Genexpression um das 1,42-fache ± 0,20 (P< 0,01). Keine signifikante Veränderung konnte für die Stimulierung mit *St. thermophilus* ST21 verzeichnet werden. Auch die Zugabe der Lyophilisate von *L. acidophilus* NCFM™ und *St. thermophilus* St21 zu den Caco-2 Zellen führte zu einen erhöhten IL-17 mRNA Spiegel um das 8,47-fache ± 1,48 und um das 15,30-fache ± 0,51. Diese Daten lassen darauf schließen, dass *B. lactis* Bt420 einen weit gefächerten entzündungshemmenden Effekt auf die Caco-2 Zellen ausübt, als die anderen beiden probiotischen Bakterien. Keinen Einfluss konnte für die Überstande auf die IL-17 Genaktivität verzeichnet werden, mit Ausnahme des Überstandes von *E. biforme*^{T}. Das der Überstand von *E. biforme*^{T} einen deutlichen Anstieg der IL-17 Expression hervor ruft ist sehr erstaunlich, weil dieses normale Darmbakterium weder die IL-8 Genexpression noch die IL-8 Konzentration in normalen und IL-1β vorbehandelten Caco-2 Zellen signifikant beeinflusst hat.

In Bezug auf die CASP3 Genexpression induzierte nur der Überstand von *L. acidophilus* NCFM™ (0,05 ± 0,01; P< 0,0001) sowie die lyophilisierten Bakterien *B. lactis* Bt420 (0,63 ± 0,14; P< 0,01) und *St. thermophilus* ST21 (0,46 ± 0,01; P< 0,0001) einen deutlichen anti-apoptotischen Effekt, der eventuell auf Grund der Hemmung der IL-8 Genexpression zurück zuführen ist.

Zusammenfassend kann man sagen, dass probiotische Bakterien wie *L. acidophilus* NCFM™, *B. lactis* Bt420 und *St. thermophilus* ST21 einen präventiven Effekt auf die Entstehung von Entzündungen haben können. Einen generellen Rückschluss auf probiotisch eingestufte Bakterien kann man aber nicht ziehen, da sich in vielen Zell- und Tierversuchen gezeigt hat, dass Immunsystem fördernde Eigenschaften nicht nur spezies-, sonder auch stammspezifisch sind und auch die Dosis der Bakterien Ausschlag gebend ist.

7 ANNEX

IL-8 ELISA without IL-1β

	mean	standard deviation	significant
Control	1.00		
Butyrate 5 µM	1.07	0.22	P> 0.05
Genistein 100 µM	0.53	0.05	P< 0.05
Genistein 200 µM	0.44	0.06	P< 0.05
Folic acid 200 µM	1.15	0.13	P> 0.05
Zebularin 100 µM	2.13	0.49	P< 0.05
Chrysin 50 µM	4.54	0.41	P< 0.0001
EGCG 100µM	1.10	0.15	P> 0.05

Annex 1: IL-8 ELISA of Caco-2 cells after stimulation with standards

IL-8 ELISA with IL-1β

	mean	standard deviation	significant
Control	1.00		
Butyrate 5 µM	0.90	0.04	P> 0.05
Genistein 100 µM	0.66	0.13	P< 0.05
Genistein 200 µM	0.48	0.03	P< 0.0001
Folic acid 200 µM	0.66	0.04	P< 0.001
Zebularin 100 µM	1.60	0.11	P< 0.001
Chrysin 50 µM	0.91	0.03	P< 0.01
EGCG 100µM	0.38	0.14	P< 0.001

Annex 2: IL-8 ELISA of IL-1β pretreated Caco-2 cells after stimulation with standards

	IL-8 gene expression			IL-17 gene expression		
	mean	standard deviation	significant	mean	standard deviation	significant
Control	1.00			1.00		
Butyrate	1.38	0.37	P> 0.05	3.72	1.23	
Genistein	5.86	2.38	P< 0.01	7.08	2.48	
Zebularin	1.33	0.42	P> 0.05			
Folic acid	1.27	0.09	P< 0.01			
Chrysin	1.97	0.23	P< 0.001			
EGCG	1.31	0.13	P< 0.01			

Annex 3: gene expression of IL-8 and IL-17 in Caco-2 cells after standard treatment

	CASP3 gene expression		
	mean	standard deviation	significant
Control	1.00		
Butyrate	1.88	0.07	P< 0.01
Genistein	1.74	0.03	P< 0.001
Zebularin	2.04	0.12	P< 0.001
Folic acid	0.88	0.12	P> 0.05
Chrysin	1.09	0.12	P> 0.05
EGCG	1.08	0.07	P> 0.05

Annex 4: CASP3 gene expression in Caco-2 cells after standard treatment

	CPT1A gene expression			CPT1B gene expression		
	mean	standard deviation	significant	mean	standard deviation	significant
Control	1.00			1.00		
Butyrate	5.22	0.27	P< 0.001	0.66	0.02	P< 0.001
Genistein	7.06	0.69	P< 0.01	1.33	0.27	P> 0.05
Zebularin				1.40	0.19	P> 0.05
Folic acid				1.00	0.01	P> 0.05
Chrysin				0.99	0.15	P> 0.05
EGCG				1.16	0.04	P< 0.05

Annex 5: gene expression of CPT 1A and CPT 1B in Caco-2 cells after treatment with standards

	CPT1A gene expression		
	mean	standard deviation	significant
Control	1.00		
supernatant Lactobacillus acidophilus NCFM™	0.37	0.04	P< 0.001
supernatant Bifidobacterium animalis subsp. lactis 420	0.70	0.05	P< 0.05
supernatant Streptococcus salivarius subsp. thermophilus ST21	0.52	0.05	P< 0.001
supernatant Escherichia coli XL-blue	2.14	0.02	P< 0.01
supernatant Eubacterium biforme^T	1.36	0.10	P< 0.05

Annex 6: CPT 1A gene expression in Caco-2 cells after treatment with bacterial supernatants

CPT1B gene expression

	mean	standard deviation	significant
Control	1.00		
Lactobacillus acidophilus NCFM™	0.71	0.09	
Bifidobacterium animalis subsp. lactis 420	0.44	0.03	
Streptococcus salivarius subsp. thermophilus ST21	0.86	0.09	
supernatant Lactobacillus acidophilus NCFM™	0.94	0.04	P> 0.05
supernatant Bifidobacterium animalis subsp. lactis 420	1.63	0.06	P< 0.01
supernatant Streptococcus salivarius subsp. thermophilus ST21	0.50	0.02	
supernatant Escherichia coli XL-blue	1.15	0.07	P> 0.05
supernatant Eubacterium biforme^T	0.82	0.04	P> 0.05

Annex 7: CPT 1B gene expression in Caco-2 cells after treatment with bacteria and bacterial supernatant

	ERS1 gene expression		
	mean	standard deviation	significant
Control	1.00		
supernatant Lactobacillus acidophilus NCFM™	0.55	0.07	P> 0.05
supernatant Bifidobacterium animalis subsp. lactis 420	1.32	0.12	P> 0.05
supernatant Streptococcus salivarius subst. thermophilus ST21	0.39		
supernatant Eubacterium biforme^T	0.72	0.10	P> 0.05
supernatant Escherichia coli XL-blue	0.78	0.16	P> 0.05

Annex 8: ESR1 gene expression in Caco-2 cells after stimulation with bacterial supernatant

	ERS1 gene expression		
	mean	standard deviation	significant
Control	1.00		
Butyrate	1.25	0.08	P> 0.05
Genistein	3.76	0.61	P< 0.01

Annex 9: ERS1 gene expression in Caco-2 cells after stimulation with standards

	DMNT3a gene expression			HDAC2 gene expression		
	mean	standard deviation	significant	mean	standard deviation	significant
Control	1.00			1.00		
Butyrate	1.45	0.2	P> 0.05	1.09	0.12	P> 0.05
Genistein	0.34	0.04	P< 0.001	1.44	0.05	P< 0.01
Zebularin	1.40	0,05	P< 0.01	1.11	0.07	P> 0.05
Folic acid	0.92	0.10	P> 0.05	0.96	0.02	P> 0.05
Chrysin	0.95	0.07	P> 0.05	1.07	0.06	P> 0.05
EGCG	0.93	0.14	P> 0.05	1.29	0.01	P< 0.001

Annex 10: gene expression of DNMT3a and HDAC2 in Caco-2 cells after stimulation with standards

	DMNT 3a gene expression		
	mean	standard deviation	significant
Control	1.00		
Lactobacillus acidophilus NCFM™	0.75	0.07	P< 0.001
Bifidobacterium animalis subsp. lactis 420	0.45	0.01	P< 0.0001
Streptococcus salivarius subsp. thermophilus ST21	0.77	0.06	P< 0.01
supernatant Lactobacillus acidophilus NCFM™	0.79	0.04	P< 0.0001
supernatant Bifidobacterium animalis subsp. lactis 420	0.59	0.07	P< 0.0001
supernatant Streptococcus salivarius subst. thermophilus ST21	1.72	0.03	P< 0.0001
supernatant Eubacterium biforme^T	1.47	0.15	P< 0.0001
supernatant Escherichia coli XL-blue	0.42	0.02	P< 0.0001

Annex 11: DNTM3a gene expression in Caco-2 cells after treatment with bacteria and bacterial supernatant

	HDAC2 gene expression		
	mean	standard deviation	significant
Control	1.00		
Lactobacillus acidophilus NCFM™	0.95	0.07	P> 0.05
Bifidobacterium animalis subsp. lactis 420	0.61	0.01	P< 0.01
Streptococcus salivarius subsp. thermophilus ST21	1.02	0.08	P> 0.05
supernatant Lactobacillus acidophilus NCFM™	0.44	0.01	P< 0.0001
supernatant Bifidobacterium animalis subsp. lactis 420	0.67	0.05	P< 0.0001
supernatant Streptococcus salivarius subst. thermophilus ST21	1.55	0.02	P< 0.0001
supernatant Eubacterium biforme^T	1.28	0.02	P< 0.001
supernatant Escherichia coli XL-blue	0.86	0.05	P< 0.01

Annex 12: HDAC2 gene expression in Caco-2 cells after stimulation with bacteria and bacterial supernatant

8 PROTOCOLS

8.1 Total RNA Isolation- User Manual NucleoSpin® RNA II (Macherey-Nagel)

Total RNA purification from cultured cells and tissue with NucleoSpin® RNA II

1. Homogenization of sample
    - Disrupt up to 30 mg of tissue (for sample amounts see section 2.3; for homogenization methods see section 2.4)
    - Up to 5 x 106 eukaryotic cultured cells are collected by centrifugation and lysed by addition of RA1 directly

2. Cell lyses
    - Add 350 µl Buffer RA1 and 3.5 µl β-mercaptoethanol to the cell pellet or to ground tissue and vortex vigorously

3. Filtration of the lysate
    - Reduce viscosity and clear the lysate by filtration through NucleoSpin® Filter Columns (violet ring): Place NucleoSpin® Filter Columns in a Collection Tube, apply the mixture, and centrifuge for 1 min at 11,00 x g.
    - In case of visible pellet formation (depending on sample amount and nature) transfer supernatant without any formed pellet to a new 1.5 ml microcentrifuge tube (not supplied)

4. Adjust RNA binding conditions
    - Discard the NucleoSpin® Filter Column and add 350 µl ethanol(70 %) to the homogenized lysate and mix by pipetting up and down (5 times).
    - Alternatively transfer flow-through into a new 1.5 ml microcentrifuge tube (not provided), add 350 µl ethanol(70 %), and mix by vortexing (2 x 5 sec).

5. Bind RNA
    - For each preparation, take one NucleoSpin® RNA II Column (light blue

ring) placed in a Collection Tube. Pipette lysate up and down 2-3 times and load the lysate to the column. Centrifuge for 30 sec at 11,000 x g. Place the column in a new Collection Tube (2 ml).

6. Desalt silica membrane
    - Add 350 µl MDB (Membrane Desalting Buffer) and centrifuge at 11,000 x g for 1 min to dry the membrane.

7. Digest DNA
    - Prepare DNase reaction mixture in a sterile 1.5 ml microcentrifuge tube (not provided): for each isolation, add 10 µl reconstituted rDNase (also see section 3) to 90 µl Reaction Buffer for rDNase. Miss by flicking the tube.
    - Apply 95 µl DNase reaction mixture directly onto the center of the silica membrane of the column. Incubate at room temperature for 15 min.

8. Wash and Dry silica membrane

1st wash
    - Add 200 µl Buffer RA2 to the NucleoSpin® RNA II Column. Centrifuge for 30 sec at 11,000 x g. Place the column into a new Collection Tube (2 ml).

2nd wash
    - Add 600 µl Buffer RA3 to the NucleoSpin® RNA II Column. Centrifuge for 30 sec at 11,000 x g. Discard flow-through and place the column back into the Collection Tube.

3rd wash
    - Add 250 µl Buffer RA3 to the NucleoSpin® RNA II Column. Centrifuge for 2 min at 11,000 x g to dry the membrane completely. Place the column into a nuclease-free Collection Tube (1.5 ml, supplied).

9. Elute highly pure RNA
    - Elute the RNA in 60 µl RNase-free Water (supplied) and centrifuge at 11,000 x g for 1 min.

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Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.

10 PUBLICATIONS

10.1 Abstract for poster presentation

10.1.1 NuGOweek 2009, Montecatini Terme, Aug. 31-Sep. 3 2009

EPIGENETIC REGULATION OF IL-8 AND E-CADHERIN GENE EXPRESSION BY BUTYRATE, FOLIC ACID, AND GENISTEIN INCLUDES DNA-METHYLATION AND HISTONE ACETYLATION

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The expression of IL-8 and E-Cadherin regulates the interactions between immune cells and epithelia in the GI-tract involving epigenetic mechanisms. Because of the epigenetic activities of the SCF butyrate, produced by intestinal microbiota, as well as genistein and folic acid we analyzed effects on histone acetylation and DNA-methylation on the expression of IL-8 and E-Cadh.
Gene expression was analyzed by real time PCR, DNA-methylation by Methylation Specific PCR (MSP), bisulfite sequencing PCR (BSP) and histone acetylation by Chromatin immunoprecipitation in the CACO-2 cell line model.
IL-8 and E-Cadh expression was increased by genistein, butyrate, folic acid and the DNA methyltransferase inhibitor zebularine. Whereas zebularine decreased DNA methylation of E-Cadh and IL-8 (on the sites -1342 and -1412), butyrate enhanced the histone acetylation on H3 lysine 9 at IL-8. Genistein and butyrate also stimulated the apoptose relevant caspase3. Changes in butyrate producing GI-microbiota (*Clostridium* cluster IX and XIVa) were found due to nutrition.
As the levels of genistein, folic acid, and butyrate in the GI-tract can be modulated by nutrition, nutritional concepts may be feasible to interfere with epigenetic mechanisms in inflammatory GI diseases.

10.1.2 Epigenetic World Congress, Berlin, Sep. 17-18 2009

Food ingredients modulate epigenetic control of expression of estrogen receptor and tumor suppressor genes

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Estrogen receptor alpha (ESR1) plays vital roles in the development and progression of breast cancer and metabolic disorders. The expression is known to be regulated by epigenetic mechanisms. Also the tumor suppressor genes P15 and P16 are aberrantly methylated in several cancer types.

To evaluate possible effects of dietary compounds on the epigenetic gene expression Caco2-cells were treated with butyrate, resveratrol, genistein, zebularine, folic acid, chrysin, and EGCG, which are known to have estrogenic or epigenetic effects. Gene expression was analyzed for ESR1, P15, and P16 by quantitative real-time-PCR. Methylation of specific CpG-sites was determined by methylation-specific PCR.

Treatment with genistein, folate, EGCG, and resveratrol increased and zebularine and chrysin decreased methylation of ESR1. P15 methylation was enhanced by genistein and folate and decreased by chrysin, zebularine, butyrate, EGCG, and resveratrol. Treatment with folate resulted in P16 hypermethylation whereas all other agents resulted in hypomethylation of this gene. Changes in methylation corresponded with altered gene expression. In contrast to CACO cells human blood cells show less methylation on the investigated genes. These results suggest that food ingredients may influence epigenetic modification of cancer relevant gene expression and might be considered for supplemental treatment and prevention.

10.1.3 19th International Congress of Nutrition, Bangkok, Oct. 4-9 2009

REGULATION OF IL-8 AND IL-17 EXPRESSION BY BUTYRATE, FOLIC ACID, AND GENISTEIN INCLUDES DNA METHYLATIONAND HISTONE ACETYLATION

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IL-8 and IL-17 regulate interactions between immune cells and epithelia in the GI-tract, involving epigenetic mechanisms. Because of the epigenetic activities of the SCF (short chain fatty acid) butyrate, produced by intestinal microbiota, genistein and folic acid we analyzed effects on histone acetylation and DNA-methylation on interleukin-8 and -17 genes.

We analyzed gene expression by real time PCR, DNA-methylation by bisulfite sequencing PCR, and histone acetylation by Chromatin immunoprecipitation in the CACO-2 cell line model.

IL-8 and IL-17 expression was increased by genistein, butyrate, and folic acid. Folic acid stimulated the IL-8-DNA-methylation on the sites -1342 and -1412. Butyrate stimulated the histone acetylation on H3 lysine 9 at IL-8. Genistein and butyrate also stimulated apoptosis relevant caspase-3. Changes in butyrate producing GI-microbiota (*Clostridium* cluster IX and XIVa) were found due to nutrition.

As the levels of genistein, folic acid, and butyrate in the GI-tract can be influenced by nutrition, nutritional concepts may be feasible to interfere with IL-8 and IL-17 expression in inflammatory GI diseases.

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	Wahlschwerpunk: Ernährung und Umwelt	
	Diplomandin am Institut für Ernährungswissenschaften der Universität Wien, Arbeitsgruppe Uni-Doz. Dr. Alexander G. Haslberger (Bereich Epigenetik)	11/2008 bis 06/2009
	ERASMUS- Austausch an der Universität Kuopio/ Finnland	09/2009 bis 12/2009

Praktika	
3-wöchiges Praktikum im Lebensmittelüberwachung- und Veterinäramt des Landkreises Sächsische Schweiz/ BRD	08/2007
Ferialjob bei der AMA (Agrarmarkt Austria), Wien/AT - 6 Wochen Datenerfassung und 2 Wochen AMA-Marketing Abteilung Fleisch- und Fleischwaren	06/2008 bis 07/2008

		08/2008
	5- wöchiges Praktika im Laboratorium für Bakteriologie und Lebensmittelhygiene Dr. Jesche, Dresden/ BRD	
	Praktika am Institut für Ernährungswissenschaften der Universität Wien/ AT; Arbeitsgruppe Uni.-Doz. Dr. Alexander G. Haslberger (Bereich Epigenetik)	10/2008
	Ferialjob bei der AMA (Agrarmarkt Austria), Wien/AT im Bereich Datenerfassung und Aushilfe für archivarische Aufgaben bei der AMA- Marketing, Abteilung Qualitätsmanagement Fleisch- und Fleischprodukte	Ende 05/2009 bis 07/2009

Publikationen	
Poster:	Aumueller E., Gnauck A., Berner C., Klein P., Haslberger A.G.: Regulation of Il-8 and Il-17 expression by butyrate, folic acid, and genistein includes DNA methylation and histone acetylation. International Congress of Nutrition, Oct. 4-9 2009, Bangkok/ THA
	Aumueller E., Berner C., Gnauck A., Just A., Haslberger AG.: Food ingredients modulate epigenetic control of expression of estrogen receptor and tumor suppressor genes. Epigenetic World Congress, Sep. 17-18 2009; Berlin/BRD
	Aumueller E., Gnauck A., Berner C. Klein P., Haslberger A.G.: Epigenetic regulation of IL-8 and E-Cadherin gene expression by butyrate, folic acid, and genistein includes DNA methylation and histone acetylation. NUGOweek 2009, Aug. 31- Sep.3 2009, Montecatini Terme/ITA