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Characterization and utilization of faecal microflora components in experimental models of human civilization diseases

Ph.D. Thesis

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Statement of originality

I hereby declare that the material in this thesis has not been previously submitted for a degrees or diploma at any other higher education institution. To the best of my knowledge this thesis is the product of my own work and contains no material previously published or written by another person, except where due references are made.

Nový Hrádek, 30. 9. 2015 Dagmar Šrůtková

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ABSTRACT

Interaction between intestinal microbiota and host mucosal immune system plays crucial role in maintenance of mucosal homeostasis. Dysbiosis, altered composition of microbial communities, has been shown to be associated with life-style diseases such as inflammatory bowel disease (IBD) or allergies. In this regard, probiotics are valuable tool for the improvement of gut microbiota disbalance and proper stimulation of the immune system.

In this thesis we focused on taxonomical classification of *Bifidobacterium longum* human origin strains by PCR-based methods, *in vitro* characterization of immunomodulatory properties of selected *Bifidobacterium* and *Lactobacillus* strains and evaluation of the beneficial effect of selected bacterial strains in IBD and allergy experimental mouse models.

We investigated four different PCR-based methods and biochemical analysis for the taxonomical classification of twenty-eight *B. longum* isolates from the healthy human faeces. The Amplified Ribosomal DNA Restriction Analysis was the only method to be able to differentiate the analyzed strains into the *B. longum/infantis* subspecies.

We showed that the analyzed immunostimulatory properties of bifidobacterial strains are strictly strain-specific. In a mouse model of acute ulcerative colitis, we have demonstrated that prophylactic administration of *B. longum* ssp. *longum* CCM 7952 prevented development of severe forms of intestinal inflammation which was associated with the preserved tight junction proteins expression and improved epithelial barrier function.

We demonstrated the butyrate-producing *Clostridium tyrobutyricum* DSM 2637 prophylactic effect on dextran sodium sulphate (DSS)-induced colitis in immunocompetent BALB/c and immunodeficient SCID mice.

In a mouse model of birch pollen allergy, we demonstrated that neonatal mother-to-offspring mono-colonization of germ-free (GF) mice with *B. longum* ssp. *longum* CCM 7952 prevented the allergic sensitization development, likely by Treg response activation.

We have revealed that colonization of GF mice with the mixture of *Lactobacillus rhamnosus* LOCK0900, LOCK0908 and *L. casei* LOCK0919 enhanced the gut mucosa integrity and ameliorated allergic sensitization to birch pollen.

Taken together, determination of the precise probiotic effect mechanism has to come from the correlation of *in vitro* data with the outcomes *in vivo*. This thesis brings better understanding of the probiotic strains immunomodulatory potential that have important implication for their use in IBD and allergy prophylaxis or therapy.

ABSTRAKT

Interakce mezi střevní mikroflórou a slizničním imunitním systémem hostitele hraje klíčovou roli při udržování slizniční homeostázy. Bylo prokázáno, že dysbióza, změna ve složení mikrobiálních společenstev, může vést k rozvoji civilizačních onemocnění, jakými jsou např. zánětlivá střevní onemocnění (IBD) nebo alergie. V tomto ohledu jsou probiotika cenným nástrojem pro zmírnění nerovnováhy střevní mikroflóry s vhodnou stimulací imunitního systému.

V této práci jsme se zaměřili na taxonomickou klasifikaci kmenů *Bifidobacterium longum* lidského původu metodami založenými na PCR, *in vitro* charakterizaci imunomodulačních vlastností vybraných bakterií rodu *Bifidobacterium* a *Lactobacillus* a stanovení příznivého účinku vybraných bakterií v experimentálních myších modelech IBD a alergie.

Pro taxonomickou klasifikaci dvaceti osmi izolátů *B. longum* získaných ze stolice zdravých lidí jsme využili metody založené na PCR a biochemické analýze. K diferenciaci analyzovaných kmenů do poddruhů *B. longum*/*infantis* byla nejúspěšnější metoda ARDRA. Potvrdili jsme, že imunostimulační vlastnosti analyzovaných kmenů bifidobakterií jsou přísně kmenově specifické. V myším modelu akutní ulcerózní kolitidy jsme ukázali, že profylaktické podávání kmene *B. longum* ssp. *longum* CCM 7952 je schopné zabránit rozvoji závažné formy střevního zánětu zachováním exprese proteinů těsných spojů a tudíž zlepšením funkce epitelové bariéry.

Dále jsme prokázali profylaktický účinek bakterie *Clostridium tyrobutyricum* DSM 2637 produkující butyrát na kolitidu indukovanou podáváním roztoku dextran sulfátu sodného (DSS) u imunokompetentních BALB/c myší a imunodeficitních SCID myší.

V myším modelu alergie k březovému pylu jsme ukázali, že neonatální monokolonizace bezmikrobních (GF) myší bakterií *B. longum* ssp. *longum* CCM 7952 zabrání rozvoji alergické senzibilizace, pravděpodobně aktivací T regulační odpovědi.

Zjistili jsme, že kolonizace GF myší bakteriální směsí *Lactobacillus rhamnosus* LOCK0900, LOCK0908 a *L. casei* LOCK0919 zlepší integritu střevní sliznice a zmírní alergickou senzibilizaci k březovému pylu.

V dizertační práci jsme ukázali, že stanovení přesného mechanismu probiotického účinku musí vycházet z korelace dat *in vitro* studií s výsledky získanými v *in vivo* pokusech. Tato práce rozšiřuje naše znalosti o imunomodulačním potenciálu probiotických kmenů, což má významný dopad pro jejich praktické využití v profylaxi nebo terapii IBD a alergie.

1. INTRODUCTION

1.1 Host-microbe relationships

Mammals live in an environment of continuous interaction with the huge number of microorganisms involving bacteria, viruses, fungi and protozoa. Commensal microflora represent open ecosystem formed by resident and transiently presented microbes, which interact with its host on body surfaces covered by epithelial cells [1]. The number of bacteria colonizing the mucosal and skin surfaces of human body (approximately 10^{14} microbes) exceeds the number of host cells by at least 10 times and express at least 100-fold more unique genes than their host's genome [2]. The colonization of the epithelial surfaces with the bacteria has been shown to contribute to development of the immune system and results in symbiotic relationship of diverse bacterial population with the host [3]. However, under specific conditions, the microflora is able to overcome host protective barrier, which may lead to deleterious parasitic or opportunistic infections [1]. Which form of relationship predominates depends on many factors, such as life-style, diet, host genotype, use of antibiotics, co-infection and disease [4]. All these factors directly influence the resilience and diversity of the microbiota which is required for balanced immune responses. A disturbed balance of beneficial and detrimental bacteria (dysbiosis) could result in number of autoimmune and inflammatory diseases, particularly prevalent in the 21st century [5, 6]. Mounting evidence suggests that commensal microflora plays a crucial role in health and disease in humans. Clarification of the role of microflora in human disorders using animal models will allow us to get insight into the mechanisms involved in the initiation and maintenance of these human civilization diseases.

1.1.1 The development of the host microbiota

The first microbial colonization of humans occurs during and after birth, although emerging evidence suggests that the *in utero* environment may not be strictly sterile, as originally thought [7, 8]. Immediately upon the birth, the mucosal and skin surfaces of babies' body is gradually colonized by the microbes, either from their mother's vagina or from skin microbes and hospital environment depending on delivery mode [9]. Factors such as type of nutrition (breast milk *vs.* formula), early use of antibiotics and environmental conditions further influence the composition of intestinal microflora [3]. The intestinal microflora of newborns shows low diversity and a relative dominance of the phyla *Actinobacteria* (primarily representatives of the genus *Bifidobacterium*) and *Proteobacteria*. After the first

year of age, the microflora of infants starts to resemble that of adult and becomes more diverse and fully corresponds to the adult microbiota by 2.5 years of age [10]. During the first years of life, the primary colonization contributes to the development of both local and systemic immune system. The disruption in the natural succession of colonizing microbes and/or deficiency of the microflora in early infancy may be a critical determinant of disease expression in later life [11]. According to this microbiota deficiency hypothesis, the correct microbial colonization during this developmental period could have effect on decreased susceptibility to diseases such as allergy and inflammatory bowel disease. On the other hand, dysbiosis or absence of some species caused by antibiotic treatment in childhood may have reverse effect [12].

1.1.2 The composition of intestinal microbiota

The human intestine represents the largest mucosal surface for microbial colonization which is tightly packed into numerous villi and crypts. Additionally, the gastrointestinal tract (GIT) provides environment rich in molecules that can be used as nutrients by microbes and therefore, the greatest number of bacteria is found in the host GIT [13].

The majority of the intestinal microbiota is composed of obligate anaerobes (90%) which dominate over the facultative anaerobes and aerobes. Human intestinal microbiota shows enormous microbial diversity represented from 500 to 2000 bacterial species [2]. Nevertheless, up to 80% of the intestinal microbial population may be uncultivable [14]. Therefore our current knowledge about the diversity of the human gastrointestinal microflora originates from both culture-based and molecular biological studies involving cloning and sequencing of individual bacterial rRNA genes [15, 16]. Based on these metagenomic analysis, the human intestinal microbiota of adults consists of two major phyla, *Bacteroidetes* and *Firmicutes*, and four other prominent phyla, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, and *Tenericutes*, although significant inter-individual variations are present [16]. The intestinal *Firmicutes* are Gram-positive bacteria, dominated by species belonging to the *Clostridia* class, but also include *Enterococcaceae* and *Lactobacillaceae* families and *Lactococcus* spp. Intestinal *Bacteroidetes* are Gram-negative bacteria including species *Bacteroides thetaiotaomicron*, *Bacteroides fragilis* and *Bacteroides ovatus*. The remaining intestinal bacteria, accounting for less than 10% of the total population, belong to the *Proteobacteria*, *Fusobacteria*, *Actinobacteria*, *Verrucomicrobia* and *Spirochaetes* phyla and a bacterial group that is closely related to *Cyanobacteria* [17]. Although there is a huge variability in the microbial composition presented in the intestine of individuals, recently it

has been suggested that the microbiota of most individuals can be categorized into one of three enterotypes based on the dominant genera (*Prevotella*, *Ruminococcus*, or *Bacteroides* respectively) [18]. Nevertheless, the distribution of the number and composition of predominant species of intestinal microflora is also not homogenous throughout whole gastrointestinal tract (GIT), but goes from 10^1 to 10^2 bacteria per gram of content in the stomach and duodenum, progressing to ileum to 10^8 and cumulating in 10^{11} to 10^{12} in colon content (Fig. 1) [19]. Colon alone is estimated to contain over 70% of all the microbes in the human body [2].

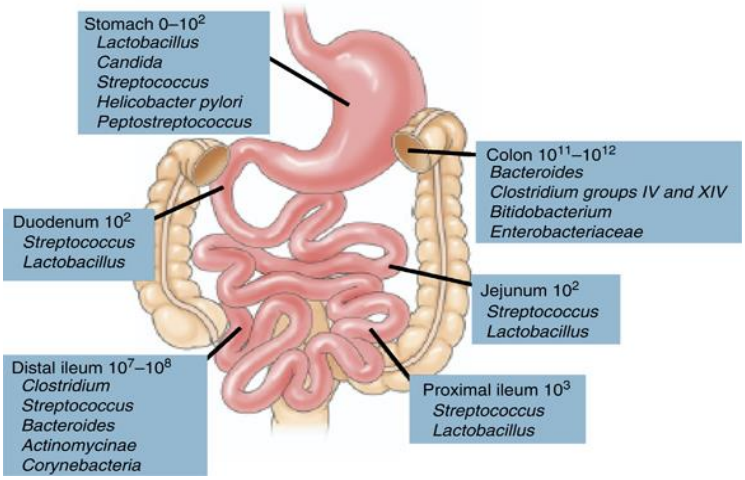

Fig. 1: Composition and numbers of dominant microbial species in the human gastrointestinal tract. Small intestine of healthy adult human is enriched for the *Bacilli* with the phyla *Firmicutes* and *Actinobacteria*. There are more prevalent the *Bacteroidetes* and the *Lachnospiraceae* family in colon of human. Adopted from Sartor [19].

1.1.3 The role of intestinal microbiota in health and disease

The critical role of the microbiota on the development of the host immune system and physiology is amply demonstrated by the example of the germ-free (GF) animals. Compared to conventional animals, GF mice have underdeveloped mucosal-associated and systemic immune systems, affected development and function of the epithelium, vasculature, neuromuscular apparatus, and gut endocrine system [20]. Impairment of peristaltic activity, reduced intestinal surface area due to smaller villus thickness and reduction in the villus capillary network in GF animals have implication for nutrient absorption and cholesterol and bile acid metabolism [21]. Intestinal bacteria as a large fermentative organ also play an important role in numerous metabolic processes of the intestine such as fermentation of dietary residues (non-degradable saccharides and proteins), metabolism of xenobiotics, activation or destruction of mutagenic metabolites, vitamin and short chain fatty acids (SCFA) synthesis [22]. Butyrate, one of produced SCFA, is not only the main energy source for colonic epithelial cells, but also prevents the accumulation of potentially toxic metabolic

by-products [23]. Recently it has been proposed that microbiota can influence the development and function of the central nervous system (microbiota-gut-brain axis) [12].

Interaction between intestinal compartment and microbiota play another fundamental role in promotion of physiological and homeostatic functions by immunomodulation, regulation of apoptosis and maintenance of barrier function [24]. Commensal microbiota provides a physical barrier against incoming pathogens by competitive exclusion, such as occupation of attachment areas, consumption of nutrient sources and production of antimicrobial substances (nonspecific fatty acids, peroxides or specific bacteriocins). On the other hand, the microflora is able to stimulate the host immune system to produce various antimicrobial compounds such as defensins, cathelicidins, C-type lectins, immunoglobulin A, mucin and other compounds of immune system [25]. It has been shown that the intestinal microbiota interacts with the innate and adaptive immune system, providing signals to promote the maturation of immune cells and the development of immune functions [26].

1.2 Mucosal immune system

Mucosal surfaces and skin mediate everyday contact between the organism and its external environment, during which organism meet many antigenic, mitogenic and toxic stimuli presented in food, microflora and air [1]. The mucosal surfaces (digestive, respiratory, urogenital tract and some others) are covered mostly by a single-layered epithelium, which compose the surface around 300 m^2. The largest epithelial surface (around 200 m^2) covers the intestine. Therefore, the mucosal surfaces are protected by a complex of mechanisms by which heterogeneous agents are degraded and removed. One of them is mucosa-associated lymphoid system (MALT), a highly specialized immune system that involves around 80% of immunologically active cells. The majority of these cells are present in the tissue of GIT, where is the highest prevalence of immunogenic stimuli from food and components of microbiota [1, 27].

The essential role of the mucosal immune system is the protection against penetration of pathogenic microorganism and other components into internal environment of the organism (barrier function), as well as the ability to distinguish between dangerous and non-dangerous agents. Thus, the induction of unresponsiveness of the systemic immunity to antigens presented on mucosal surfaces (oral, mucosal tolerance) and maintenance of the homeostasis of mucosal immunity are other important features [28, 29]. Nevertheless, the mucosal balance can be disturbed by the attack of microbial pathogens or their toxins or by inadequate function of components of the immune system which can caused disorders such as

infectious diseases, inflammatory disease, allergies, multi-organ failure or some autoimmune disease (such as rheumatoid arthritis, type I diabetes, etc.) [1, 30].

1.2.1 Barrier function of mucosal immune system

Physical and chemical barriers of mucosal immune system limit the contact of microbes and the intestinal epithelial cells (Fig. 2) [31].

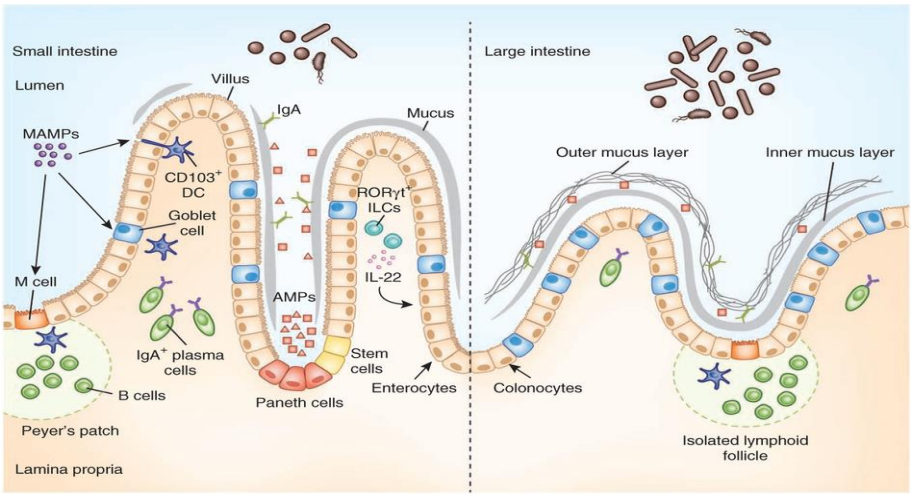

Fig. 2: Immune mechanisms of mucosal immune firewall limiting the contact between luminal microbial community and the intestinal epithelial cell surface. Goblet cells secrete mucin glycoproteins that assemble into inner and outer layer of mucus. Paneth's cells and goblet cells secrete antimicrobial proteins that help to eliminate bacteria penetrating the mucus layer. Plasma cells secrete IgA, that is transcytosed through epithelial layer and limit the number of mucosa-associated bacteria. There are some differences in the compartment of physical barrier in the small and large intestine. Adopted from Brown *et al.* [31].

The first barrier encountered the intestinal microbes is **mucus**, a thick layer of mucin glycoprotein secreted by specialized goblet cells in the epithelium. This mucus assembles into two viscous gel-like layers: outer layer contains large numbers of bacteria, whereas inner layer delineates a protected zone at the apical epithelial cell surface where it limits the contact of bacteria and their components with epithelial cells [32]. In contrast to the colon, the mucus layer of small intestine is thinner and more penetrable for the contents of the intestine (Fig. 2) [33]. Mucus consists of two main glycoproteins, which production is encoded by *Muc1* and *Muc2* genes. It has been shown that *Muc1* is one of the newly implicated loci in Crohn´s disease pathogenicity [34]. Mice deficient for the gene *Muc2* do not have the bacteria-free zone and suffer from spontaneous chronic intestinal inflammation and colitis-associated colorectal cancer [35, 36]. These findings highlighted the importance of the mucus barrier in maintaining intestinal homeostasis. Moreover, the recent studies have shown that mucus also promotes tolerogenic response to food and commensal antigens [37].

Antimicrobial proteins (AMPs) secreted from epithelial cells (such as enterocytes, Paneth's cells and goblet cells) provide protection against pathogenic and commensal microbes that penetrate the mucosal barrier. Into this protein family belong defensins, cathelicidins and C-type lectins that can kill bacteria through enzymatic attack on bacterial cell wall, through disruption of inner membrane or by depriving of essential metals such as iron [38]. Nevertheless, the expression of different subset of AMPs is regulated by distinct mechanisms. While the most defensins are expressed constitutively without any bacterial signal, the production of another subset of antimicrobial proteins (e.g. β-defensin) is governed by bacterial signals through the specific activation of pattern recognition receptors such as Toll-like receptors (TLRs) and nuclear oligomerization domain (NOD)2 [38-40]. In addition, AMPs can exert modulatory functions on chemotaxis, TLR signaling and wound healing [41].

The intestinal epithelium is composed of a 30 μm single layer of intestinal epithelial cells (IECs), including absorptive enterocytes (in colon called colonocytes), goblet cells, Paneth's cells and enteroendocrine cells. They collectively cover a huge surface area, tightly packed into numerous villi and crypts. The epithelium acts as selectively permeable barrier, permitting absorption of nutrients, electrolytes and water, while maintain a defence against intraluminal toxins, antigens and microbiota. The integrity of this physical barrier layer is crucial to protect the host from penetration of commensal microbiota and food antigens from the intestinal lumen and to prevent pathologic antigen-specific immune responses, as evident in IBD and celiac disease [1, 42].

The epithelial layer is constantly renewed through cycles of IEC proliferation, migration, and apoptosis along the crypt-villus axis. As the cells migrate upwards from the crypt, they differentiate into three different cell lineages: enterocytes, enteroendocrine cells and goblet cells [43]. This dynamic renewal process also serves as a protective mechanism to remove cells that may be damaged or invaded by pathogens. This process occurs without compromising the integrity of cell-to-cell junctions and intestinal permeability which could lead to inflammatory disease [42].

Each epithelial cell contributes to integrity of barrier through distinct specialized mechanisms. Goblet cells reinforce the enterocyte barrier by secretion of mucus and trefoil peptides, whereas Paneth's cells play an important homeostatic role by production of antimicrobial peptides. The enterocytes migrate out of the crypts to form a surface epithelium which provide the barrier by apical brush border and intercellular junctional complexes represented by adherens junctions (AJs), tight junctions (TJs), gap junction and desmosomes

(Fig. 3A). They are formed by complex of protein-protein networks that mechanically link adjacent cells and seal the intercellular space (Fig. 3B) [44, 45].

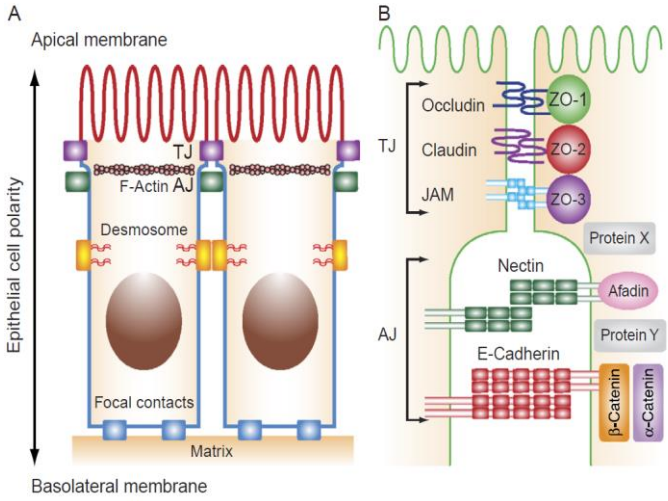

Fig. 3: Intracellular junctional complex. The epithelium consists of a single layer of polarized epithelial cells. (**A**) Adjacent cells are connected by three main structures: desmosomes, adherens junctions (AJs) and tight junctions (TJs). (**B**) AJs and TJs are connected through adaptor proteins to the actin cytoskeleton. TJ are located on the apical-lateral membrane junction and consist of integral transmembrane proteins, such as occludin, claudins and junctional adhesion molecules (JAMs) that interact in the paracellular space with adjacent proteins of adjacent cell. The adaptor proteins (ZO-1, ZO-2 and ZO-3) mechanically link the TJ complex to the actin cytoskeleton. Adopted from Miyoshi and Takai [44].

Adherens junctions (zonula adherens) are formed by interactions between transmembrane proteins, intracellular adaptor proteins and cytoskeleton. The major AJs are formed by cadherin-catenin interactions, where E-cadherins (calcium-dependent adhesion molecules) are transmembrane-spanning glycoproteins. They interact with cadherins of neighbouring cells, whereas catenins link the AJ to the cytoskeletal network. Cadherin-catenin complexes are also important for maintaining cell polarity, regulating epithelial migration and proliferation and formation of adhesive complexes, such desmosomes [46, 47].

Tight junctions (TJs) are the apical adhesive junctional complexes at the border between apical and lateral membrane regions (Fig. 3). TJs represent a selective/semipermeable paracellular barrier which enable the transport of ions and solutes through intercellular space and prevent the translocation of luminal antigens, microorganisms and their toxins. They form an interconnected network that act as dynamic and strictly regulated port of entry in response to signals such as cytokines, bacterial components and other signals from lumen, lamina propria and epithelium and that also participate in preserving of cellular polarity [27, 48]. TJs consist of four unique families of transmembrane proteins – occludin, claudins, junctional adhesion molecules (JAMs), and tricellulin [49]. Occludin, claudins and JAM proteins contain extracellular loops responsible for TJ protein-protein interactions and regulation of selective paracellular permeability, whereas intracellular

domain interact with specific scaffold protein zonula occludens (ZO-1, ZO-2 and ZO-3), which is required to link occludin to the actin cytoskeleton of the cell [50, 51].

Expression of these junctional proteins in the intestine is tightly regulated and depends on the intestinal compartment, villus/crypt localization, cell membrane specificity and phosphorylation [52]. Altered expression and/or localization of tight junction proteins and intestinal barrier dysfunction is thought to be critical in the predisposition and exacerbation of numerous autoimmune and inflammatory conditions, including IBD, food allergy, celiac disease and diabetes. For example, central mediators of intestinal inflammatory disease, cytokines IFN-γ and TNF-α, promote the reorganization or disruption of several TJ proteins such as ZO-1, JAM-A, occludin, claudin-1 and claudin-4 through deregulated expression of these proteins [48, 53]. Whereas, stimulation of colonic epithelial cells with Th2 cytokines (IL-4 or IL-13) induced an increase in intestinal permeability, the anti-inflammatory cytokine IL-10 has been shown to have protective role in intestinal barrier function [54].

Some enteric pathogens (such as *Vibrio cholerae*, enteropathogenic *Escherichia coli*, *Clostridium perfringens* and others) have evolved mechanisms to disrupt the TJs of epithelial cells and to cross the epithelial barrier or to gain nutrients. They could bind directly to cell-surface molecules and induce changes in TJ protein expression, or generate toxins and proteases, which can promote cell damage and apoptosis, alter epithelial ion transport, and disrupt TJs and the cytoskeleton [55]. In contrast to enteric pathogens, some commensal and probiotic bacteria have been reported to enhance barrier function and/or protect against barrier disruption by pathogens [45]. Studies investigating the effects of probiotics on tight junction proteins have shown that live *Streptococcus thermophilus* and *Lactobacillus acidophilus* alter phosphorylation of several related tight junction proteins [56], whereas live *Escherichia coli* Nissle 1917 has been shown to increase ZO-2 production [57]. Enhancement of epithelial barrier integrity by lactobacilli or bifidobacteria strains has been observed in *in vitro* and *in vivo* models [58-60] as well as in biopsy of healthy humans [61]. Several studies indicated that TJ modification is mediated by TLR2 ligands through modulation of activity of specific isoform protein kinase C (PKC-ζ) [62-64].

1.2.2 Recognition of microbes in the intestine

The mammalian intestine is an extremely complex and rich ecosystem and provides an extensive platform for intercellular signaling between the components of microbiota, the host epithelial cells and incoming pathogens. The interaction between immune system of the host and its microbiota is mediated by a variety of proteins called as pattern recognition receptors

(PRRs), which are expressed by IECs (e.g. enterocytes and Paneth's cells), immune cells such as dendritic cells (DCs) and many hematopoietic or nonimmune cells (Table 1) [65, 66]. The well-characterized PPRs families include Toll-like receptors, nuclear oligomerization domain (NOD)-like receptors, retinoic acid inducible gene I (RIG-I)-like receptors that recognize viral RNAs, C-type lectin receptors and AIM2-like receptors. All these receptors recognize microbe-associated molecular patterns (MAMPs), consisting of conserved structures found both on pathogenic and non-pathogenic microorganisms. The innate sensing mediated by PRRs is essential in detection of pathogens and initiation of immune responses, but also in maintaining homeostasis towards commensal microbiota [67, 68]. Moreover, interaction between PRR-MAMP is crucial in promotion of mucosal barrier function and regulation of the production of mucin glycoproteins, AMPs, IgA and cytokines [69]. Several studies have shown that loss of specific PRRs can lead to an altered microbial composition and intestinal barrier defects leading to microbial invasion into systemic organs [70, 71].

1.2.2.1 Microbe-associated molecular patterns

The term microbe-associated molecular pattern (MAMP) or pathogen-associated molecular pattern (PAMP) have been referred to as small molecular motifs conserved within a class of microbes, that are present on all microorganisms but not on the body´s own cells. The immune system is thus able to distinguish self (body) from pathogenic motifs and responds against the microorganisms. They include peptidoglycans, lipopolysaccharides (LPS) and lipoproteins, lipoteichoic acid, wall teichoic acid, flagellin, glycan, mannose-rich oligosaccharides, oligopeptides and unmethylated CpG DNA of bacteria or double-stranded RNA of viruses and others (Table 1). Although each MAMP have a similar basic structure, the variability of bacterial ligands showed that the interaction with PRR is highly bacterial strain specific, and can induce distinct cytokine response of the host [40, 72]. Moreover, different levels of bioactivity have been described for Gram-positive and Gram-negative bacterial surface components. LPS possess 1000-times higher immunomodulatory activity than peptidoglycan of Gram-positive bacteria [73]. naïve

1.2.2.2 Pattern recognition receptors

Recognition of MAMPs by PRRs at cell surface or in intracellular membrane vesicles induces a signaling cascade that can result in the production of cytokines, chemokines and other effector molecules and thus activation of the immune response of the host (Fig. 4).

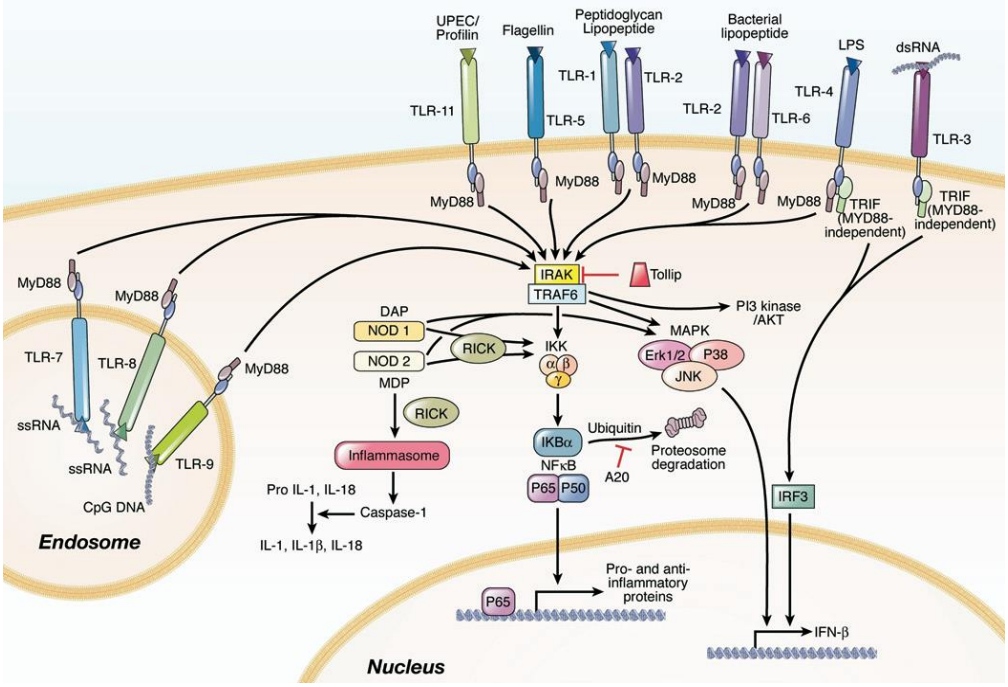

Fig. 4: Recognition of bacterial ligands by membrane-bound toll-like receptors (TLR) and NOD-like receptors (NLR), with signaling through NF-κB pathways and mitogen-activated protein kinases (MAPK) signal transduction pathways. The signaling cascade is mediated through one or combination of four TIR-containing adaptor proteins (MyD88, TRIF, MAL or TRAM), IRAK and TRAF family members as upstream components of signaling cascade [40, 74]. Diversification, amplification and activation of MAP kinase (JNK, p38, and ERK) lead to degradation of NF-κB inhibitor (IKB) and the translocation of NF-κB into nucleus where this factor activates transcription of genes encoding pro-inflammatory cytokines and chemokines, AMPs and initiate the inflammatory response [75, 76]. TLR3 and in some cases TLR4 signaling cascade is mediated through the adaptor protein TRIF, which is initiated by dsRNA or LPS, respectively, and lead to the expression of type 1 interferons [77]. Expression of TLRs on different subcellular locations seems to correspond to the chemical nature of the ligands, which they recognize. TLR 1, 2, 4, 5, 6, and 11 which recognize mainly microbial membrane components (lipids and proteins), are expressed at the plasma membrane. TLR 3, 7, 8 and 9 sensing microbial nucleic acids are found in endocytic compartments, such as the endoplasmatic reticulum, endosomes, lysosomes and endolysosomes [76, 78]. Adopted from Sartor [19].

Toll-like receptors (TLRs) are transmembrane glycoproteins, that share a conserved structure with and extracellular domain that contains multiple leucine-rich repeats (LRR), and a highly conserved intracellular domain, the Toll-interleukin 1 receptor (TIR) domain. The extracellular LRR domains are responsible for the binding to MAMP and contribute to the homo- and hetero-dimerization of the TLRs, which is required for their function [78]. All TLRs, except TLR3, signal through adaptor protein MyD88 [79]. Full ligand sensitivity of TLRs may depend on the activity of another co-receptors and molecules. For example, CD14 and LPS-Binding Protein (LBP) are known to facilitate the presentation of LPS to MD-2 co-receptor in TLR4 signalization pathway. Some studies suggest that LPS from pathogenic bacteria and commensals is recognized differentially by TLR4 to induce inflammation, although the structural differences between pathogenic and non-pathogenic LPS remain uncertain [80].

TLRs play a central role in both innate and adaptive immune responses in host defence against pathogenic infection. TLRs have also critical function in the activation of naïve T

cells and development of Th1 immune response. TLR recognition by antigen presenting cells, such as dendritic cells and macrophages, lead to their migration from the periphery to secondary lymphoid organs, upregulation of costimulatory molecules (such as B7), enhanced antigen processing and presentation, and the secretion Th1-polarizing cytokines such IL-12 [76, 81].

Table 1: Toll-like and NOD receptors, their ligands and localization.

Receptor	Ligand(s)	Ligand location	Cell types
TLR 1/2	multiple triacyl lipopeptides	Bacteria	MO/MF, DC, B-l
TLR 2	multiple glycolipids, lipopeptides, lipoproteins	Bacteria	MO/MF, N, MyDC, MC
	lipoteichoic acid	G+ bacteria	
	HSP70	Host cells	
	zymosan (β-glucan)	Fungi	
TLR 3	double-stranded RNA	Virus	DC, B-l
TLR 4	LPS	G- bacteria	MO/MF, N, MyDC, MC, B-l, Intestinal epithelium
	several heat shock proteins	Bacteria and host cells	
	fibrinogen and hyaluronic acid fragments	Host cells	
TLR 5	flagellin	Bacteria	MO/MF, DC, Intestinal epithelium
	profilin	T. gondii	
TLR 6/2	multiple diacyl lipopeptides	Mycoplasma	MO/MF, MC, B-l
TLR 7	small synthetic compounds		MO/MF, pDC, B-l
	single-stranded RNA	RNA virus	
TLR 8	small synthetic compounds; single-stranded RNA		MO/MF, DC, MC,
TLR 9	unmethylated CpG Oligodeoxynucleotide DNA	Bacteria, DNA virus	MO/MF, pDC, B-l
TLR 10	Unknown		Only human
TLR 11	Profilin	T. gondii	MO/MF, liver cells, kidney urinary bladder epithelium
TLR 12	Profilin	T. gondii,	MF, Neurons, DC; only mouse
TLR 13	bacterial rRNA sequence CGGAAAGACC	Virus, bacteria	MO/MF, DC ; only mouse
NOD 1	meso-diaminopimelic acid from peptidoglycan	G- bacteria, mycobacterium	Epithelial cells and others
NOD 2	muramyl dipeptides from peptidoglycan	G+ and G- bacteria, mycobacterium	DC, MF, epithelial cells, Paneth´s cells, T-l

MO, monocyte. MF, macrophage; DC, dendritic cell; N, neutrophil; B-l, B lymphocyte; T-l, T lymphocyte; MyDC, myeloid DC; pDC, plasmacytoid DC; MC, mast cell [40, 77, 78, 82].

Nucleotide-like receptor (NLR) proteins belong to a diverse family of cytoplasmic microbial sensors, consisting of 22 members in humans and about 34 in mice [83]. NLR proteins likewise TLRs recognize a wide range of bacterial ligands, toxins or extracellular danger signals, that play role in defence of pathogens, as well as certain damage-associated molecular patterns (DAMPs) of the host cells (Table 1) [84]. These receptors could signal through different pathways using caspase activation and NF-κB, which lead to cytokine, chemokine and/or defensin expression or cell death (Fig. 4). Whereas NOD1 is expressed throughout the epithelium, NOD2 is predominantly expressed in Paneth's cells where it acts as one of the critical factors in regulation of the bacterial load in the intestine through secretion of antibacterial compounds and maintaining the normal microbial population [85]. Dysregulated host-microbe interaction due to NOD2 mutations may increase the susceptibility

to abnormal gut inflammation and, in combination with other genetic or environmental factors (e.g. altered microflora), may result in the development of inflammatory bowel disease [86].

TLR and NLR signaling with subsequent inflammatory responses are required for protection the host from pathogenic infection. However, PRR signaling needs to be carefully regulated to avoid excessive immune responses to non-pathogenic microbiota by the mechanisms involving the regulation of TLR expression, TLR localization, differential apical and basolateral TLR signaling, negative regulation of the NF-κB pathway and attenuation of NF-κB activation by commensal bacteria [87]. In normal condition, TLRs are expressed at low levels on the epithelial surface and their expression is highest in the intestinal crypts. TLR2 and TLR4 are located on apical side of intestinal epithelial cells, whereas TLR5 is mainly expressed on basolateral side in the human colonic epithelial cells, where it detects flagella on pathogens crossing the epithelium [65, 88]. The crucial role of NF-κB as intestinal homeostasis regulator has been clarified by studies using knockout mice that are not able to activate NF-κB signaling and develop severe chronic intestinal inflammation [89]. Mice lacking TLR2, TLR4, TLR5 and TLR9 and MyD88 are more susceptible to experimentally induced colitis with higher level disease severity and mortality [90, 91]. On the other hand, mice lacking TLR4, NOD1 or the common adaptor protein MyD88 have impaired immunity to infectious agents [92-94].

1.2.3 Mucosa-associated lymphoid tissue of the gut

Mucosal surfaces of the organisms are protected by a strongly developed and highly specialized immune system, the mucosa-associated lymphoid tissue (MALT) that comprises about 80% of immunologically active cells. The most of these cells are found in GIT, where they compose the so called gut-associated lymphoid tissue (GALT). The GALT consists of organized tissue representing solitary and multiple lymphatic follicles (Peyer's patches, appendix), mucosa-draining mesenteric lymph nodes, cryptopatches, lymphoglandular complexes in the large intestine and freely dispersed lymphocytes throughout the epithelium (intraepithelial or lamina propria lymphocytes) [28, 29, 95]. Peyer's patches and lymphatic follicles cells represent an inductive site of the mucosal immune response, where antigens sampled from mucosal surfaces stimulate associated naïve T and B lymphocytes. The germinal centers of these follicles compose mainly of B cells, whereas T lymphocytes are prevalent in interfollicular space. Organized lymphoid tissue is covered by an epithelial layer (follicle-associated epithelium, FAE), which contains a specialized type of epithelial cell – microfold (M) cells (Fig. 5) [96, 97].

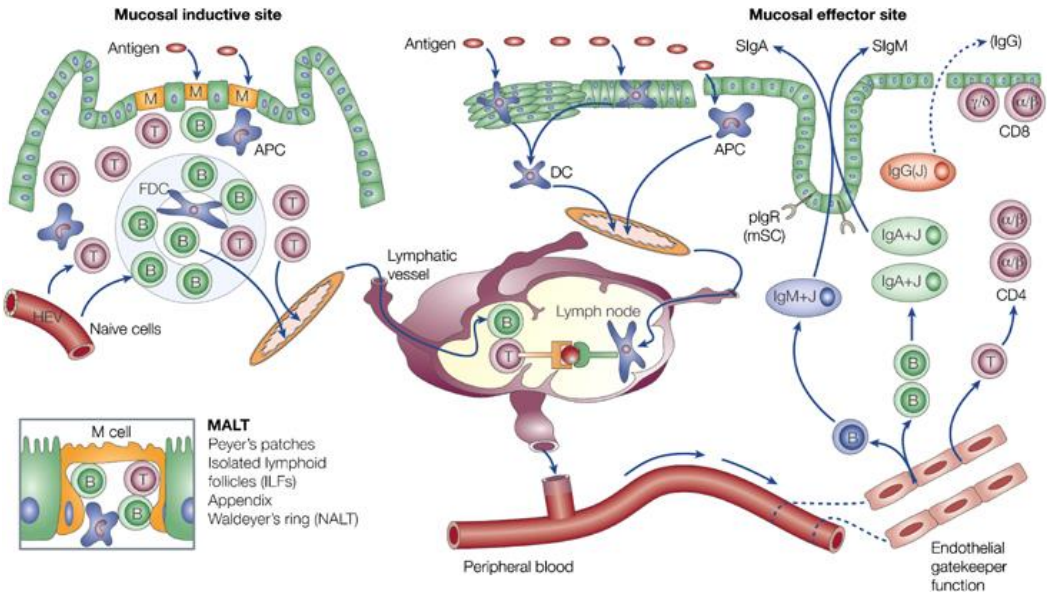

Fig. 5: The human mucosal immune system. Inductive sites of mucosal immunity are constituted by regional MALT with their B-cell follicles and M-cell-containing follicle-associated epithelium through which exogenous antigens are transported actively to reach APCs, including DCs, macrophages, B cells, and FDCs. DCs may capture antigens at the effector site and migrate via draining lymphatics to local/regional lymph nodes where they stimulate T cells for productive or suppressive immune responses. Naïve B and T cells enter MALT and lymph nodes via HEVs. Generated memory/effector B and T cells migrate from MALT and lymph nodes to peripheral blood for subsequent extravasation at mucosal effector sites, which is directed by the local profile of vascular adhesion molecules and chemokines. The gut lamina propria contains few B lymphocytes, IgA (dimers/polymers) and IgM (pentamers) plasmablasts and plasma cells, rare amount of IgG plasma cells and many T cells (mainly $CD4^+$). APCs, antigen-presenting cells; DCs, dendritic cells; FDCs, follicular dendritic cells; HEVs, high endothelial venules; MALT, mucosa-associated lymphoid tissue; SIgA, secretory IgA; SIgM, secretory IgM. Adopted from Brandtzaeg, *et al*. [96].

The effector part of the GALT is represented by diffuse lymphocytes which after extravasation, retention, and differentiation contribute to the formation of SIgA antibodies (Fig. 5) [96]. IELs on the basolateral side of enterocytes represent predominantly CD8+ T cells differing from T cells found in periphery. They could bear either αβ or γδ T-cell receptor (TCR), adhesive molecules (integrin αEβ7) and cytoplasmic granules containing cytolytic proteins [98]. It has been shown, that commensal bacteria modulate the abundance and activation of γδ T cells, which can produce some AMPs in response to the penetrating bacteria [99]. Diffuse lymphocytes of lamina propria, predominantly represented by CD4+ T cells and B cells producing polymeric IgA, are the most abundant and active effector cells of mucosa. These T cells have the αβ TCR receptor and are generated in the PP as a result of antigen stimuli [28].

1.2.4 Innate immune response - induction of the immune response to antigens

The intestinal immune system constantly samples the bacterial antigens in order to distinguish which ones require an immune response and which can be safely tolerated. Antigen sampling and handling in the intestine comprise of three distinct mechanisms. First,

enterocytes transport bacterial antigens from intestinal lumen to the lamina propria and act as antigen presenting cells (APC) due to expression of MHC II class molecules. Second, antigen sampling occurs in specialized follicular associated epithelium (FAE) of Peyer's patches, which is composed by enterocytes and M cells on the surface, T cells, B cells, DCs and macrophages inside the lymphoid follicle (Fig. 5). M cells do not possess the brush border or mucus production, but they play an important role in transporting antigens across the epithelium to the lymphoid follicle. Some pathogens also use M cells to penetrate the intestinal mucosa and invade the host. Third, intestinal DCs use their dendrites to sample the antigens from lumen without distortion of the integrity of epithelial tight junctions [100]. Macrophages and DCs plays important role in both innate and adaptive immunity, and participate in compartmentalization of the systemic and mucosal immune system [101].

Macrophages are present in high numbers in the mammalian GIT in close contact with the epithelium. Commensal microorganisms that cross the intestinal epithelial barrier undergo rapid phagocytosis and elimination by these cells in lamina propria. Although macrophages from many tissue sites (such as the bone marrow) secrete pro-inflammatory mediators that recruit neutrophils and activate T cells, intestinal macrophages recognizing the bacteria do not mediate strong pro-inflammatory response. They efficiently kill the phagocyted microorganisms through mechanisms that include antimicrobial proteins and reactive oxygen species reflecting an adaptation to the high bacterial content of the gut [102, 103]. Injury of gut epithelial cell can quickly lead to bacterial penetration areas that trigger the expression of a specific repair pathway in macrophages, inducing them to migrate to the damaged areas and to produce growth factors that help to vigorous enterocyte proliferation and replacement of damaged epithelium with new cells [104].

Mucosal DCs are the crucial APCs responsible for initiation of adaptive immune response to pathogens and commensals and for maintaining of immune tolerance to self antigens [105]. After sampling, the live bacteria are not immediately killed but persist within the activated DCs and are transported to the mesenteric lymph nodes (MLNs) (Fig. 5). Short lifespan of DCs laden with live bacteria do not allow them to reach central systemic lymphoid structures. This mechanism limits the induction of immune response against commensals but works against pathogenic bacteria that have penetrated epithelial defences [25, 106].

Most tissue resident DCs are immature with low expression of major histocompatibility complex (MHC) molecules and co-stimulatory molecules (CD40, CD80, and CD86). When they contact with MAMPs or DAMPs from damaged or stressed cells, they undergo maturation, after that they express high levels of MHC, costimulatory molecules and

cytokines which are needed for antigen presentation and T cell activation, clonal expansion and differentiation (Fig. 6). Non-dangerous luminal antigens are presented to T cells by immature dendritic cells possessing low expression of costimulatory molecules, thus limiting the activation of T cells and initiation of adaptive immune responses [107].

Two distinct subsets of DCs have been defined in the intestine on the basis of the expression of exclusive molecules, the integrin CD103 and the fractalkine receptor CX3CR1 [108]. In the lamina propria, two subsets of CD11c+ DCs are derived from two different blood cell precursors with distinct function. The CD103- DCs have features of macrophages and are in close contact to the epithelium. The subset CD103+ DCs which express CX3CR1, can continuously sample the intestinal microbiota by extending transepithelial dendrites through the epithelium by CX3CR1-dependent mechanism including interaction with IECs [109]. DCs with sampled commensals are transported to MLNs, where they potentially induce the production of IgA by B cells to limit the penetration of commensals into host tissue [110].

The major subset of lamina propria (LP) DCs is the CD103+CX3CR1- that migrate from LP to MLNs to present locally administered antigen to naïve CD4+ T cells and promote their conversion into inducible FoxP3+ regulatory T cells [111, 112]. Recently it has been shown, that CD103+ DCs produce the enzyme indoleamine 2,3-dioxygenase (IDO), which is required for their tolerogenic function and is a key enzyme that controls the balance between regulator and effector functions of T cells [113]. Different DC subsets used different PPRs and molecular pathways to induce FoxP3+ T cells. For example monocyte-derived DCs and primary myeloid DCs required the activation of retinoic acid (RA) metabolism, TLR2 and DC-SIGN signaling, while IDO and TLR9 are involved in regulatory profile of plasmocytoid DCs [114]. Interestingly, these regulatory factors are also induced by IECs after interaction with some bacteria showing that bacteria can potentially modulate DC function in LP. Moreover, Wang *et al*. [115] showed, that administration of probiotic mixture VSL#3 alter the distribution and phenotypes of dendritic cells within the intestinal mucosa of mice. Also the capacity of selected lactobacilli to protect mice from colitis was recently shown to be linked to their cell wall structure and to correlate with the induction of regulatory pathways including mucosal CD103+ DCs and IDO activation [116, 117]. In the Peyer's patches (PP) and isolated lymphoid follicles are DCs considered to be primarily responsible for T cell-dependent IgA response through TNF-α and inducible nitric oxid synthase (iNOS) production [118]. On the other hand, CD11b+ subset of DCs in PP play an important role in IgA isotype switching by secretion of IL-10, TGF-β, IL-6 and RA [119].

Innate lymphoid cells (ILCs) are cells generated from common lymphoid precursors, which do not express an antigen-specific receptor. ILCs of the intestinal lamina propria are classified into three groups based on the phenotype and function: ILC1, ILC2 and ILC3 [120]. ILCs reflect many functions of CD4+ T helper cells especially by production of some cytokines. In contrast to the T cells, they are not selected on the basis of antigen specificity, and expand and act shortly after stimulation. Therefore, ILCs play fundamental roles early in responses to infection and injury, in the maintenance of homeostasis, and possibly in the regulation of adaptive immunity. It has been shown that their dysregulation may lead to immune pathology such as allergy and autoimmune disease [121].

1.2.5 Adaptive immune response – effector site of immune response

Essential players of adaptive immune responses are T and B lymphocytes. The development of adaptive immune response to antigen stimuli takes longer than the innate response. Adaptive immune system provides a more effective protection against pathogens through its ability to recognize and remember an impressive number of antigens. Memory T and B lymphocytes provide more effective immune responses against secondary infections. Both lymphocytes possess specific antigen receptors (TCR and BCR) in which unique lymphocyte specificity is created by genetic rearrangements of variable areas during lymphocyte development. While B cells contribute to the immune response by secreting antibodies (humoral immunity), T cells play their role in cell-mediate immunity. On the other hand, the processing of food antigens by intestinal DCs with absence of activating molecules (such as TLR ligands from microorganisms) promotes the peripheral tolerance of T and B cells [100, 122].

1.2.5.1 Effector T-cells

T cells recognize the antigenic determinant in the complex with appropriate MHC molecule through their TCR receptor (consisting of αβ or γδ chains), whereas co-receptor CD3 mediates T cell activation signal. CD8+ T cells recognize their antigens in the form of a peptide/MHC class I complex, while CD4+ cells interact with complex peptide/MHC class II. MHC class I molecules are expressed at the surface of all nucleated cells. MHC class II are presented only on professional APCs (DC, macrophages, B lymphocytes, enterocytes), engage with the T cell receptor and provide co-stimulatory signals through other molecules on DC (e.g. CD80 or CD86) and CD28 on the T cell (Fig. 6) [123]. DCs can also stimulate T cells indirectly by production of IL-12 that promotes the differentiation of the CD4+ T helper

(Th) 1 cell subset. Depending on the phenotype of APCs and cytokine environment, T cells differentiate into different subsets of effector or regulatory T cells (Fig. 6). CD4+ Th cells control the immune response by activating and regulating other immune cells such macrophages and B lymphocytes through the secretion of specific cytokines, whereas CD8+ cells become cytotoxic and targets the infected cells. The differentiation of naïve CD4+ T cells is generally dependent on environmental conditions (DCs, cytokines milieu, nature and dose of antigen, etc.) and transcription factors and proceeds within a few days after naïve T cells recognized antigen presented by APCs (Fig. 6) [122, 124].

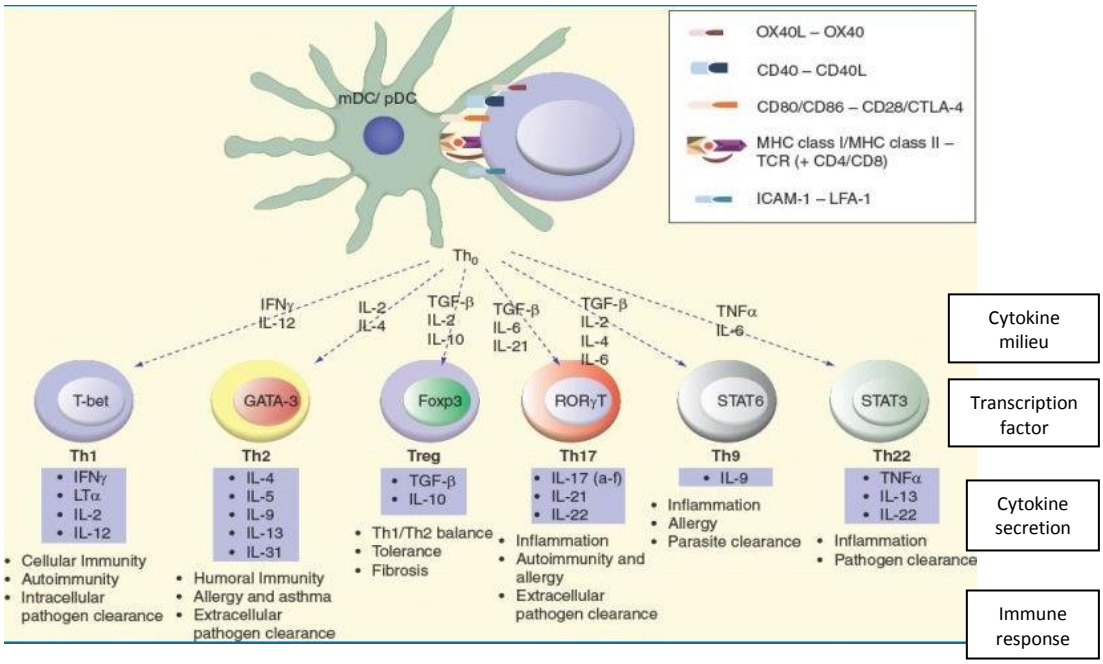

Fig. 6: Activation and differentiation of CD4+ Th lymphocytes upon interaction with dendritic cell. Depending on the encountered antigen and cytokine milieu, T cells may differentiate through transient state Th0 into Th1, Th2, Th9, Th17, Th22 or Tregs for appropriate immune response. The interaction between dendritic cell and T cell is dependent on many stimulatory and co-stimulatory molecules to exert various physiological effects. Adopted from Gaurav and Agrawal [124].

CD4+ T cells are mainly found in two distinct cell types, Th1 and Th2. These subsets are distinguished by the production of specific cytokine pattern and they are involved in specific immune response. **Th1 cells** produce pro-inflammatory cytokine milieu like IFN-γ, TNF-α and IL-2, which can stimulate the phagocytosis and destruction of intracellular microbial pathogens. Th1 differentiation is reliant on presence of IL-12 and IFN-γ, which induces a STAT–1 dependent signaling cascade. This pathway up-regulates expression of the transcription factor T-bet that regulates the signaling towards enhancement of the production of IFN-γ and Th1 cell differentiation. T-bet also prevents differentiation towards Th2 and Th17 by suppressing the expression of the factors required for these subsets [122, 125].

Th2 cells produce IL-4, IL-5, IL-6 and IL-13, which generally stimulate B cells to the secretion of antibodies directed toward large extracellular parasites (IL-4 or eosinophil-stimulating IL-5). Th2 cell differentiation is induced by TCR/IL-4 receptor signaling in presence of IL-4 in the extracellular milieu. Induction of STAT-6-dependend pathway leads to the expression of transcription factor GATA-3 (master regulator toward Th2 cells). GATA-3 also suppresses the critical elements for the Th1 differentiation process [126, 127]. Likewise other cytokines have been found to be associated with Th1 type responses (e.g., IL-2, lymphotoxin) or Th2 type responses (e.g. IL-6, IL-9, IL-10), but their secretion does not primary characterize the Th1 or Th2 type response [122].

In recent years other subsets of differentiated T lymphocytes have been described. In presence of IL-4 and TGF-β, the Th2 cells can further differentiate into a **Th9 cells**, which produce IL-9 and IL-10, whose pathophysiological meaning as well as the possibility that they can also directly originate from the naïve Th cell are still unclear. In the presence of TNF-α and IL-6, naïve Th cells differentiate into **Th22 cells** expressing aryl hydrocaron receptor. They secrete IL-22 and may play a role in skin homeostasis and pathology [128]. Another subset of CD4+ T helper cells with pro-inflammatory properties which is able to respond to extracellular bacteria and fungi is called **Th17 cells**. In the presence of TGF-β, IL-6 and IL-21 (in mice) or IL-1β and IL-23 (in humans), the naïve Th cells express retinoic acid - related orphan receptor (ROR)γt that lead to differentiation into Th17 cells. These cells are characterized by the production of IL-17, IL-21, IL-22, TNF-α and CCL20 [128-130]. These cells can be also responsible for autoimmune disorders. **Follicular helper T cells** (Tfh) are located in follicular areas of lymphoid tissue, where they participate in the development of antigen-specific B-cell immunity. They express CXCR5 receptor and cytokines IL-6 and IL-21 are involved in the differentiation of these cells [122]. According to predominant cytokine secreted, they could be classified as Tfh1 (IFN-g), Thf2 (IL-4), or Thf10 (IL-10) and they promote the secretion of IgG2a, IgG1 and IgE, or IgA, respectively [131].

1.2.5.2 B cells and secretory IgA

Secretory immunoglobulins play an important role in specific humoral defense of mucosal surfaces. The most abundant is IgA that constitutes approximately 75% of the total antibody production in mammals [132]. The secretory IgA (sIgA) antibodies in the intestine have dimeric structure and are differentiated into two subclasses. While sIgA1 is produced systemically and at mucosal surfaces, sIgA2 is produce at mucosal surfaces only. SIgA2 is

more resistant to degradation by bacterial proteases and so it is main IgA subclass produced in the intestinal lamina propria [133].

The studies in germ-free mice showed, that the development of the gut-resident B-cells and the production sIgA in intestine is strongly regulated by exposure to commensal microflora [132, 134]. Commensal-specific IgA is produced with the help of intestinal DCs that sample bacteria associated with the epithelium and interact with B cells through T cell-dependent or independent mechanism. The process of class switch recombination to IgA occurs mainly in the Peyer's patches, isolated follicles and MLNs under control of the TGF-β, retinoic acid, B cell activating factor of the TNF family and a proliferation-inducing ligand (APRIL) [101, 132]. IgA^+ plasma cells translocate to intestinal lamina propria and secrete J-chain-linked IgA dimers that are subsequently transcytosed across epithelial cells into lumen of the gut (Fig. 5) [135]. In the lumen of the gut, secreted IgA limits bacterial attachment with the intestinal epithelial cell surface and restrict the penetration of microorganisms and other antigens across the gut epithelium [132]. Whereas nonspecific IgA binds to microbial surface glycans causing bacterial agglutination, microbe-specific IgA control the microbiota by trapping of the organisms in the mucus, prevention of epithelial-cell invasion and alteration of bound bacteria including abrogation of bacterial resistance to oxidative burst response [136]. In addition to crucial role of IgA in mucosal protection against pathogens and neutralization of their toxins, enzymes or LPS, these antibodies promote homeostasis by the shaping of the intestinal microbiota, preventing mucosal inflammation by immune exclusion, removal of antigen-antibody complexes in the lamina propria and transport several non-specific bactericidal substances contained in mucosal secretions (lactoperoxidase, lactoferrin) to bacterial surfaces [137, 138].

Secretory IgM (SIgM) is of greater importance in newborns and adults with selective IgA deficiency [139]. Healthy individuals also spontaneously produced small amount of IgE that protect the epithelium against the adhesion of bacteria and play a regulatory role [140].

1.2.6 Regulation of the immune response in the gut

The induction and maintenance of tissue homeostasis is crucial for survival of the organism. This process relies on a complex of coordinated innate and adaptive immune responses that selects and regulates or actively suppress the responses against self, food, commensals, metabolites and pathogens in the appropriate manner. Specialized populations of cells play the integral part in the induction of the regulatory response. Failure to regulate these

responses can lead to severe pathological outcomes ranging from inflammatory bowel disease to allergies [5].

1.2.6.1 Regulatory T cells

Regulatory T (Treg) cells are predominantly of CD4+ T cell subtype and represent 5% of all CD4+ T cells. Their major role is regulation of the function of other T lymphocytes to avoid excessive immune activation. T cells with regulatory properties comprise: natural Treg (nTreg), induced Treg (iTreg), Tr1 (IL-10 dependent), Th3 (TGF-β dependent) and CD8+ Treg [141]. CD4+CD25+ Treg cells specifically express the Forkhead transcription factor FoxP3, which is induced downstream to TGF-β signaling [142]. **Natural Tregs** originated in the thymus are CD4+ CD25+ FoxP3+, while **inducible Treg** (iTregs) are induced in peripheral lymphoid tissues such as GALT and MLN by interaction with APCs and dietary antigens and are CD4+, CD25+ or CD25- and FoxP3+ or FoxP3- [143]. It has been shown that the differentiation of Tregs is determined by local tissue environment involved epithelial derived TGF-β, RA and thymic stromal lymphopoietin (TSLP). TGF-β is the critical cytokine responsible for the initiation of the iTreg cell lineage, but also IL-10 and IL-35 are other potent inhibitory cytokines with the ability to suppress pro-inflammatory response and thus limit tissue damage by inflammatory process or induces oral (mucosal) tolerance. IL-10 and TGF-β also potently suppress IgE production, thereby may have important role in attenuating allergic inflammation [122]. **Tr1** are IL-10-producing cells that importantly suppress inflammation and autoimmune processes. Cytokines IL-10 and IL-27 are involved in their differentiation [144]. In the lamina propria of colon, the majority of the IL-10 producing cells are FoxP3+ Tregs, while in the small intestine is IL-10 produced by CD4+FoxP3- intraepithelial lymphocytes and by Tr1 cells of lamina propria [143]. Other cells with regulatory function **- Th3 cells -** are able to supress excessive immune activation. These cells express the latency-associated peptide on the surface and secrete TGF-β and are especially relevant in the induction of oral tolerance [141].

Commensal bacteria are critical and active inducer of regulatory responses. Antigens derived from the commensal microbiota specifically influence the proportion of Tregs in lamina propria. These iTregs express a unique subset of TCR receptors, which are distinct from those expressed by naïve and effector CD4+ T cells in the LP [145]. Induction of Tregs or influence of mucosal DCs toward a pro-regulatory function is proposed as one of the mechanisms of action of probiotics [5, 116, 146]. Mazmanian *et al.* [147, 148] have demonstrated that unique symbiont molecule – polysaccharide A, which is produced by a

human symbiont *Bacteroides fragilis*, can induce and expand IL-10 producing Treg cells and thus protect mice from experimental colitis induced through TLR-2 dependent manner. Atarashi *et al.* [149] have shown that some indigenous *Clostridium* species also promotes Treg cells accumulation by the capacity to create a TGF-β rich environment. Moreover, recent studies have reported that SCFA metabolites derived from commensal microbiota, particularly butyrate, can directly induce the differentiation of intestinal FoxP3+ iTregs in the colon [150-152]. It appears that ability to increase the frequency of Tregs in colon is not restricted to defined commensal species, but could be common to large number of species of resident microbiota [153]. Aside from their suppressor function, Treg cells can act as helper cells to induce microbiota-specific IgA responses in TGF-β dependent mechanism [154].

1.2.6.2 Mucosal (oral) tolerance

Mucosal immune system is able to respond in two opposite manners, i.e. development of immunity against pathogens and maintaining the systemic unresponsiveness (tolerance) or active suppression against non-pathogenic antigens from food and commensal bacteria. Oral application of antigens leads to the inhibition of systemic immune response to parenterally administered antigens so called "oral tolerance". Oral tolerance to food antigens and commensal flora is established during the first weeks after the birth. Intestinal and nasal mucosa surfaces are the most sensitive sites for induction of the tolerance [1, 141]. Dose of antigen is one of the main factors determining which mechanism of tolerance induction will be involved in. Low doses favour the generation of CD4+ regulatory cell-driven active suppression, whereas high doses of antigen favour deletion or anergy and apoptosis of T cells [155]. The mechanism of suppression is triggered by inhibitory cytokines TGF-β and IL-10, which are produced by regulatory Tr1 and Th3 cells. Disruption of the tolerance against food and commensal antigens may lead to pathological conditions including celiac disease, allergies and inflammatory bowel disorders [28].

1.3 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is the common term for a group of idiopathic chronic relapsing disorders associated with uncontrolled inflammation within the gastrointestinal tract [156]. Ulcerative colitis (UC) and Crohn's disease (CD) are the two major forms of IBD caused by failure of intestinal homeostasis and dysregulated immune responses to the microbiota in genetically susceptible individuals [157, 158]. However, both diseases show their distinct pathogenesis, symptomatology, inflammatory profiles and gut

microbiota composition. Inflammation associated with CD is discontinuous, may extend deeply into the submucosal regions and occurs anywhere along the alimentary tract. This form of IBD is predominantly associated with a Th1 and Th17 immune responses, characterized by increased production of cytokines IL-12, IL-23, IL-27, IFN-γ and TNF-α. In UC, inflammation involves only the superficial layers of the intestinal mucosa and is located into regions of the gut most highly colonized by bacteria, starting at the distal colon and leading proximally along the large bowel. Diversely, UC seems to be associated with a Th2 type of immune response, mainly leading to elevated levels of IL-5 and TGF-β [19, 158].

The incidence and prevalence of IBDs is higher in more developed Western countries, such as the United States, Northern Europe and the United Kingdom, with the average number of cases ranging from 100 to 500 cases per 100,000 persons [159, 160]. The increasing IBD incidence in developing countries highlights the significance of environmental factors on IBD development. Industrialization and urbanization of societies are associated with changes in the microbial exposures, sanitation, occupations, diet, lifestyle behaviours, medications, and pollution exposures, as well as elimination of helminth parasitic infections, which have all been implicated as potential environmental risk factors for IBD [160]. However, the aetiology of IBD is more complex and multifactorial and is believed to be the consequence of genetic, environmental and immunological factors [161].

Familial aggregation suggests that IBD is heritable, with 5–20% of patients with IBD having a family history of the disease. A positive IBD family history is generally observed more frequently in patients with Crohn's disease than in patients with ulcerative colitis with concordance rates between monozygotic twins 6-14 % in UC compared to 44-50 % in CD [162-164]. Over 160 genetic loci have been linked with the development of IBD, and many of the identified gene products are involved in mediating interactions between the immune system and intestinal microorganisms [165]. One of the strongest links is between NOD2 mutations and ileal Crohn's disease showing, that NOD2-deficient mice have greater bacterial loads in the terminal ileum due to lower expression of α-defensin [39].

Nevertheless, IBD pathogenesis includes multiple defects in different upstream pathways, initiated by barrier dysfunction that brings increased bacterial translocation through the lamina propria. Microbial antigens elicit a strong inflammatory response due to basolateral TLR stimulation (as opposed to physiological apical stimulation of TLR9 receptors), activation of the NF-κB pathway and consequent induction of pro-inflammatory chemokine and cytokine secretion. This inflammatory process is aggravated by the reduction of luminal defensins (mainly in CD patients) and IgA and defective phagocytosis, which

amplifies the bacterial translocation through the injured epithelial layer. Disease mainly progress as a results of defective immunoregulation and immunotolerance in response to the initial inflammatory insult, due to overaggressive T cell reaction, dysfunctional regulatory T cells and antigen presenting cells (APC) (Fig. 7) [31, 166].

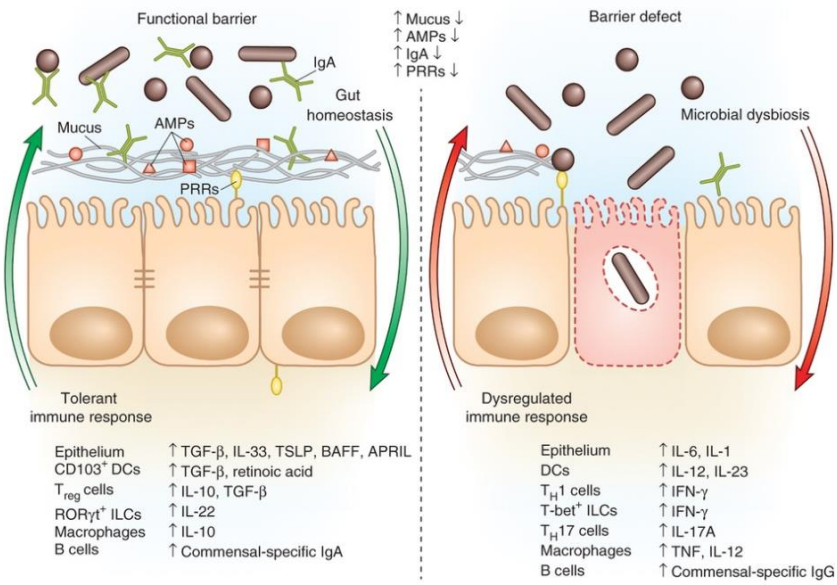

Fig. 7: Innate barriers and their defect in inflammatory bowel disease. Functional barrier is represented by normal amounts of PRRs, mucus, AMPs and secreted IgA that promotes intestinal homeostasis with the microbiota. MAMPs-PRRs interactions stimulate the secretion of cytokines, chemokines and IgA from immune and epithelial cells, which contribute to tolerant immune response. In inflammatory disorders are physical barriers defective. The intestinal immune system directs a potentially harmful pro-inflammatory response by secretion of various mediators of inflammation to microbial dysbiosis, which lead to dysregulated immune response [31].

One of the first steps in IBD pathogenesis is characteristically increased epithelial permeability due to under-expression of certain tight junction proteins (e.g. occludin, ZO-1, JAM-A) concomitant with up-regulation of other pore-forming proteins (especially claudin-2) [167, 168]. In IBD, reduction of intestinal IgA is compensated by increased secretion of IgG, which induces pro-inflammatory cytokine production and mounting of adaptive immune responses to the resident microbiota [19]. Failure of bacterial killing in IBD is also linked to dysfunctional autophagy that plays a key role in innate and adaptive immunity against intracellular and extracellular invading bacterial pathogens (such as *Legionella*, *E. coli*, *Mycobacterium*, etc.). Mutations in some genes coding this process have recently been shown to be associated with CD [169]. Defective autophagy pathway could also influence antigen presentation by APCs, epithelial cells and immune surveillance, and has been shown to be implicated in the regulation of T cell death and proliferation explaining the pathologic behavior of T cells in IBD. Defective interaction between Treg cells in the lamina propria and epithelial cells is crucial in the process of loss of tolerance to microbial exposure. This

mechanism involves activation of NF-κB signaling, which is in healthy individuals normally suppressed by anti-inflammatory cytokines such as TGF-β and IL-10. While in IBD, Th1- and Th17-type immune responses are predominant and lead to chronic inflammation and worsening of the epithelial layer damage [166]. This leads to a cycle of aberrant immune response, mucosal inflammation, and altered microbiota composition with increased mucosal permeability that results to persistent and recurrent nature of IBD.

1.3.1 Role of microbiota in inflammatory bowel disease

Clinical and experimental studies investigated the relationship of microbiota and IBD show that the immune dysregulation can drive microbial changes, and also that shifts in the microbiota can drive immune dysfunction (Fig. 7) [31]. Damage of the intestinal epithelium in IBD patients could lead to establishment of leaky gut wall, which permits bacteria from gut lumen to gain access to the submucosal compartments or even to the systemic circulation, with the potential to cause the sepsis [11]. Studies of the gut microbiota in patients with IBD have revealed quantitative and qualitative changes with an increase of pathogenic bacteria such as adherent/invasive *E. coli*, *Mycobacterium avium* ssp. *paratuberculosis*, *Yersinia*, *Campylobacter*, *Klebsiella*, *Desulfovibrio piger*, *Clostridium difficile* and others. On the other hand, molecular characterization reveals decreased biodiversity of commensal bacteria, most notably the phyla Bacteroidetes and Firmicutes, including the clinically-relevant *Faecalibacterium prausnitzii*, bifidobacteria and lactobacilli or some butyrate producing *Clostridia* [170-174]. However, it is still unclear whether the IBD-associated alteration in the gut microbiota is the cause or the consequence of the disease. Animal experiments showed that transfer of colitogenic microflora from RAG2-/- mice (deficient in both T-bet and adaptive immunity) to another RAG2-/- or wild-type mice could induce colitis in both types of mice. This fact demonstrates that abnormal microflora could initiate inflammatory response in individuals not genetically predisposed [175]. Moreover, aberrancies in bifidobacterial microbiota, including decreased numbers or an atypical composition have been identified in patients with UC and CD [176, 177].

Many clinical trials supported the efficiency of administration of selected microorganisms in IBD prevention and/or treatment. Especially the probiotic bacteria including nonpathogenic *E. coli*, *F. prausnitzii*, *Saccharomyces boulardii*, *Bifidobacterium* and *Lactobacillus* strains have shown the positive effect in maintaining remission and in treating mild to moderate relapses in ulcerative colitis [178-182]. Some preliminary studies have revealed that fecal transplantation, a strategy successfully used in patients with recurrent

C. difficile infection, may be also promising in ulcerative colitis treatment [183]. All these evidence suggest that microbiota play one of the most important role in IBD pathogenesis, including the fact that inflammation mainly occurs in the intestinal sites with the highest bacterial colonization (in UC), and antibiotic treatment often results in amelioration of disease symptoms [184]. Moreover, the colitis does not spontaneously initiate even in genetically modified IL-10- or IL-2-defficient GF animals [185].

1.4 Allergy

Allergy is defined as immunological hypersensitivity that can lead to a variety of diseases via different pathological mechanisms (Table 2). Allergic reactions are symptomatic response to normally innocuous environmental antigen [186]. The **hypersensitivity reaction** is excessive and undesirable immune response to antigen resulting in local tissue injury or systemic manifestations of allergic symptoms including anaphylactic shock or even death. Gell and Coombes (1963) classified hypersensitivity reactions according to immune response and effector mechanisms into four types [187] (Table 2).

Table 2: Classification of hypersensitivity reactions according to Gell and Coombes [187]

Type	Mediators	Mechanism	Associated diseases
I (Immediate hypersensitivity)	IgE	IgE is bound to basophils and mast cells via its high-affinity Fc portion. When an allergen binds to these antibodies, crosslinking of IgE induces degranulation.	Atopic dermatitis, Anaphylaxis, Asthma, food allergies, seasonal allergies Localized and systemic anaphylaxis
II (Antibody-mediated cytotoxic hypersensitivity)	IgG or IgM and complement	Host cells or tissues are destroyed by bound antibodies on the surface, either by activation of complement or by a cytotoxic T cell with and Fc receptor for the antibody (antibody-dependent cellular cytotoxicity)	Blood transfusion reaction Autoimmune hemolytic anemia Goodpasture´s disease Erythroblastosis fetalis
III (Immune complex-mediated hypersensitivity)	IgG or IgM and complement	Antigen-antibody complexes are deposited in tissues, causing activation of complement, which attracts neutrophils to the site	Serum sickness Arthus´reaction Lupus erythematosus Rheumatoid arthritis
IV (T-cell mediated, delayed hypersensitivity)	T cells (CD4+ or CD8+), macrophages, histiocytes	Th1 cells secrete cytokines, which activate macrophages and cytotoxic T cells which mediate direct cellular damage	Transplant rejection Contact dermatitis Tuberculin reaction

The allergic reaction starts after the first exposure with an antigen (allergen), and after the subsequent antigenic contact, the sensitized immune system initiate exaggerated and uncontrolled immune reaction [188]. **Allergen** is the small protein molecule (mainly of molecular weight 10-50 kDa) that is capable to instruct the immune system to initiate IgE – specific immune response (primary sensitizer). Nevertheless, some homologous protein molecules may not act as primary sensitizer but due to the shared similar IgE and T cell

epitopes they can cross-react with specific IgE antibodies and activated allergic Th2 immune response in allergic patients. One example is the birch pollen-related food allergy, where sensitization by the major birch pollen allergen Bet v 1 often leads to allergic cross-reaction to a variety of fruits, nuts and vegetables [189, 190]. Many known allergens are lipid binding proteins (e.g. Bet v 1 and homologues, house dust mite allergens, lipocalins of pets, plant lipid transfer proteins) or glycoproteins (e.g. peanut Ara h 1 and grass pollen Phl p 1). Their lipid ligands and conjugated glycans have been shown to interact with PRR such as TLR and C-type lectins on APCs, and skew the immune system towards Th2-type responses and IgE production [191].

During the development of allergic reaction, professional APCs such as dendritic cells (DCs) come into contact with allergens at mucosal surfaces of the lungs, intestine and skin, directly by extending their dendrites across the epithelial barrier or after insult of the barrier layer. After antigen uptake, DCs process the allergen to peptide fragments and present them via MHC II to naïve T cells in appropriate lymph nodes, leading to clonal expansion and antigen-specific T cells differentiation into CD4+ Th2 or CD8+ T cells [192]. DCs also closely communicate with neighbouring epithelial cells via PRRs that triggers the production of cytokines and chemokines (e.g IL-25, IL-33, TSLP) [193].

The allergic reaction compose of two phases: sensitization related to development of memory T and B cell responses with IgE production and effector phase related to tissue inflammation, injury, remodeling and chronicity (Fig. 8). During the development of **the sensitization phase** of allergic reaction, effector Th2 cells produce cytokines such as IL-4, IL-5, and IL-13. It was shown, that ILCs type 2 also provide Th2-type cytokines which can then activate eosinophils (IL-5, IL-13) and mast cells (IL-9) as well as T and B lymphocytes [194]. In addition, secretion of IL-13 induces mucus production, smooth muscle contractility, and alternative activation of alveolar macrophages, which leads to amplification of IL-33 production. TSLP also drives the maturation of immature lung DC to the presentation of antigen to T cells that drives their differentiation into Th2 cells [195]. Th2 cytokine milieu induces class switching to the ε immunoglobulin heavy chain in B cells and the production of allergen-specific IgE antibodies. IgE binds to the high-affinity FcεRI on the surface of mast cells, basophils and activated eosinophils, thus leading to the patient's sensitization. Re-exposure to the allergen in **the effector phase** induces activation of these cells by cross-linking of the allergen specific IgE antibodies on their surface and leads to secretion of anaphylactogenic pro-inflammatory mediators (including histamine and heparin; prostaglandins, leukotrienes and platelet-activating factor; tryptase and mast cells protease;

and cytokines TNF-α, IL-4, IL-5 and IL-13) responsible for the immediate acute allergic reaction. Mast cells degranulation with a more sustained inflammation is responsible for the late phase in chronic allergic reaction (Fig. 8) [196, 197].

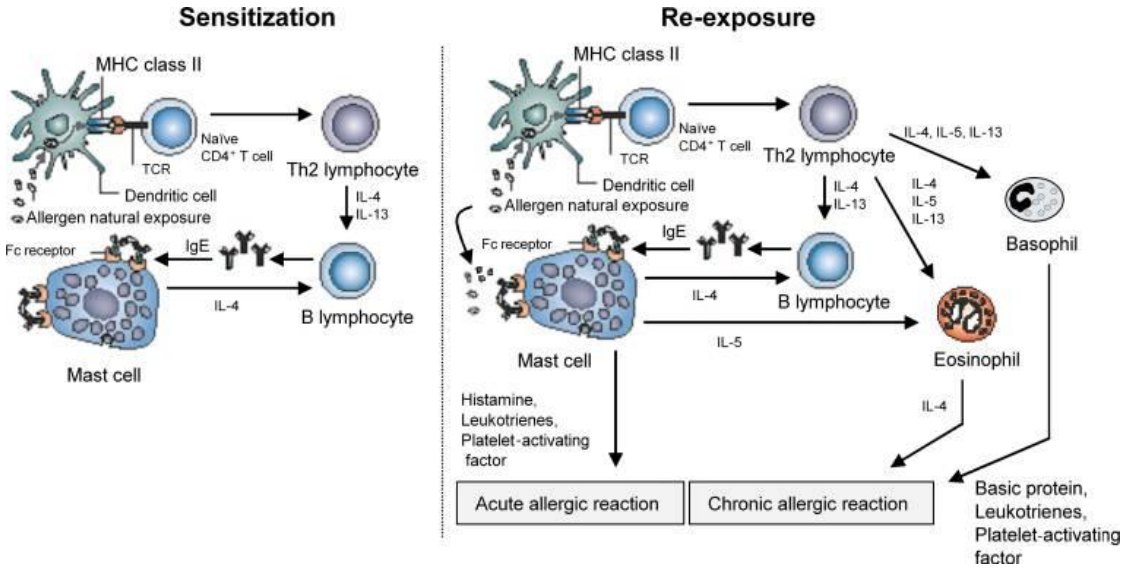

Fig. 8: Mechanism of allergic reaction. During the sensitization phase, initial exposure of APCs to allergen leads mainly to the activation of specific Th2 cells and to the production of allergen-specific IgE which bind to the high-affinity FcεRI on the surface of mast cells and basophils. Subsequent re-exposure to the allergen induces activation of these cells by cross-linking of the allergen specific IgE antibodies on their surface that leads to secretion of pro-inflammatory mediators and cytokines responsible for acute allergic reaction. Chemokines released by these mast cells and other types of cells recruit the Th2 cells and eosinophils, that release large number of pro-inflammatory mediators which are responsible for the late phase of response in chronic allergic reaction. The recruitment of effector cells, such as neutrophils, eosinophils, basophils and Th2 cells to the site of inflammation leads to prolonged inflammatory reactions. Adopted from Taher *et al.* [197].

Allergic diseases affect more than 25% of the population in western countries and include asthma, rhinoconjunctivitis, atopic dermatitis (eczema), contact dermatitis, granulomatous reactions, anaphylaxis, urticaria, angioedema, as well as food- or drug – induced hypersensitivity reactions and others, which can occur alone or in combination. Although almost half of the urban population worldwide is genetically predisposed to produce IgE in serum and the most of allergic patients are atopic, some allergies can developed without this atopy predisposition (insect allergies) [198]. The increase in the incidence of allergic diseases over the past decades and the contrast in the rate of allergic disease between industrialized and developing countries suggest that environmental and lifestyle changes are a major factor in the development and progression of allergies [199]. These environmental factors such as pollution, timing and dose of allergen, dietary changes, exposure to tobacco smoke, antibiotic overuse and changes in exposure to infectious diseases and parasitic infections in early childhood due to sanitation seems to play a general role in early immune programming and development [200]. Prenatal exposure to these elements is postulated to

cause epigenetic changes in genes and signaling pathways of fetal immunity that may have effects during the child's life on skewing them towards an allergic phenotype [201].

1.4.1 Role of microbiota in development of allergy

The *"hygiene hypothesis*" was originally proposed by Strachan in 1989 and describes the lack of early microbial stimulation resulting in aberrant immune responses to innocuous antigens later in life. Infection and microbial stimuli induce Th1 immune responses which balance the Th2 predominant immune response in early childhood. This theory suggests that the factors influencing the extent of an individual's exposure to microorganisms such as household size, sanitation, birth order, and antibiotic use are the reasons why the prevalence of atopic diseases like eczema, hay fever, and asthma have dramatically increased in countries that have developed the "westernized" style of life [202]. Moreover, allergic diseases are more prevalent in children and young adults living in the city, whereas children living in farms with heavy exposure to farm animals and their microbiota have less abundance of allergic disease [198, 203]. The *"microflora hypothesis"* which was initially proposed by Noverr and Huffnagle (2005), suggests that perturbations in the gastrointestinal microbiota composition (due to changes in diet, life-style and antibiotic overuse) lead to delayed maturation of the immune system, disruptions in the mechanisms of mucosal immunological tolerance and increasing incidence of allergic hypersensitivity [204]. Several epidemiological and clinical studies have indicated associations between antibiotic use, which can augment Th2 type immune response, and increased risk of allergy and asthma [199, 205, 206]. The broader term "*old friends"* suggests the effect of prenatal, neonatal and adult exposures to organisms that were associated with humans throughout the evolution (e.g. helminths, saprophytic Mycobacteria, Toxoplasma, gut microbiota and others) and that exerted potent immunoregulatory effects. Their depletion by modern lifestyle correlates with the rise in chronic inflammatory disorders such as allergies, IBD, type 1 diabetes and autoimmunity [207]. Particular changes in the composition of the intestinal microbiota and significant reductions in certain groups of the microbiota are also correlated with allergic symptoms early in life. In detail, the lower levels of *Bifidobacterium* and *Enterococcus spp.* were shown to correlate with allergic symptoms in the first month of life. A higher ratio of *Bacteroidetes* to *Bifidobacterium spp.* was reported at 2 years of age in infants that showed symptoms of atopic dermatitis [208]. Quantitative real-time PCR revealed that children who developed allergies were less frequently colonized with *Lactobacillus spp.* and *Bifidobacterium spp.* at 2 month of age and that children belonging to larger households with one or more siblings consistently

generated more diverse microbiota profiles [209]. A decline of microbial diversity was proposed as a major cause of the allergy epidemic. This area of the hygiene hypothesis, now defined "*biodiversity hypothesis*", has found specific support in several epidemiological studies, where respiratory allergies are inversely related to the number of different foodborne infections. Moreover, lower diversity of the gut microflora in the first week of life is associated with atopic eczema at 18 months and the probability of developing asthma in farming children is inversely related to the range of exposure to environmental bacteria and fungi [210]. Perturbations in the microbiota composition may be especially important during early-life when the immune system is still developing, resulting in a critical "window of opportunity" for instructing the immune system. A sufficiently high antigenic burden in early life, provided by diversified microbiota, infections and nutrition, is therefore necessary to properly "educate" the immune system and to prevent childhood allergic diseases [211]. The importance of the healthy intestinal microbiota on prevention and/or treatment of allergic diseases has been shown by clinical trials demonstrating the effects of probiotics, oral or intranasal administration of bacterial extracts and earlier introduction of foods [212, 213].

1.5 Animal models of IBD and allergy

Animals models clarify the mechanisms of the disease, the activity of a variety of genes and cellular pathways, define the role of environmental factors (such as the microbiota), predict the safety of new drugs or probiotics before being used in clinical studies, define the pathogenic pathways and suggest new therapeutic options [214, 215].

1.5.1 Experimental mouse models of ulcerative colitis

Much of our current understanding of IBD pathogenesis has come from the studies of various animal models. Mouse models of spontaneous or induced intestinal inflammation are essential tools to investigate physiological mechanisms and immunological processes underlying mucosal inflammation. Moreover, these models provide evidence that commensal enteric bacteria continuously drive chronic, immune-mediated colitis and ileitis [19]. Although there are some differences, these models resemble to human disorders [216]. Basically, mouse models of IBD are categorized into four main classes: a) chemically induced, b) genetically modified (gene knockouts or transgenic, e.g. IL-$2^{-/-}$, IL-$10^{-/-}$, c) adoptive transfer models (CD4+CD45RB high T-cells transferred into T and B cells deficient severe combined immunodeficient (SCID) mice), and d) mice spontaneously developing colitis (SAMP1/Yit mice with chronic terminal ileitis) [216-218].

Chemically induced mouse models of intestinal inflammation such as dextran sodium sulfate (DSS) treatment (UC-like) and trinitrobenzene sulfonic acid (TNBS) treatment (CD-like) are the most commonly used systems in animal studies due to many histopathological and immunological similarities to human IBD. They are characterized by developed colonic mucosal inflammation with ulcerations, changes in morphology of inflamed colon (loss of basal crypts and surface epithelium), increased inflammatory cell infiltration, body weight loss, bloody diarrhoea, and aberrant regulatory mechanisms in colon followed by cytokine and chemokine dysregulation [219, 220]. The acute phase of DSS colitis is induced by administration of 2 to 5% DSS (molecular weight 40-50 kDa) solution in water for 5 to 7 days, whereas administration of three to five cycles of DSS followed by cycles of water induced chronic inflammation [221]. Proposed mechanism of DSS treatment consists of toxic effects on the epithelium and increased exposure to luminal microbiota and other antigens due to destruction of mucin content, altered macrophage function and permeabilization of epithelial layer [221, 222]. The disruption of epithelial barrier function, bacterial translocation to lamina propria of the intestine and subsequent activation of non-lymphoid cells such as macrophages and polymorphonuclear cells with the release of pro-inflammatory cytokines is the main initial mechanism of these IBD model. This initial phase is independent from lymphocyte-mediated adaptive immune response as demonstrate the development of DSS-induced colitis in SCID mice lacking T and B cells [223, 224]. Poritz *et al.* [225] showed that the loss of ZO-1 and increased permeability preceded the development of significant intestinal inflammation suggesting that in DSS colitis alteration in TJ complex occurs before the inflammatory reaction. In acute stages of DSS colitis the secondary T-cell response consist of a polarized Th1 response, but in later and more chronic phases of the inflammation, a mixed Th1/Th2 response occurs [215]. In both cases, the DSS-treatment induces the production of a large amount of TNF-α and IL-6 that are mainly responsible for the tissue damage in the disease [226]. These changes in colonic mucosa have been shown to result in the breakdown of tolerance to the microflora which could lead to, or perpetuate, inflammation in GIT [227, 228]. The importance of microbiota in the development of DSS colitis was clearly shown in the GF conditions where mice developed only mild form of acute DSS colitis compared to conventionally reared counterparts [224].

1.5.2 Experimental mouse model of allergy

Animal models have been developed for almost all types of allergic disease such as asthma, allergic rhinitis, food allergy, anaphylaxis, atopic dermatitis and allergic

conjunctivitis. Experimental mice are not able to spontaneously develop allergies, therefore a range of sensitization and challenge protocols have been developed. The number of sensitizations and challenges is crucial for the development of acute or chronic allergic forms. The nature of the allergic disease and inflammatory response is directly influenced by the genetic background of the mice, type and dose of allergen, type of the sensitization and challenge protocol and contamination of the allergen with substances (e.g. LPS), which stimulate the innate immune response. Certain protocols require the combination of allergen with an appropriate adjuvant. Alum (aluminium hydroxide) is one of the preferred adjuvants in respiratory allergy models [229]. Although, animal models are particularly useful for identifying novel cellular and molecular immunological mechanisms of allergy, no model completely resembles all aspects of an allergic response. Mainly, the role of Th2 type cytokines and Treg cells in the pathogenesis of allergy have been well-characterized in animal models [230]. Most commonly used antigens in mouse models of allergic airway inflammation are ovalbumin, house dust mite or pollen allergens (e.g. Bet v 1) or combination of multiple antigens. In acute model of allergic airway inflammation, antigen is co-administered together with adjuvant to enhance its antigenicity. The chronic form is induced by inhalation of antigen without adjuvant for repeated exposure [231, 232].

1.5.3 Germ-free and gnotobiotic animal models

Animal models reared in germ-free conditions (mice, rats, pigs, zebrafish, fruit fly etc.) bring us an invaluable tool for evaluating the role of microbiota and for differentiating genetically determined spontaneously developing immune responses from mechanisms induced by environmental agents [1, 233]. The word "gnotobiology" comes from the Greek words "gnosis" (knowledge) and "bios" (life) and means the science concerned with the study of organisms living in the absence or presence of defined microorganisms. Gnotobiological techniques were developed at the end of the 19th century. In the Czech Republic gnotobiology has long-lasting tradition. Gnotobiological laboratory of the Institute of Microbiology was established by Professor Jaroslav Šterzl more than 50 years ago as a second laboratory of gnotobiology in Europe [27]. The first generation of animals is usually born by caesarean section, transferred into isolators for germ-free rearing and fed by cow milk-based formulas, foster-mother or by sterile diet and water. The sterility of the isolators and GF animals must be checked by microscopy, aerobic and anaerobic cultivation, and methods of molecular microbiology [27, 234].

GF animals exhibit pronounced defects in multiple aspects of their organs, structure and function. In this regard, the intestinal immune system in GF mammals is underdeveloped, deficient in many immune components including enterocyte brush border compartment of the intestine, barrier function, circulating antibodies and mucosal T cells, and does not produce some AMP [27, 235, 236]. Colonization of GF animals with defined microbial species/strains allows the study of individual members of the microbiota (one specific strain or mixture of defined microbiota strains) in the context of different health and disease status [21]. Microbial colonization influences immune maturation resulting in an increase of immunoglobulin levels, the production of specific antibodies, changes in MALT and innate and adaptive immune cell populations and increase in the systemic immunological capacity [1, 27]. It has been shown that microbiota also contribute to physiological maturation of enterocytes, resulting in the specific activities of enterocyte brush-border enzymes [237]. Moreover, disease models studying the contribution of intestinal microflora in GF animals alongside their conventional counterparts have been evaluated for disorders such as IBD [216, 224] or allergy [238-241]. These gnotobiological models have also been successfully used for studying the effects of probiotics on the host and screens for beneficial probiotic strains in prevention and therapies of specific human disease [27, 240-242].

1.6 Probiotics in prevention and treatment of human civilization diseases

The beneficial association of lactic acid bacteria on the human host was probably first suggested by Döderlein in 1892, who proposed that vaginal bacteria produced lactic acid from sugars to prevent or inhibit the growth of pathogenic bacteria. Health benefits of these bacteria were documented by Metchnikoff in 1908 [243], who considered the longevity of Bulgarian people to be related to their high intake of fermented milk products such as yogurt. Today probiotics are defined as live microorganism that, when administered in adequate amounts, confer a health benefit on the host [244, 245]. In last few years, the role of these probiotic bacteria in modulating of intestinal microbial balance and mucosal immune responses has been widely investigated. Therefore, there is a great interest in their preventive and therapeutic potential for many immune and metabolism disorders, IBDs, infections or allergies [246].

1.6.1 Beneficial effect of probiotic bacteria

Although the exact molecular mechanisms of probiotic action are largely unknown, they can act in a number of ways. Probiotics directly inhibit the growth of pathogens by

producing antibacterial substances (including bacteriocins, different organic acids, hydrogen peroxide, biosurfactans) or they inhibit adherence and translocation of pathogens to the intestinal mucosa. Among the most important effect of probiotic bacteria belongs the modulation of innate and adaptive immune response on local or systemic level. Probiotics interact with the gut mucus and the epithelium and enhance the integrity of the intestinal barrier by increasing expression of tight junctions or by promoting the survival of intestinal epithelial cells. A nonspecific antiviral effect is mediated through the stimulation of innate immune cells (cytotoxic activity of NK cells and macrophage phagocytosis). Specifically individual probiotic strains can activate pro-inflammatory (Th1 cells) or anti-inflammatory (Th2 cells and Tregs) responses and production of IgA by B cells of the adaptive immune system [247]. They can also improve digestive processes of the host (lactose intolerance, food allergies), influence the enteric nervous system, function of liver and other organs [246, 247] and they have been shown to possess the anti-carcinogenic activity [248]. Some beneficial mechanisms, such as inhibition of potential pathogens, the production of useful metabolites or gut barrier reinforcement are widespread across taxonomic groups or certain probiotic species, whereas neurological, immunological or endocrinological effects are present in only a few strains of given species [245].

Commensal bacteria of the human gut are often the source of probiotic strains, but until these strains are isolated, characterized and their health effects are demonstrated, they cannot be called as probiotics [245]. Therefore, probiotic bacteria need to possess some specific criteria for their use in humans. They should be identified at the genus, species and strain level; safe for food and clinical use; resistant to gastric acid, bile salts and pancreatic enzymes; able to adhere to intestinal mucosa and colonize the intestinal tract or vagina (at least temporarily); produce antimicrobial substances; be able to antagonize pathogens; possess clinically validated health effects and be stable during processing and storage [249].

1.6.2 Identification of lactic acid bacteria and bifidobacteria

It is widely documented that the effect of probiotics is strictly strain specific, and therefore proper strain identification is highly required [250, 251]. For the identification of lactic acid bacteria and bifidobacteria on genus, species and strain level, different molecular microbiological techniques based on specific polymerase chain reaction (PCR) using 16S rRNA sequences are widely applied. Species and subspecies discrimination could be achieved by most fingerprinting techniques, especially with combining PCR. Fingerprinting methods based on restriction fragment length polymorphism (RFLP) analysis is essentially a profiling

tool based on patterns obtained from DNA restriction digest by rare-cutting restriction enzymes and subsequent pulsed-field gel electrophoresis (PFGE) [251, 252]. Amplified ribosomal DNA restriction analysis (ARDRA) combines amplification of 16S rDNA region by PCR-based method followed by digestion with one or more selected restriction enzymes. This method was successfully used in several studies for discrimination of some *Bifidobacterium* species and even subspecies [253-256]. Other PCR-based approaches using specific primers could be applied for genus, species and even subspecies identification. They include repetitive extragenic palindromic PCR (rep-PCR) or randomly amplified polymorphic DNA (RAPD), which are the most accurate methods for analysis of interspecies variation and differentiation of lactic acid bacteria on strain level. These culture-independent methods based on the 16S rRNA molecule are nowadays widely used for assessment of the composition of the intestinal microbiota, in relation to host health or probiotic efficacy [17, 257, 258].

1.6.3 Commonly used probiotics and their beneficial effect in IBD and allergy

Among widely used probiotic bacteria belong species of the genus *Lactobacillus*, *Lactococcus*, *Enterococcus* and *Streptococcus* (commonly called as lactic acid bacteria) and *Bifidobacterium*, but also some strains of commensal *Escherichia coli* (e.g. *E. coli* Nissle, *E. coli* O83) and certain yeast strain *Saccharomyces boulardii*.

Bacteria of the genus *Lactobacillus* are gram-positive rod-shape, nonsporing, facultative anaerobic or microaerophilic, acid-tolerant and strictly fermentative (lactic acid is product of their fermentation) [252]. These bacteria are often found in the human oral cavity, intestinal tract and vagina, where they beneficially influence these human ecosystems. Some *Lactobacillus* species are used for the production of yogurt, cheese, sauerkraut, pickles, beer, wine, cider, kimchi, cocoa, kefir, and other fermented foods, as well as animal feeds [259]. Some strains of *Lactobacillus* spp. may possess therapeutic potential in inflammatory, allergic, cancer or metabolic diseases [87, 260] or in restoration of physiological balance in the vaginal eco-system [261]. Species such as: *L. acidophilus*, *L. casei* with subspecies *casei* or *paracasei*, *L. rhamnosus*, *L. johnsoni*, *L. plantarum*, *L. gasseri*, *L. fermentum*, *L. delbrueckii* subsp. *bulgaricus*, *L. brevis*, etc. are commonly used in probiotic food and supplements [245, 259, 262].

Members of the genus *Bifidobacterium* are Gram-positive, non-motile, non-sporulating, anaerobic (some species can tolerate oxygen) and saccharolytic microorganisms with the typical morphology of bifid or multiple-branching rods. They produce lactic and acetic acid as the main product of their unique fermentative pathway which serves as a

taxonomic tool for their identification. All species are inhabitant of different ecological niches, such as human intestine, oral cavity, food, animal and insect GIT and sewage [263]. The most frequently detected species in faeces of breast-fed infants are *B. breve*, *B. longum* ssp. *longum* or *infantis* and *B. bifidum*, whereas in adults predominate *B. adolescentis* and *B. catenulatum* [264]. Bifidobacteria, that are usually used in probiotic food and supplements, belong to the species *B. longum* ssp. *longum* or ssp. *infantis*, *B. animalis* ssp. *lactis*, *B. bifidum*, *B. breve* and *B. adolescentis* [245, 249]. Specific strains of the genus *Bifidobacterium* together with other probiotics have been proven to treat constipation, travellers' diarrhoea, antibiotic-associated diarrhoea, alleviate the disease activity of gut inflammation and moderate ulcerative colitis, prevent as well as treat the necrotizing enterocolitis in newborns, reduce the development of disease risk for eczema or allergies and have cholesterol-lowering capacities [259].

Potential of certain probiotic strains to prevent and reduce the probability of relapse in IBD have been documented in several human studies. The mechanisms involve the modulation of IBD-associated dysbiosis of gut microbiota, improvement of mucosal barrier function and modulation of immune response [249, 265]. Several clinical studies have shown that UC could be placed into remission by few probiotics including *E. coli* Nissle 1917, yeast *S. boulardii*, and *L. rhamnosus* LGG, however probiotic mixture VSL#3 has been the most successful [181, 266-268]. Positive results were also observed in a double-blind, randomized controlled trial with *B. breve* and *B. bifidum* strains in treatment of UC patients [269]. In general, it appears that this probiotic supplementation is more effective in reducing disease onset or recurrence, rather than diminishing active inflammatory symptoms. Furrie *et al*. [270] have shown that supplementation of the inulin-derived prebiotic together with *B. longum* in UC patients significantly decreased rectal pro-inflammatory cytokine levels and down-regulated the expression of inflammation-associated β-defensins.

It has been shown that probiotics could be also efficiently used in a prevention/treatment of some allergic diseases. Whereas, only a few clinical studies have evaluated the role of probiotics in the treatment of asthma or allergic rhinitis, their administration for treatment of eczema or atopic dermatitis seems to be more efficient. Recent studies investigating supplementation of some lactobacilli strains in adults with allergic rhinitis demonstrated improved clinical symptoms of this disease [271-273]. Administration of lactobacilli strains such as LGG, *L. reuteri* or *L. fermentum* have been shown to reduce symptoms of atopic dermatitis/eczema in high risk infants [212, 274-276]. However, the majority of studies showed that probiotics are more effective in prevention of dermatitis

and/or IgE-associated eczema during the first 2 years of live, particularly if probiotics are administered both pre- and postnatally [213, 277]. Whereas, these trials indicate a strong association between probiotic supplementation and reduction of atopic dermatitis, not all epidemiological studies have reached the same conclusions, probably due to differences in the set-up of probiotic supplementation and used bacterial strains [278-280].

Other evidence linking the use of probiotics with health benefits in IBD or allergy have come from *in vitro* and animal studies using numerous experimental models of UC and CD [281-283] and allergic sensitization [240, 284, 285]. Several studies of immunomodulatory activity of lactobacilli show that the lactobacilli-induced high ratio of IL-10/IL-12 or IL-10/TNF-α in immune cells correlate with their capacity to provide significant protection against colitis induced in animal models [286-288]. Administration of some bacterial strains of *Lactobacillus*, *Bifidobacterium* or *Streptococcus* could also drive the expansion of Treg population and thus have potential therapeutic effects in models of IBD, atopic dermatitis or rheumatoid arthritis [289]. The data from animal models indicate that administration of certain probiotic strains is capable of inhibiting total and allergen-specific IgE production when administered orally before or concomitantly with the allergen during sensitization and challenge phase. This can be associated with enhanced production of Th1 cytokines, decreased synthesis of Th2 cytokines, or both [284]. The *in vitro* and *in vivo* studies demonstrated that response to probiotic supplementation depends on many factors such as the immunological status of the host as well as strain-specific immunomodulatory capacities of used probiotic strain [249].

1.7 References

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2. SIGNIFICANCE, AIMS AND OUTLINES OF THE THESIS

Mucosal immune system of human has coevolved over million years with its associated commensal microbiota. Mounting evidence shows that both the innate and adaptive immune responses have evolved to require microbial interactions during their development. Nevertheless, in our modern life-style, many people are not exposed to appropriate microbial signals, and thus the immune system does not develop correctly. Inadequate microbial stimulation in early life or an altered composition of microbial communities (dysbiosis) might result in civilization diseases such as IBD or allergies. Their increasing incidence in Western countries in last few decades has become a significant health burden, and therefore, novel preventive/treatment strategies are highly required. In this regard, probiotics are able to improve disbalance of gut microbiota and stimulate immune system, and thus they might be valuable tools for prophylaxis or therapy of these disorders. Although commensal bacteria in the intestine are often source of probiotic strains, until these strains are isolated, characterized and their health effects are verified, they cannot be called as probiotics.

This thesis clearly demonstrates that commensal microflora play a crucial role in health and disease of the host. We show that selected bacterial strains can act on various aspects of immunity by stimulation of cytokine response, improvement of the barrier integrity, induction of IgA secretion, and modulation of T-helper balance by enhancing Treg activity. We demonstrate that the mode of action of analysed probiotic strains is strictly strain-specific. In this thesis, *in vitro* and *in vivo* studies with two different mouse models of human civilization diseases are used to generate new insights into the beneficial effect of newly selected strains of lactobacilli, bifidobacteria and *Clostridium tyrobutyricum*. Specific aims that contribute to better understanding of the host-microbiota interaction in a state of intestinal inflammation or allergic sensitization are addressed in individual chapters and are listed below.

Chapter 1 summarizes current knowledge about the gut commensal microflora and probiotics, mucosal immune system, inflammatory bowel disease, allergy and experimental models of these diseases.

Chapter 3 describes an integral part of the characterization of probiotic strains, the correct taxonomical classification and discrimination of the bifidobacterial strains into subspecies *Bifidobacterium longum* ssp. *longum* and *Bifidobacterium longum* ssp. *infantis* by PCR-based methods.

In **Chapter 4** we address the question how specific immunomodulatory properties of probiotic strains could correlate to their potential in prevention of inflammatory disorder. In co-culture *in vitro* assay we determined the immunomodulatory capacity of nine bifidobacterial strains of human origin with potential probiotic properties. According to the results of *in vitro* analysis we selected two strains of *B. longum* ssp. *longum* with completely different immunomodulatory pattern and we analyse their potential in prevention of experimentally induced ulcerative colitis. Moreover we determine which pattern recognition receptors are involved in the immune recognition of analysed *B. longum* strains.

In **Chapter 5** we investigate the prophylactic effect of butyrate-producing *Clostridium tyrobutyricum* strain on DSS-induced colitis in immunocompetent BALB/c and immunodeficient SCID mice.

In **Chapter 6** we address the question whether the neonatal mother-to-offspring colonization of germ-free mice with *Bifidobacterium longum* strain could prevent the allergic sensitization to the birch pollen allergen.

In **Chapter 7** we characterize the immunomodulatory properties of three individual *Lactobacillus* strains and their mixture (Lmix) in *in vitro* study and we determine the effects of Lmix on the development of allergic sensitization in a gnotobiotic mouse model.

And finally, **Chapter 8** completes this thesis with general discussion of the results, conclusions and future perspectives coming from individual chapters with respect to the *in vitro* and *in vivo* characterization of the strains.

3. EFFICIENCY OF PCR-BASED METHODS IN DISCRIMINATING *BIFIDOBACTERIUM LONGUM* SSP. *LONGUM* AND *BIFIDOBACTERIUM LONGUM* SSP. *INFANTIS* STRAINS OF HUMAN ORIGIN

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Efficiency of PCR-based methods in discriminating *Bifidobacterium longum* ssp. *longum* and *Bifidobacterium longum* ssp. *infantis* strains of human origin

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ABSTRACT

Bifidobacterium longum is considered to play an important role in health maintenance of the human gastrointestinal tract. Probiotic properties of bifidobacterial isolates are strictly strain-dependent and reliable methods for the identification and discrimination of this species at both subspecies and strain levels are thus required. Differentiation between *B. longum* ssp. *longum* and *B. longum* ssp. *infantis* is difficult due to high genomic similarities. In this study, four molecular-biological methods (species- and subspecies-specific PCRs, random amplified polymorphic DNA (RAPD) method using 5 primers, repetitive sequence-based (rep)-PCR with BOXA1R and $(GTG)_5$ primers and amplified ribosomal DNA restriction analysis (ARDRA)) and biochemical analysis, were compared for the classification of 30 *B. longum* strains (28 isolates and 2 collection strains) on subspecies level. Strains originally isolated from the faeces of breast-fed healthy infants (25) and healthy adults (3) showed a high degree of genetic homogeneity by PCR with subspecies-specific primers and rep-PCR. When analysed by RAPD, the strains formed many separate clusters without any potential for subspecies discrimination. These methods together with arabionose/melezitose fermentation analysis clearly differentiated only the collection strains into *B. longum* ssp. *longum* and *B. longum* ssp. *infantis* at the subspecies level. On the other hand, ARDRA analysis differentiated the strains into the *B. longum/infantis* subspecies using the cleavage analysis of genus-specific amplicon with just one enzyme, *Sau3*AI. According to our results the majority of the strains belong to the *B. longum* ssp. *infantis* (75%). Therefore we suggest ARDRA using *Sau3*AI restriction enzyme as the first method of choice for distinguishing between *B. longum* ssp. *longum* and *B. longum* ssp. *infantis*.

1. Introduction

Bifidobacteria are Gram-positive, anaerobic, non-motile bacteria that are naturally found in the human gastrointestinal tract (GIT) and vagina. They colonize the intestine of newborn children within the first few days after birth and in breast-fed infants represent up to 95% of the intestinal microflora (Harmsen et al., 2000; Trebichavsky et al., 2009). The most frequently detected species in the faeces of breast-fed infants are *Bifidobacterium longum*, *Bifidobacterium breve*, and *Bifidobacterium bifidum*. The amount as well as species distribution of bifidobacteria changes depending on age (Gomes and Malcata, 1999; Matsuki et al., 1999, 2003). The bifidobacterial community in an individual is often complex and several species and several strains representing the same species may colonise the GIT simultaneously (McCartney et al., 1996; Satokari et al., 2001; Sakamoto et al., 2003). Although not numerically dominant in the complex intestinal microflora of adults, bifidobacteria are considered as key commensals which promote a healthy GIT during the whole life (Gevers et al., 2005a). The current trend of supplementing various types of food products with health promoting probiotic bacteria has been driven by a rapidly increasing knowledge about the importance of intestinal microbiota on the health status of the host. Bifidobacteria have been also widely used in cultured milk, beverages, cheese products, cookies, (Hughes and Hoover, 1991; Kim, 1988), and powdered milk (Nagawa et al., 1988) as cell suspensions or as freeze-dried additives.

One of the human-specific bifidobacterial species widely used as a probiotic is *B. longum* with its subspecies *B. longum* ssp. *longum* and

Abbreviations: PCR, Polymerase chain reaction; RAPD, random amplified polymorphic DNA; rep-PCR, repetitive sequence-based PCR; ARDRA, amplified ribosomal DNA restriction analysis; CCM, Czech collection of microorganisms; ATCC, American Type and Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen; MRS agar, de Man, Rogosa and Sharpe agar.

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B. longum ssp. *infantis* (Martin, 1996; Gomes and Malcata, 1999). The taxonomical position of these subspecies has been controversial for quite some time (Sakata et al., 2002; Mattarelli et al., 2008), and differentiation between *B. longum* ssp. *longum* and *B. longum* ssp. *infantis* is still intricate due to more than 97% 16S rRNA gene sequence similarity (Sakata et al., 2006). Nevertheless, probiotic properties of bacteria of the species *B. longum* are strictly strain-dependent, and therefore there has been growing interest in the exact characterization of newly isolated strains with potentially probiotic properties (Gomes and Malcata, 1999; O'Sullivan, 2001; Delgado et al., 2008). Molecular-genetic techniques based on polymerase chain reaction (PCR) have become methods of choice for prompt and reliable bacterial classification (Ventura et al., 2004; Ward and Roy, 2005; Sidarenka et al., 2008). Differences in the nucleotide sequences of 16S rRNA gene are commonly used for the development of genus-, species- and subspecies-specific PCR primers for bifidobacterial identification (Matsuki et al., 1999, 2003; Roy and Sirois, 2000; Ward and Roy, 2005). DNA fingerprinting methods represent a different approach in bifidobacterial taxonomy, allowing characterization on species, subspecies and strain levels (Masco et al., 2003; Gevers et al., 2005b; Křížová et al., 2008). Amplified Ribosomal DNA Restriction Analysis (ARDRA) is based on the amplification of 16S rRNA region, followed by digestion of PCR products with restriction enzymes (Satokari et al., 2003; Ventura et al., 2004; Křížová et al., 2006). Random Amplified Polymorphic DNA (RAPD) works with short random sequence primers bound to unknown targets on the chromosomal DNA sequence (Satokari et al., 2003; Ward and Roy, 2005) and rep-PCR fingerprinting uses primers corresponding to naturally occurring repetitive sequences in the interspersed regions (Masco et al., 2003; Prodělalová et al., 2005; Gevers et al., 2005b).

In the present work we compared different PCR-based techniques and biochemical test for subspecies classification of 28 *B. longum* strains isolated from the faeces of 25 healthy breast-fed infants, 3 healthy adults and 2 collection strains.

2. Materials and methods

2.1. Bacterial strains, culture conditions, and biochemical tests

Two type (control) and two collection strains were obtained from the Czech Collection of Microorganisms (*B. longum* CCM 3764), the American Type and Culture Collection (*B. longum* ssp. *longum* ATCC 15707^T, *B. infantis* ATCC 17930), and the Deutsche Sammlung von Mikroorganismen und Zellkulturen (*B. longum* ssp. *infantis* DSM 20088^T). Altogether 70 bacterial isolates from faecal samples of healthy infants and adults were kindly provided by Prof. V. Rada (Department of Microbiology, Nutrition and Dietetics, Czech University of Agriculture, Prague, Czech Republic) and Prof. J. Nevoral (Department of Internal Medicine, 1st Faculty of Medicine, Charles University in Prague, Czech Republic). The bacterial isolates were previously classified by enzymatic methods into species of genus *Bifidobacterium* by Vlková et al. (2005). Finally, twenty-eight *B. longum* strains (twenty-five from healthy breast-fed infants and three from healthy adults) (Table 1) were selected by genus- and species-specific PCR and *B. longum*-negative strains were excluded from further analysis (data not shown).

Bacterial strains were cultivated anaerobically in MRS broth (Oxoid, Hampshire, UK) supplemented with L-cysteine-hydrochloride (0.5 g/l), pH 6.5–7.0 at 37 °C for 72 h. The purity of the strains was verified by cultivation on MRS agar plates (Oxoid, Hampshire, UK) supplemented with L-cysteine-hydrochloride (0.5 g/l) under the same conditions and by Gram staining. Biochemical tests were carried out using an API 50CHL kit (bioMérieux, Marcy L'Etoile, France) according to the manufacturer's instructions. Biochemical identification was evaluated using Apiweb software (bioMérieux, Marcy L'Etoile, France). Determinations of arabinose and melezitose were part of the API50 CHL profile.

2.2. DNA preparation

Cells in bacterial cultures (4 ml) were collected by centrifugation at 10.000× rpm for 5 min, washed and resuspended in 500 µl lysis buffer (10 mM Tris–HCl, 5 mM EDTA, pH 7.8) containing lysozyme (3 mg/ml). After 1 h incubation at room temperature, 12.5 µl of 20% SDS (0.5% final concentration) and 1 µl of proteinase K (100 µg/ml) was added to each sample and incubated at 55 °C overnight. Cell lysates were treated with 1 µl RNase A (10 µg/µl) for 30 min at 37 °C. DNA was isolated by phenol (pH 7.8) and chloroform: isoamyl alcohol (24:1) from crude cell lysates, precipitated with 96% ethanol and dissolved in 200 µl TE buffer according to Sambrook and Russell (2001). The purity, integrity and concentration of nucleic acids were confirmed by agarose gel electrophoresis and UV spectrophotometry (Sinden, 1994).

2.3. Genus-, species- and subspecies-specific identification by PCR

Genus-specific primers Pbi F1/Pbi R2 (5′ CCGGAATAGCTCC 3′/5′ GACCATGCACCACCTGTGAA 3′) (Roy and Sirois, 2000) were used for amplification of 914 bp length DNA fragments from a highly conserved 16S rDNA region. Two sets of specific primers Pbi F1/Pbi LonR4 (5′ CCGGAATAGCTCC 3′/5′ CGTATCTCTACGACC 3′) (Roy and Sirois, 2000) and BiLON-1, 2 (5′ TTCCAGTTGATCGCATGGTC 3′/5′CGAAGGCTTGCTCC-CAGT 3′) (Matsuki et al., 1999), developed from 16S rRNA gene sequences were used for identification of *B. longum* and *B. longum* ssp. *longum*. The subspecies *B. longum* ssp. *infantis* was determined by specific primers BiINF-1, 2 (5′ TTCCAGTTGATCGCATGGTC 3′/5′ GGAAACCCCATCTCTGGGAT 3′), as described by Matsuki et al. (1999). All amplification reactions were carried out in an MJ Research Programme Cycler PTC-100 (MJ Research, Watertown, USA) and the PCR cycling parameters are given in Table 2. Agarose gel electrophoreses were carried out in TBE buffer (45 mM boric acid, 45 mM Tris-base, 1 mM EDTA, pH 8.0). The genus- and species-specific PCR products were separated by electrophoresis in 1.5% agarose gel.

2.4. Random amplified polymorphic DNA and rep-PCR fingerprinting

Random amplified polymorphic DNA (RAPD) was performed using five random decamer primers: P15 (5′ CTG GGC ACG A 3′), P16 (5′ TCG CCA GCC A 3′), P17 (5′CAG ACA AGC C 3′) (Samarzija et al., 2002), PER1 (5′AAG AGC CCG T 3′) (Perez et al., 2002), and CC1 (5′AGC AGC GTG G 3′) (Cocconcelli et al., 1995). The amplifications were carried out in a total volume of 25 µl containing 50 ng template DNA, 1× PCR buffer with 2.5 mM $MgCl_2$, 0.2 mM dNTP mixture, 1 pmol of primer and an appropriate amount of PCR water. *Taq* 1.1 polymerase (Top-Bio, Prague, Czech Republic) was added into the mixture in amount of 1.5 U for RAPD with primers P16, PER1 and CC1, or 3 U for RAPD with primers P15 and P17.

Two rep-PCR primers, $(GTG)_5$: 5′ GTG GTG GTG GTG GTG 3′ (Gevers et al., 2005b) and BOXA1R: 5′ CTA CGG CAA GGC GAC GCT GAC G 3′ (Masco et al., 2003), were used for *B. longum* identification on subspecies levels. The PCR mixtures for both rep-PCRs were composed of 1× PCR buffer without $MgCl_2$, 2.5 mM $MgCl_2$, 0.2 mM dNTP mixture, and 1 pmol primer. The amount of *Taq* 1.1 polymerase (Top-Bio, Prague, Czech Republic) was 2 U for $(GTG)_5$-PCR and 1 U for BOXA1R-PCR; 30 ng of DNA template and PCR water was added up to a 25 µl volume. The PCR cycling parameters of both amplification reactions are listed in Table 2. The products of each PCR were separated by electrophoresis in one 1.4% agarose gel in TBE buffer.

2.5. Amplified ribosomal DNA restriction analysis

The amplicons (914 bp) used for restriction analysis were obtained by PCR with genus-specific primers Pbi F1/Pbi R2 (Roy and Sirois, 2000). Five restriction endonucleases, *Alu*I, *Bam*HI, *Taq*$^\alpha$I (Takara,

Table 1
Identification of *Bifidobacterium longum* strains isolated from human faeces and type/collection strains by amplification methods.

Strain	PCR primer/PCR product (bp)				Cluster analysis		Carbohydrate-fermentation/ substrate	
	PbiF1/PbiR2[a]	PbiF1/PbiLonR4[b]	BiLON-1, 2[c]	BiINF-1,2[d]	ARDRA [e]	$(GTG)_5$[f]/rep-PCR	arabinose	melezitose
	(914)	(875)	(831)	(828)	cluster	cluster		
RB17	+	+	+	−	I	Ia	+	−
RB20A V6A6	+	+	+	−	I	Ia	+	(+)
RB45P	+	+	+	−	I	Ia	+	−
RB46P	+	+	+	−	I	Ia	+	−
RB47P	+	+	+	−	I	Ia	+	+
RB50P	+	+	+	−	I	Ia	+	+
RB52P	+	+	+	−	I	Ia	+	−
B. longum CCM 3764	+	+	+	−	I	Ia	n.d.	n.d.
B. longum ssp. *longum* ATCC15707^T	+	+	+	−	I	Ib	+	+
RB4V	+	+	+	−	II	IIa	(+)	−
RB25	+	+	+	−	II	IIa	−	−
RB47	+	+	+	−	II	IIa	+	+
RB50	+	+	+	−	II	IIa	+	+
RB52	+	+	+	−	II	IIa	+	−
RB27	+	+	+	−	II	IIa	+	+
RB37	+	+	+	−	II	IIa	+	+
RB21	+	+	+	−	II	IIa	+	+
RB23	+	+	+	−	II	IIa	(+)	+
RB12MPP	+	+	+	−	II	IIa	(+)	(+)
RB14P	+	+	+	−	II	IIa	+	+
RB17P	+	+	+	−	II	IIa	+	-
RB18P	+	+	+	−	II	IIa	+	+
RB18P V6A6	+	+	+	−	II	IIa	n.d	n.d.
RB19A	+	+	+	−	II	IIa	+	+
RB19B	+	+	+	−	II	IIa	+	+
RB34P*	+	+	+	−	II	IIa	(+)	−
RB38AP*	+	+	+	+	II	IIa	+	+
RB38BP*	+	+	+	+	II	IIa	+	(+)
RB65 6A	+	+	+	+	II	IIa	n.d.	n.d.
RB65 6B	+	+	+	−	II	IIa	n.d.	n.d.
B. infantis ATCC 17930	+	−	−	+	II	IIa	−	−
B. longum ssp. *infantis* DSM20088^T	+	−	−	+	II	IIb	−	−

* *B. longum* strains isolated from faeces of adults.
\+ specific PCR/biochemical product was detected,
(+) specific biochemical product of weak intensity was detected.
− no PCR/biochemical product was detected.
n.d. not determined.
[a] Primer set specific to genus *Bifidobacterium* (Roy and Sirois, 2000).
[b] Primer set specific to *B. longum* (Roy and Sirois, 2000).
[c] Primer set specific to *B. longum* ssp. *longum* (Matsuki et al., 1999).
[d] Primer set specific to *B. longum* ssp. *infantis* (Matsuki et al., 1999).
[e] Restriction patterns obtained after cleavage analysis by *Alu*I, *Bam*HI, *Sau*3AI, *Sau*96I and *Taq*$^\alpha$I (Roy and Sirois, 2000; Křížová et al., 2006).
[f] Rep-PCR with primer $(GTG)_5$ (Gevers et al., 2005a).

Table 2
Cycling parameters of genus-, species- and subspecies-specific PCRs, rep-PCRs and RAPD.

Step	PCR											
	Genus[1]		Species[2]		Subspecies[3]		Rep-PCR[4]		Rep-PCR[5]		RAPD[6]	
	T (°C)	time (min)	*T* (°C)	time (min)	*T* (°C)	time (min)	*T* (°C)	time (min)	*T* (°C)	time (min)	*T* (°C)	time (min)
initial denaturation	94	5	92	5	94	5	94	4	94	4	94	4
denaturation	94	1	92	0.5	94	20[7]	94	1	94	1	94	1
primer annealing	50	1	56	0.5	55	20[7]	50	1	40	1	36	1
extension	72	2	72	1	72	0.5	72	2	72	2	72	2
final extension	72	10	72	10	72	10	72	9	72	9	72	9
number of cycles	30		35		30		35		35		35	

[1] genus-specific PCR: Roy and Sirois (2000), PCR primers Pbi F1/Pbi R2,
[2] species-specific PCR: Roy and Sirois (2000), PCR primers Pbi F1/Pbi LonR4,
[3] subspecies-specific PCR: Matsuki et al. (1999), PCR primers BiLON-1, 2 and BiINF-1, 2,
[4] Rep-PCR: Gevers et al. (2005b), PCR primer $(GTG)_5$,
[5] Rep-PCR: Masco et al. (2003), PCR primer BOXA1R,
[6] RAPD: PCRs primers P15, P16, P17 (Samarzija et al., 2002), PER1 (Perez et al., 2002), and CC1 (Cocconcelli et al., 1995),
[7] time in sec.

Shiga, Japan), *Sau*3AI, and *Sau*96I (Roche Diagnostic, Mannheim, Germany) were used for the cleavage of the PCR product (Křížová et al., 2006; Roy and Sirois, 2000; Venema and Maathuis, 2003). ARDRA digests (15 μl) of each enzyme were separated in 2.5% agarose gel in TBE buffer (Table 3).

2.6. Data analysis

The PCR products were visualised using ethidium bromide (0.5 μg/ml; Sigma, St. Louis, USA) staining and UV transilluminator EB-20E from UltraLum (Paramount, USA) at 305 nm UV light and photographed on a TT667 film with a CD 34 Polaroid Camera (Polaroid, Waltham, USA). The fingerprinting profiles obtained by rep-PCR and RAPD were analysed by the software programme Gel Compare II (2.0 version) and dendrograms were constructed using UPGMA analysis and Pearson similarity coefficients. The ARDRA profiles were analysed by Gel Compare II Programme (2.0 version) and a dendrogram was designed using UPGMA analysis and Jaccard similarity coefficients.

3. Results

3.1. Biochemical tests, genus-, species- and subspecies-specific identification by PCRs

Traditionally, bifidobacterial species have been identified on the basis of analysis of fermentation products. Arabinose is reported as not fermentable by *B. longum* ssp. *infantis* while *B. longum* ssp. *longum* characteristically ferments melezitose (Mattarelli et al., 2008). The results received for the set of strains tested are given in Table 1.

By means of genus-specific PCR with primers Pbi F1/Pbi R2 (Roy and Sirois, 2000), the specific amplification product (914 bp) was obtained for all bifidobacterial strains involved in this study. Thirty studied strains (two collection and twenty-eight new isolated strains) gave specific amplicons with primer sets Pbi F1/Pbi LonR4 (Roy and Sirois, 2000) and BiLON-1, 2 (Matsuki et al., 1999) developed for *B. longum* ssp. *longum* (formerly *B. longum*) identification. Five strains (one collection, one type and three newly isolated strains) gave an amplicon with the primer set BiINF-1, 2 specific to *B. longum* ssp. *infantis* (formerly *B. infantis*) (Matsuki et al., 1999). The results are summarised in Table 1.

3.2. Random amplified polymorphic and rep-PCR DNA fingerprinting

Five different RAPD primers (Cocconcelli et al., 1995; Perez et al., 2002; Samarzija et al., 2002) were used for subspecies determination of *Bifidobacterium longum* strains. However, the RAPD profiles of the studied strains did not show any discrimination potential for the resolution of *B. longum* ssp. *longum* and *B. longum* ssp. *infantis*. According to this analysis, the strains formed many separate clusters (dendrograms not shown) and showed a relatively high inter-strain heterogeneity both among tested and type/collection strains.

For evaluation of the rep-PCR fingerprinting technique, two primers BOXA1R (Masco et al., 2003) and $(GTG)_5$ (Gevers et al., 2005b) were tested for subspecies discrimination of the *B. longum* strains. Five main groups were obtained after cluster analysis of the BOXA1R-PCR products (dendrogram not shown) at a relatively low level (83%) of similarity with obvious discrimination of type/collection strains. Nevertheless, the BOXA1R profiles did not give a clear resolution of the analysed strains on the subspecies level. In contrast to the BOXA1R, $(GTG)_5$–PCR generated profiles with lower inter-strain heterogeneity between the studied strains. Based on the dendrogram (Fig. 1), the analysed isolates formed two clusters (I and II) on 85% of similarity and four clusters (Ia, Ib, IIa, IIb) with separated type strains on a 90% level of similarity. In the cluster Ia collection strain *B. longum* CCM 3764 was grouped with seven analysed strains. The cluster IIa was formed by collection strain *B. infantis* ATCC 17930 with 21 studied strains. Surprisingly, the type strains *B. longum* ssp. *infantis* DSM 20088^T and *B. longum* ATCC 15707^T were organised into two individual clusters (Ib and IIb) without any newly isolated bifidobacterial strains and produced rep-PCR patterns with greater variability compared to the more recently isolated strains. The cluster analysis of *B. longum* subspecies according to $(GTG)_5$ fingerprinting is summarised in Table 1.

3.3. Amplified ribosomal DNA restriction analysis

Digestion of genus-specific PCR products by *Alu*I, *Bam*HI, *Taq*I and *Sau*96I provided identical restriction patterns in all tested strains (Fig. 2) and confirmed that the two subspecies are closely related. Differentiation between *B. longum* ssp. *longum* and *B. longum* ssp. *infantis* strains was possible only by *Sau*3AI restriction enzyme. Seven analysed strains isolated from suckling babies (RB17, RB20V6A6, RB45P, RB46P, RB47P, RB50P, RB52P) were clearly distinguished into one cluster with type and collection strains *B. longum* ssp. *longum* ATCC 15707^T and *B. longum* CCM 3764 (cluster I). Type strain *B. longum* ssp. *infantis* DSM 20088^T and *B. infantis* ATCC 17930 collection strain formed second main cluster II together with 21 analysed strains (three from healthy adults' and eighteen from infants' faeces) (Table 1). Based on dendrogram (Fig. 2), restriction analysis of PCR products of *B. longum* ssp. *infantis* strains provided digest products of the length around 420 bp and 380 bp, whereas *B. longum* ssp. *longum* strains restriction provided only one detectable fragment of the length around 420 bp.

4. Discussion

Some phenotypic characteristics can be useful for *Bifidobacterium longum* subspecies differentiation. However, identification of the subspecies using carbohydrate fermentation patterns (arabinose and melezitose) is less suitable. Strains assigned to *B. longum* ssp. *infantis* and *B. longum* ssp. *longum* show in some cases carbohydrate patterns that are different from the type strains. These results are in agreement with those published in the literature (Bahaka et al., 1993). Numerous strains isolated from infant faeces, which fermented arabinose or failed to ferment melezitose, were recognized by means of DNA–DNA

Table 3
Conditions of genus-specific PCR product cleavage by restriction enzymes.

Component	Restriction enzymes/component volume (μl)				
	*Alu*I (10 U/μl)	*Bam*HI (12 U/μl)	*Sau*3A (1 U/μl)	*Sau*96I (1 U/μl)	*Taq*$^{\alpha}$I (20 U/μl)
PCR H_2O	9.4	9.2	9.5	9.5	9.75
Restriction buffer	1.5	1.5	1.5	1.5	1.5
Restriction enzyme	1.1	1.3	1.0	1.0	0.75
PCR product (914 bp)	3.0	3.0	3.0	3.0	3.0
Reaction conditions					
Digestion time (h)	3	3	3	3	3
Digestion temperature (°C)	37	37	37	37	65

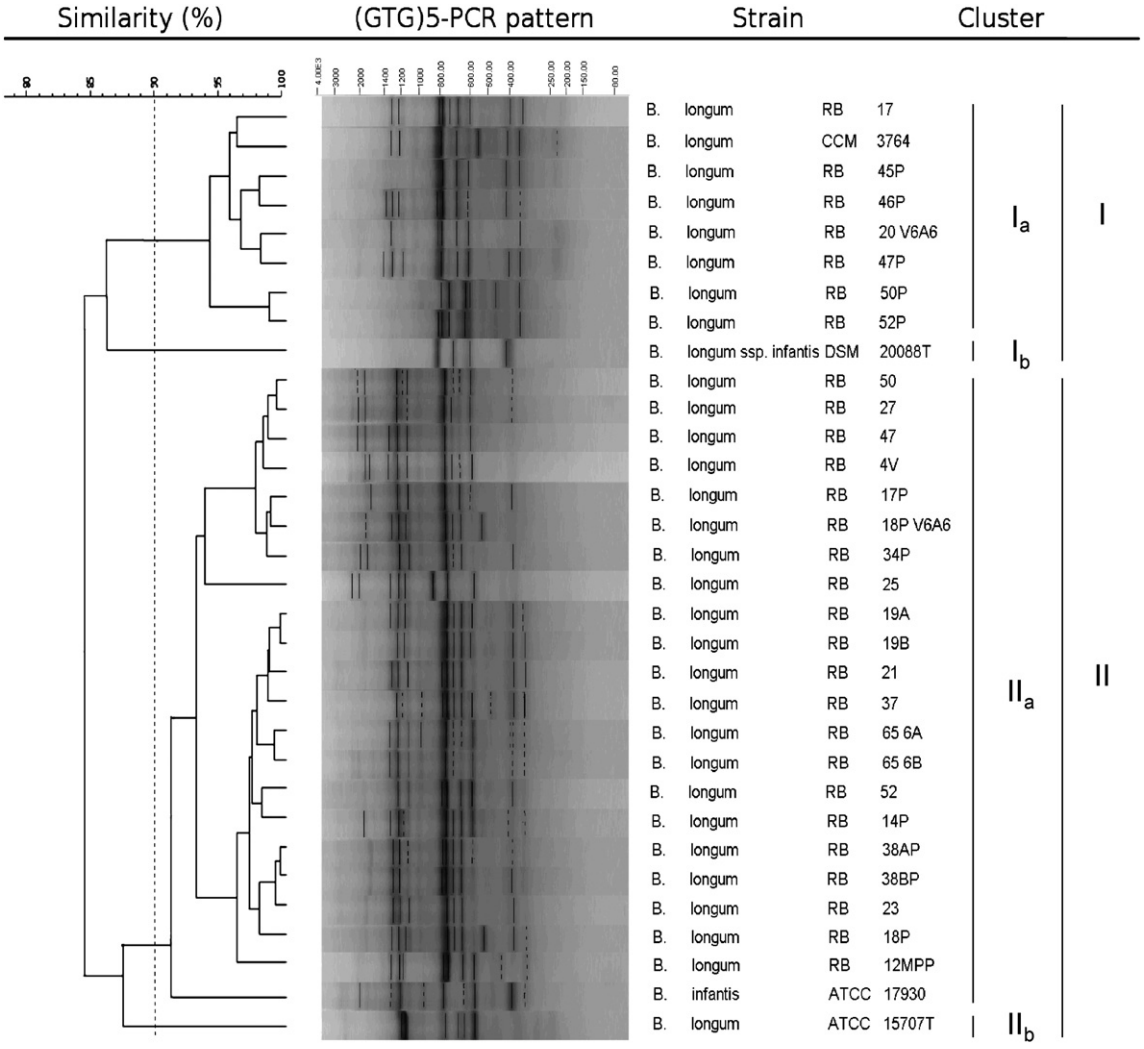

Fig. 1. Cluster and subcluster analysis of the $(GTG)_5$–PCR fingerprints of the 28 bifidobacterial strains isolated from faeces, two type (control) and two collection strains of corresponding species and subspecies. Dendrogram is based on UPGMA analysis with correlation levels expressed as percentage values of the Pearson correlation coefficient. The vertical dotted lines indicate the similarity level of 90% (minimum value for delimitation of relevant subcluster with analysed and individual reference strains).

hybridization as *B. longum* ssp. *infantis* or *B. longum* ssp. *longum*, respectively (Scardovi, 1986).

Bacterial taxonomy based on ribosomal 16S rRNA gene sequences is considered to provide powerful approach to the investigation of phylogenetic relationships. Therefore, these sequences were frequently used for design of specific primers for rapid identification of bifidobacterial species (Matsuki et al., 1999, 2003; Roy and Sirois, 2000). According to Frothingham et al. (1993), the 16S rRNA gene sequences of *B. longum* and *B. infantis* subspecies differ in only seven bases. However, Roy et al. (1996) showed that these subspecies could be differentiated by PCR with respective primers differing in only as much as three bases. In our study, only the type and collection strains were clearly distinguished into subspecies using primer sets BiLON-1, 2 and BiINF-1, 2 designed by Matsuki et al. (1999) from 16S rRNA gene sequence of type strains *B. longum ssp. longum* ATCC 15707^T and *B. longum* ssp. *infantis* ATCC 15697^T (DSM 20088^T). However, PCR analysis by subspecies-specific primer sets did not clearly differentiate analysed strains originally isolated from faeces of healthy breast-fed infants and adults. In this study, only 4 strains (RB38AP, RB38BP, RB65 6A, *B.* infantis ATTC 17930) provided the same PCR product lengths as type strain *B. longum* ssp. *infantis* DSM20088^T. In the previous study (Vlková et al., 2005); species/subspecies-specific PCR identification according to Matsuki et al. (1999) of *B. longum* isolates did not correspond to the DGGE results targeting the transaldolase gene. As described by Gevers et al. (2005a), procaryotic species are considered to be a group of strains (including the type strain) that are characterised by a certain degree of phenotypic consistency, showing 70% homology of DNA-DNA hybridization and over 97% of 16S rRNA gene sequence identity. These results are in agreement with the data summarised and discussed by Mattarelli et al. (2008), who proposed to reclassify the three biotypes of *B. longum* as three subspecies on the basis of genotypic and phenotypic studies.

In order to evaluate subspecies identification of strains, RAPD and rep-PCR fingerprinting methods were investigated. In this study, RAPD and rep-PCR showed various level of heterogeneity of strain-specific profile. Unfortunately, in both RAPD and rep-PCR the analysed strains isolated from humans did not generate a profile similar to the type strains *B. longum* ssp. *infantis* DSM 20088^T and *B. longum* ssp. *longum* ATCC 15707^T.

Applying ARDRA method (Křížová et al., 2006) for identification of 25 strains isolated from faeces of healthy breast fed infants and 3 from healthy adults, we observed that the majority of the strains belong to the *B. longum* ssp. *infantis* (75%). According to our results, we proposed the reclassification of two collection strains into the subspecies level: *B. longum* CCM 3764 as *B. longum* ssp. *longum* CCM 3764 and *B. infantis* ATCC 17930 as *B. longum* ssp. *infantis* ATCC 17930.

Taken together, in this study PCR-based methods and the biochemical analysis were compared for the classification of 28 strains of human origin on *B. longum/infantis* subspecies level. Fermentation tests, subspecies-specific PCR and rep-PCR clearly differentiated only

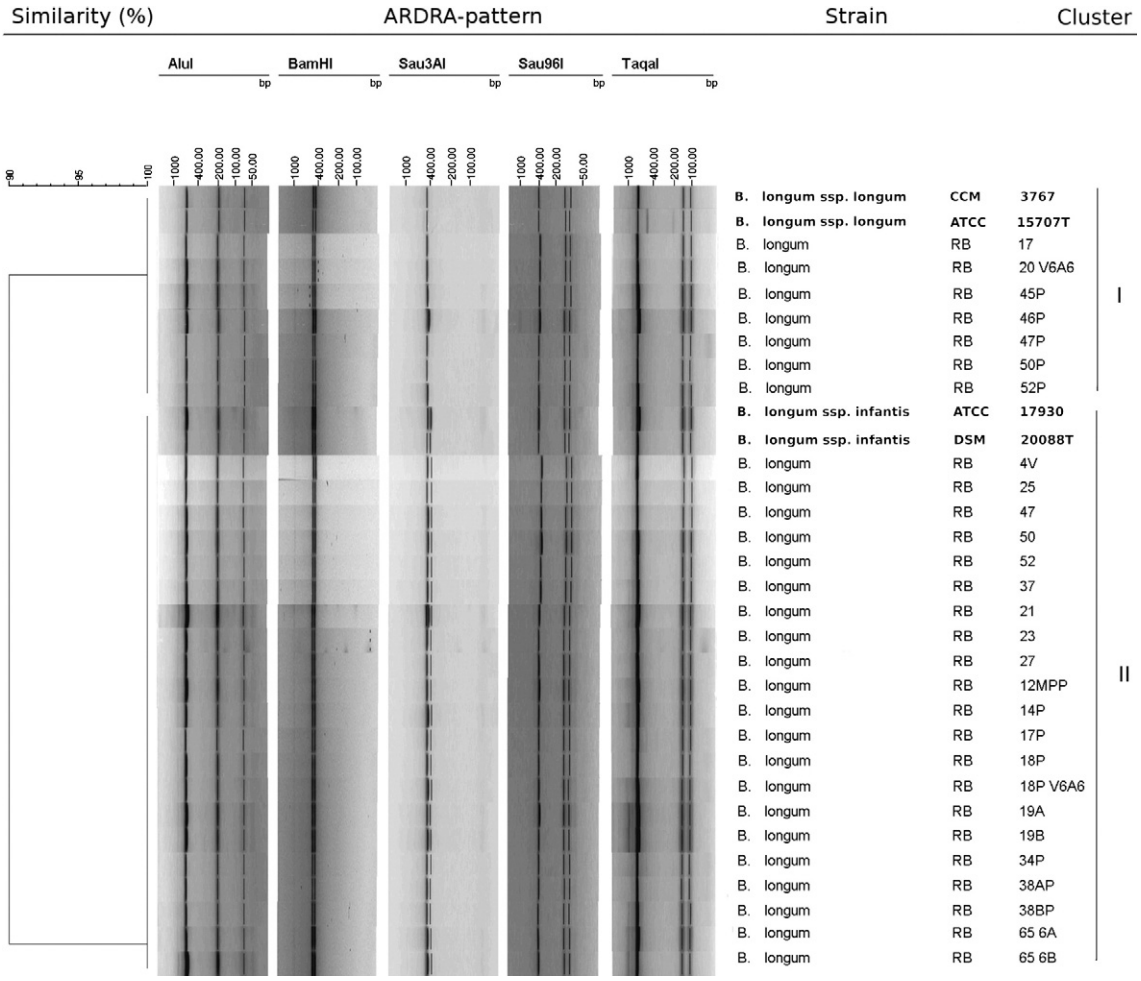

Fig. 2. Cluster analysis of ARDRA patterns generated from restriction analysis of genus-specific amplicon (914 bp) of the 28 bifidobacterial strains isolated from human faeces, two type (control) and two collection strains of corresponding species and subspecies. The dendrogram is based on UPGMA analysis of Jaccard correlation coefficients.

type and collection strains into *B. longum* and *B. infantis* subspecies level but they were not sufficient for discrimination of newly isolated strains. On the other hand, ARDRA succeeded in differentiating analysed strains into the *B. longum/infantis* subspecies using the cleavage analysis of genus-specific amplicon with just one enzyme, *Sau3*AI. We showed that this easy-to-perform molecular technique has discriminatory power for rapid identification of the strains on the respective subspecies level without necessity of DNA sequencing of the analysed strains. Therefore we suggest ARDRA using *Sau3*AI restriction enzyme as the first method of choice for distinguishing between *B. longum* ssp. *longum* and *B. longum* ssp. *infantis*.

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4. *BIFIDOBACTERIUM LONGUM* CCM 7952 PROMOTES EPITHELIAL BARRIER FUNCTION AND PREVENTS ACUTE DSS-INDUCED COLITIS IN STRICTLY STRAIN-SPECIFIC MANNER

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RESEARCH ARTICLE

Bifidobacterium longum CCM 7952 Promotes Epithelial Barrier Function and Prevents Acute DSS-Induced Colitis in Strictly Strain-Specific Manner

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Abstract

Background

Reduced microbial diversity has been associated with inflammatory bowel disease (IBD) and probiotic bacteria have been proposed for its prevention and/or treatment. Nevertheless, comparative studies of strains of the same subspecies for specific health benefits are scarce. Here we compared two *Bifidobacterium longum* ssp. *longum* strains for their capacity to prevent experimental colitis.

Methods

Immunomodulatory properties of nine probiotic bifidobacteria were assessed by stimulation of murine splenocytes. The immune responses to *B*. *longum* ssp. *longum* CCM 7952 (BI 7952) and CCDM 372 (BI 372) were further characterized by stimulation of bone marrow-derived dendritic cell, HEK293/TLR2 or HEK293/NOD2 cells. A mouse model of dextran sulphate sodium (DSS)-induced colitis was used to compare their beneficial effects *in vivo*.

Results

The nine bifidobacteria exhibited strain-specific abilities to induce cytokine production. BI 372 induced higher levels of both pro- and anti-inflammatory cytokines in spleen and dendritic cell cultures compared to BI 7952. Both strains engaged TLR2 and contain ligands for NOD2. In a mouse model of DSS-induced colitis, BI 7952, but not BI 372, reduced clinical symptoms and preserved expression of tight junction proteins. Importantly, BI 7952 improved intestinal barrier function as demonstrated by reduced FITC-dextran levels in serum.

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Conclusions

We have shown that BI 7952, but not BI 372, protected mice from the development of experimental colitis. Our data suggest that although some immunomodulatory properties might be widespread among the genus *Bifidobacterium*, others may be rare and characteristic only for a specific strain. Therefore, careful selection might be crucial in providing beneficial outcome in clinical trials with probiotics in IBD.

Introduction

Inflammatory bowel disease (IBD), such as ulcerative colitis (UC) and Crohn's disease (CD), comprises a variety of chronic immune-mediated inflammatory disorders of the gastrointestinal tract. Although their aetiology and pathogenesis are not completely understood, it is becoming clear that the combination of genetic, immunological, environmental, and microbial factors play an important role in the development and progression of these conditions [1, 2].

Once considered rather rare, IBD has been raising dramatically over the past few decades in the high income countries [3, 4]. It has been suggested that modern style of life, such as modern sanitation systems, modifications in diet, decline in endemic parasitism, smaller family size or overuse of antibiotics changed the structure and function of the intestinal microbiota [5]. Indeed, dysbiosis, such as reduction in mucosa-associated *Bifidobacterium* spp. or *Lactobacillus* spp., along with an increased relative abundance in pathogenic *Escherichia coli* was observed in IBD patients [6–8]. Current pharmaceutical treatment of IBD, which includes anti-inflammatory and immunosuppressive drugs, biological agents and antibiotics, induces or maintain remission, but is not curative [9]. Moreover, long-term use of these drugs can lead to substantial side effects such as allergic reactions or liver problems [10]. Since IBD is clearly multifactorial and results from complex host-microbiota interactions, preventive strategies targeting the aberrant composition of the intestinal microbiota may have the potential to open new possibilities to tackle these diseases.

Probiotic bacteria, most notably the *Bifidobacterium* and *Lactobacillus* genera have been used for the prevention and/or treatment of gastrointestinal inflammatory diseases [11, 12]. Application of multispecies product VSL#3, which is a mixture of *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* strains, has been used successfully to treat UC [11, 13, 14]. Controversially, clinical trials using different strains have not confirmed the beneficial effects [15–18]. It is getting clear that not only the type of disease and the immunological status of the host, but also the selection of probiotic strain and the mode of application are important factors to be taken into consideration. Essentially, the immunomodulatory potential and beneficial effect of probiotic bacteria seems to be strictly strain-specific and cannot be automatically applied to another strain, or even if to another species.

Several animal models have been developed to understand aetiology and pathogenesis of IBD and to evaluate novel prophylactic/therapeutic strategies [19–21]. Colitis induced by dextran sulphate sodium (DSS) is one of the most extensively used experimental models due to its simplicity, reliability and applicability. Acute or chronic disease can be induced by administration of adaptable concentration of DSS in drinking water [19, 22, 23]. Despite certain differences, DSS model resembles crucial clinical and histopathological features of human IBD; such as changes in morphology of inflamed colon, body weight loss, bloody diarrhoea and aberrant regulatory mechanisms in colon followed by cytokine dysregulation [19, 21, 22, 24].

Other important factors which are shared by human IBD and DSS-induced colitis are reduced expression and/or reorganization of tight junction proteins (e.g. zonulin-1 or occludin) in the epithelium, and increased intestinal permeability for luminal bacteria [25]. The breakdown of the gut barrier function have been shown to precede the clinical relapses in UC patients and also the development of intestinal inflammation in DSS-induced colitis [26].

The genus *Bifidobacterium* is considered as a key member of the human gut microbiota which has been shown to exert a range of beneficial effects on the immune system [27–29]. Notably, the representatives of the species *B. longum* are one of the dominant bacterial members of the gut microflora of healthy breast-fed infants [30, 31]. Interestingly, a double blinded randomised controlled clinical trial showed that *B. longum* reduced clinical appearance of chronic mucosal inflammation in patients with active UC [32]. Moreover, Fujiwara *et al.* described inhibitory effect of *B. longum* strain on DSS-induced experimental colitis [33]. Along these lines, we have shown recently that mucosal application of *B. longum* prevented the development of experimental allergy in mice [34, 35]. Although these data suggest that *B. longum* might be a promising candidate for prevention/treatment of immune-mediated inflammatory diseases, question remains whether different *B. longum* strains, belonging to one subspecies, are equal in their health-beneficial effect.

In the present study, nine different probiotic strains of the genus *Bifidobacterium*, which were originally collected from healthy children and adults, were tested for their ability to induce cytokine production by murine splenocytes. Based on the cytokine profiles, two candidates of one subspecies: i) *Bifidobacterium longum* ssp. *longum* CCDM 372 (Bl 372), the strain with high stimulatory capacity and ii) *B. longum* ssp. *longum* CCM 7952 (Bl 7952), the strain with low stimulatory capacity, were selected for further comparative *in vitro* and *in vivo* studies. In experimental model of acute ulcerative colitis, administration of Bl 7952, but not Bl 372, prevented the disruption of gut barrier function by enhanced expression of tight junction proteins in epithelial layer which was associated with reduced development of DSS-induced symptoms.

Materials and Methods

Bacterial strains and culture conditions

Nine *Bifidobacterium* strains: *B. longum* ssp. *longum* CCDM 372 (Bl 372) and CCM 7952 (Bl 7952); *B. longum* ssp. *infantis* CCDM 369 (Bi 369); *B. animalis* CCDM 218 (Ban 218) and CCDM 366 (Ban 366); *B. adolescentis* CCDM 368 (Bad 368), CCDM 370 (Bad 370), CCDM 371 (Bad 371) and CCDM 373 (Bad 373) were isolated from faecal samples of healthy adults and breast-fed infants and kindly provided by Prof. V. Rada (Department of Microbiology, Nutrition and Dietetics, Czech University of Agriculture, Prague, Czech Republic) and Prof. J. Nevoral (Department of Internal Medicine, 1st Faculty of Medicine, Charles University in Prague, Czech Republic). These strains were deposit in Culture Collection of Dairy Microorganisms (Milcom, Prague, Czech Republic) and Czech Collection of Microorganisms (Brno, Czech Republic). The isolates were cultivated in MRS medium (Oxoid, Hampshire, UK) supplemented with 0.05% L-cysteine-hydrochloride (MRSC) at 37°C in anaerobic conditions for 48 to 72 hours.

Selection and identification of probiotic strains

Nine bifidobacterial isolates were positively selected on the basis of the resistance to bile salt and low pH (data not shown), further identified and analysed for their immunomodulatory properties in *in vitro* assays. Identification of these isolates was performed by the genus-, species- and subspecies-specific PCR and Amplified Ribosomal DNA Restriction Analysis (ARDRA) as previously described [36, 37]. Please see S1 Materials and Methods for details.

Inactivation of bacterial strains

Nine probiotic *Bifidobacterium* strains were cultivated in MRSC medium at 37°C in anaerobic condition to the end of exponential growth phase. Bacterial cells were inactivated with 1% phosphate-buffered formalin for 3 h at room temperature, washed 3 times with sterile phosphate buffered saline (PBS) and stored at 4°C as previously described [38].

Stimulation of mouse splenocytes with inactivated *Bifidobacterium* strains

Immunomodulatory potential of *Bifidobacterium* strains was tested *in vitro* on splenocytes derived from naïve BALB/c mice (8 weeks of age; n = 5) in two independent experiments. Spleens were removed aseptically and single cell suspensions were prepared by disruption of the tissues through a cell strainer into culture medium (RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 10mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin). Spleen cells (6 x 10^5/well) were stimulated with formalin-inactivated *Bifidobacterium* strains (6 x 10^6/well), Pam3CSK4 (1 μg/ml, InvivoGen, USA) or media alone in 96-well plates at 37°C and 5% CO_2 for 48 h. Concentration of IFN-γ, TNF-α, IL-6 and IL-10 was determined in cell supernatants by the MILLIPLEX MAP Mouse Cytokine/Chemokine Panel (Millipore Corporation, Billerica, MA, USA) according to manufacturer's instructions and analysed with Bio-Plex System (Bio-Rad Laboratories, USA).

Preparation and activation of mouse bone marrow-derived dendritic cells

Mouse bone marrow-derived dendritic cells (BM-DC) derived from naïve BALB/c mice (8 weeks of age; n = 3) were prepared as previously described [34, 39]. Briefly, bone marrow precursors isolated from femurs and tibias were seeded at 2 x 10^5 cells/ml in RPMI 1640 culture medium containing 10% FCS, 150 μg/ml gentamycin, and 20 ng/ml mouse GM-CSF (Sigma-Aldrich, Germany) and incubated for 8 days. BM-DC (10^6 cells/well) were stimulated with formalin-inactivated *B. longum* strains Bl 7952 and Bl 372 (10^7 CFU/well), Pam3CSK4 (1 μg/ml), ultrapure LPS (1 μg/ml, InvivoGen, USA) or left untreated for 18 h. The levels of IL-10, IL-12p70, IL-6 and TNF-α were analysed in supernatants of stimulated cells by ELISA using Ready-Set-Go! kits (eBioscience, USA) according to manufacturer's instructions. For cell surface marker analysis, BM-DC were labelled for 30 min at 4°C with anti-mouse FITC-conjugated CD11c, APC-conjugated MHC II and PE-conjugated CD40, CD80 or CD86 monoclonal antibodies (eBioscience, USA). The data were acquired on a BD FACSAria III flow cytometer (BD Biosciences, USA) and analysed with FlowJo software 7.6.2 (TreeStar, USA).

Stimulation of Human embryonic kidney 293 cells stably transfected with TLRs and NOD2

Human embryonic kidney (HEK) 293 cells stably transfected with plasmid carrying human (h) TLR2/CD14 gene were kindly provided by Prof. M. Yazdanbakhsh (Leiden, Netherlands), hTLR4/MD2/CD14 were a gift of Prof. B. Bohle, PhD (Vienna, Austria) and hNOD2 expressing cells were purchased from InvivoGen (USA). Cells were stimulated for 20 h with Pam3CSK4 (1 μg/ml), LPS (1 μg/ml), muramyl dipeptide (100 ng/ml, InvivoGen) and formalin-inactivated Bl 7952 and Bl 372 at concentrations of 10^6, 10^7, or 10^8 CFU/ml in 96-well plates. Concentrations of IL-8 were analysed in cell supernatants by ELISA (Thermo Scientific, USA) according to the manufacturer's instructions.

Animals

All experimental mice (female 8-week-old BALB/c) were kept in IVC cages (Tecniplast, Italy), exposed to 12: 12-h light-dark cycles, and fed with standard pellet diet (ST1, Bergman, Kocanda, Czech Republic) and tap water *ad libitum*. All experiments were approved by the Animal Experimentation Ethics Committee of the Institute of Microbiology of the Academy of Sciences of the Czech Republic and conducted in accordance with the "European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (CETS No.: 123)".

Experimental design and induction of colitis

Female 8-week-old BALB/c mice were divided into 4 experimental groups. Two groups were treated with 200 μl of live bacterial suspension containing 2 x 10^8 CFUs of Bl 7952 (Bl 7952/DSS; n = 10) or Bl 372 (Bl 372/DSS; n = 8) in PBS by intragastric gavage. Controls (PBS/DSS; n = 10) received PBS only. The administration of bacterial suspensions or PBS was repeated daily for 10 days followed by treatment with 2.5% w/v DSS in drinking water in order to induce acute colitis. Age-matched untreated mice (Naïve; n = 5) were used as healthy controls. Dextran sulphate sodium (DSS, molecular weight 40 kDa; ICN Biomedicals, Cleveland, OH, USA) was administered in the drinking water (2.5% w/v) for 7 days. Clinical symptoms of inflammation were evaluated daily, degree of colitis was determined as disease activity index (DAI) according to Cooper *et al.* [40] with minor modifications (S1 Table). Animals were sacrificed by CO_2 inhalation and cervical dislocation. The colon was aseptically removed, the length was measured and segments of colon descendens (approximately 0.5 cm in length located 1 cm proximal to the anus) were fixed in 4% buffered paraformaldehyde (Sigma Aldrich, Germany) for histological and immunohistochemical analysis.

Determination of cytokine response of mesenteric lymph node cells

Mesenteric lymph nodes (MLN) were excised aseptically from all experimental mice; cell suspensions in concentration 6 x 10^6 cells/ml were prepared and cultivated as described previously [34, 39]. Production of IFN-γ, TNF-α and IL-10 was determined in cell supernatants by the MILLIPLEX MAP Mouse Cytokine/Chemokine Panel (Millipore Corporation, Billerica, MA, USA) according to manufacturer's instruction and analysed with Bio-Plex System (Bio-Rad Laboratories, USA).

Histopathological evaluation of inflammation in colonic mucosa

Colon descendens from all experimental mice (one segment from each mouse) were fixed in 4% paraformaldehyde and processed to paraffin blocks as previously described [41]. For determination of inflammation in colonic mucosa and mucin production, the tissue specimens were sliced to 5-μm thickness, deparaffined and stained with haematoxylin and eosin (H&E) or Alcian Blue with post-staining by Nuclear Fast Red (All from Vector, Burlingame, CA, USA). The degree of pathophysiology of the tissue was characterized by presence of ulcerations, damage to the surface epithelium, crypt distortion, signs of oedema, infiltration of inflammatory cells into lamina propria or submucosa and reduction of goblet cells and mucin production according to Cooper *et al.* [40]. The histopathological evaluation was performed blindly by two investigators.

Immunohistochemical determination of zonulin-1 and occludin in colon

For immunohistochemical staining, the 5-μm deparaffined colon sections (3 sections from each mouse) were treated with protease type XIV (1 mg/ml; Sigma-Aldrich, Germany) for 8

min at 37°C. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in 100% methanol for 15 min. Nonspecific adsorption was eliminated by incubation of the sections in 10% normal goat serum in PBS for 30 min. Samples were incubated overnight with polyclonal rabbit anti-zonulin-1 (2.5 μg/ml) or anti-occludin (2.5 μg/ml) (ZYMED Laboratories Inc., San Francisco, CA, USA) at 4°C. After washing in PBS, section were incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (1: 200 in PBS) (Jackson, ImmunoLabs.,West Grove, PA, USA) for 1 hour and stain by AEC chromogen solution (Dako, Carpinteria, CA, USA) for 5 min. The counterstain was carried out with haematoxilin and samples were mounted in the Histotec Aqueous Mountant (Serotec, UK) and viewed under an Olympus BX 40 microscope equipped with an Olympus DP 70 digital camera. Photographs were taken on proposal of Camedia Master 2.5 and DP-Soft (Olympus, Germany).

Western blot analysis of zonulin-1 and occludin

Segment of colon descendens (approximately 0.5 cm in length located 1.5 cm proximal to the anus) from all experimental mice was homogenized on ice in protein extract buffer (Pierce, Rockford, IL, USA) with a protease inhibitor cocktail (Pierce) for 10 min and sonicated. Samples were centrifuged at 10,000 x rpm for 10 min at 4°C and stored at −80°C until use. Protein concentration was measured using the BCA Protein Assay Kit (Pierce). Western blotting was performed as described by Cinova *et al.* [42] using antibodies against occludin (1:1000) (Invitrogen, Carlsbad, CA, USA), zonulin-1 (1:1000) (ZYMED Laboratories Inc., San Francisco, CA, USA), and β-actin (1:5000) (Abcam, Cambridge, CA, USA). After incubation with the respective primary antibodies, secondary staining was performed using horseradish peroxidase-conjugated species-specific antibodies (1:1000) (ZYMED Laboratories). The reaction was developed using the SuperSignal Weat Femto Maximum Sensitivity Substrate (Thermo Scientific, USA) and the signal intensities were measured on the G:BOX (Syngene, UK) and processed with Adobe Photoshop CS5.

Evaluation of the intestinal permeability *in vivo*

The intestinal permeability was measured by determination of the amount of FITC-dextran in blood after oral administration as described previously [43]. Briefly, female BALB/c mice were intragastrically gavaged by Bl 7952 (Bl 7952/DSS; n = 5) or PBS (PBS/DSS; n = 5) for ten consecutive days prior to intestinal inflammation was induced by drinking of 2.5% DSS in water for 7 days. Naïve mice served as healthy controls (Naïve; n = 5). On the last day of DSS administration, each mouse received 360 mg/kg of the body weight of FITC-dextran (molecular weight 4.0 kDa; Sigma-Aldrich) by intragastric gavage. Blood samples were obtained after 5 hours, centrifuged at 3,000 x rpm for 30 min, and serum was collected. The concentration of FITC-dextran was determined by spectrophotofluorometry (Safire2, Tecan Group Ltd., Mannedorf, Switzerland) with an excitation wavelength of 483 nm and an emission wavelength of 525 nm using serially diluted FITC-dextran as standard.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). For the *in vitro* assays, data were analysed by One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison post-hoc test. For the *in vivo* assays, unpaired Student's t-test was used. Statistical analysis was performed using GraphPad Prism 5.0 Software (San Diego, CA, USA).

Results

Identification of probiotic *Bifidobacterium* isolates to the species, subspecies and strain level

Nine bacterial isolates of the genus *Bifidobacterium* from healthy children and adults have been selected for their probiotic properties based on resistance to low pH and resistance to bile salt (data not shown). These isolates were identified to the species, subspecies and strain level. PCR-based methods (S1 Materials and Methods) let to identification of three species and two subspecies of the genus *Bifidobacterium*: *B. longum* ssp. *longum* CCDM 372 (Bl 372) and CCM 7952 (Bl 7952); *B. longum* ssp. *infantis* CCDM 369 (Bi 369), *B. animalis* CCDM 218 (Ban 218) and CCDM 366 (Ban 366) and *B. adolescentis* CCDM 368 (Bad 368), CCDM 370 (Bad 370); CCDM 371 (Bad 371), and CCDM 373 (Bad 373). Discrimination of the *B. longum* strains into subspecies *longum* and *infantis* was performed on the basis of Amplified ribosomal DNA restriction analysis by the enzyme *Sau*3AI (S1 Fig) as described previously [36].

Immunostimulatory properties of probiotic strains from the genus *Bifidobacterium* are strictly strain-specific

The *in vitro* stimulation of spleen cells collected from naïve BALB/c mice with nine *Bifidobacterium* strains revealed distinct and strain-specific pattern of cytokine production (Table 1). Production of IL-10 by probiotic strains has been associated with their protective effect on inflammatory diseases [44]. Various levels of IL-10 were detected in spleen cultures with values ranging between 100–700 pg/ml, depending on the used strain, where Bi 369, Bad 371, and Bl 372 were the most robust inducers of this anti-inflammatory cytokine (Table 1). On the other hand, Ban 218 and Bad 373 induced low levels of IL-10, but high levels of TNF-α, IL-6 and IFN-γ. Strains Bl 372 and Bad 370 induced substantial production of pro-inflammatory cytokines TNF-α, IL-6, and IFN-γ. Strains Bl 7952 and Ban 366 led to only moderate production of TNF-α and IFN-γ, but significantly elevated levels of IL-10. According to this *in vitro* analysis, we selected two strains of one subspecies *B. longum* ssp. *longum*: Bl 372 and Bl 7952, which provided contrasting cytokines pattern and used them for further characterization.

Table 1. Cytokine production by splenocytes stimulated with inactivated bacteria of different *Bifidobacterium* strains.

Control/ *Bifidobacterium* strain		IL-10	TNF-α	IL-6	IFN-γ
Medium		7 ± 1	6 ± 3	13 ± 4	12 ± 11
PAM3C		628 ± 57**	166 ± 20	175 ± 84**	790 ± 332
B. longum	**Bl 7952**	270 ± 25**	138 ± 12	452 ± 35	173 ± 80
B. longum	**Bl 372**	532 ± 39**	451 ± 37**	638 ± 80**	744 ± 183
B. infantis	**Bi 369**	542 ± 85**	384 ± 56**	493 ± 117	108 ± 59
B. animalis	**Ban 218**	187 ± 14**	935 ± 198**	650 ± 62**	1865 ± 422**
B. animalis	**Ban 366**	268 ± 14**	200 ± 22	229 ± 13	55 ± 24
B. adolescentis	**Bad 368**	92 ± 4	189 ± 17	334 ± 52	154 ± 91
B. adolescentis	**Bad 370**	243 ± 16**	357 ± 36**	923 ± 114**	669 ± 151*
B. adolescentis	**Bad 371**	606 ± 52**	290 ± 8*	675 ± 70**	145 ± 65
B. adolescentis	**Bad 373**	186 ± 14*	495 ± 60**	987 ± 87**	1819 ± 275**

Splenocytes isolated from naïve mice (n = 5) were stimulated with formalin-inactivated bifidobacteria (6 x 10^7 CFU/ml) for 48 h. Pam3CSK4 (PAM3C, 1μg/ml) was use as a positive control. Non-stimulated splenocytes (Medium) were evaluated as control of basal cytokine levels. Concentration of cytokines in supernatants was determined by multiplex assay. Data are expressed as mean ± SEM. Results are representatives of two repeat experiments. Significant difference to medium was calculated using One-way ANOVA and Dunnett's multiple comparison post-hoc test *p < 0.05; **p < 0.01.

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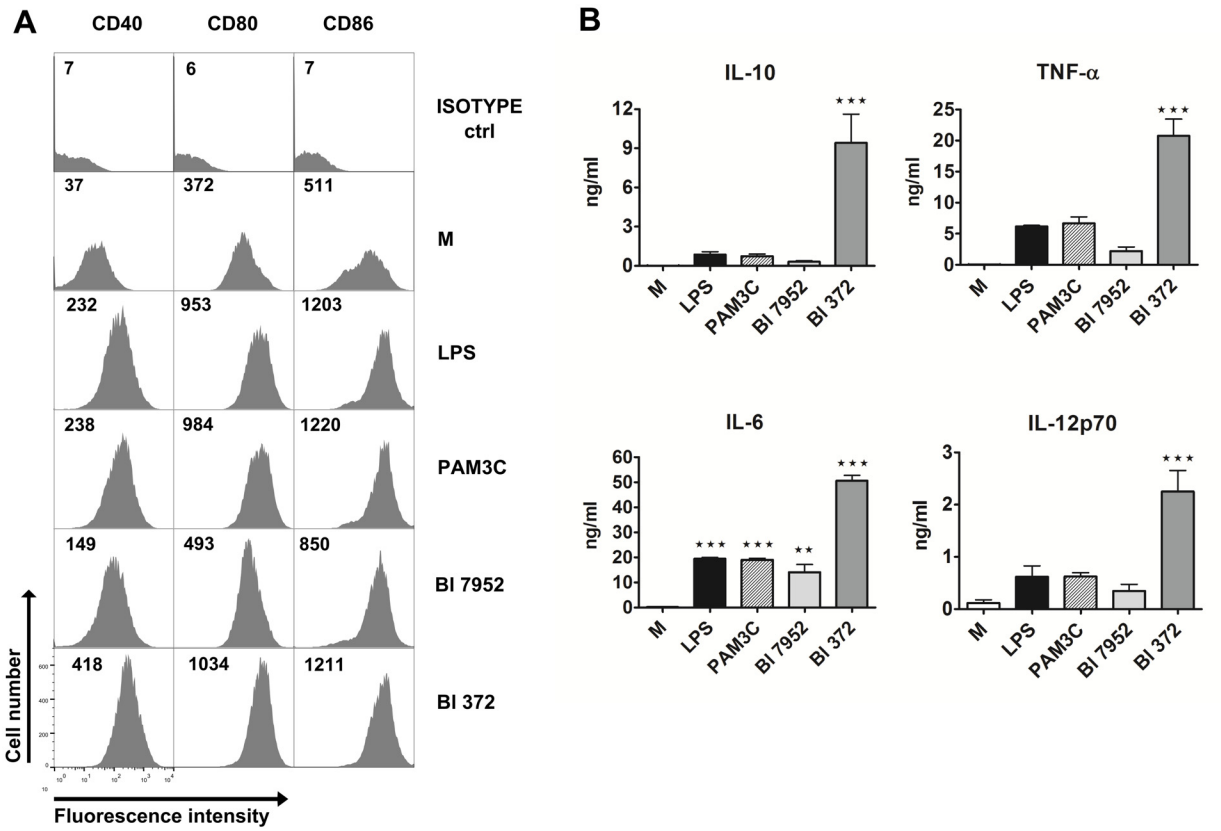

Fig 1. Stimulation of bone marrow-derived dendritic cells with BI 7952 and BI 372. Bone marrow-derived dendritic cells (BM-DC) from naïve mice were cultured with formalin-inactivated BI 7952 or BI 372 (10^7 CFU/ml) for 18 h. Ultra-pure lipopolysaccharide from *E. coli* (LPS, 1 μg/ml) and Pam3CSK4 (PAM3C, 1 μg/ml) were used as positive controls. Untreated cells (M) served as negative control. (A) Expression of CD40, CD80 and CD86 was assessed by means of flow cytometry. BM-DCs were gated as MHCII+CD11c+. Numbers represent florescence units from one representative experiment out of three. (B) Cytokines in cell culture supernatants were determined by ELISA. Results are representative of three repeat experiments. Data are expressed as mean ± SEM. Significant differences between cytokine levels of experimental group to negative control (M) was calculated using One-way ANOVA and Dunnett's multiple comparison post-hoc test (**p < 0.01, ***p < 0.001).

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Strains BI 7952 and BI 372 have differential ability to activate dendritic cells *in vitro*

Dendritic cells (DC) have been shown to play a central role in regulating intestinal immune homeostasis by induction of tolerance to harmless antigens and commensals or initiating protective immunity against pathogens, contributing to control of intestinal diseases such as inflammatory bowel diseases [45]. In our study, BM-DCs derived from naïve BALB/c mice were used as *in vitro* model to investigate the immunostimulatory potential of both *B. longum* spp. *longum* strains. Expression of co-stimulatory markers CD40, CD80, and CD86 were measured to investigate the activation status of BM-DC after stimulation with each bacterial strain. The induction of these surface markers differed between the tested strains. Higher levels of CD40, CD80 and CD86 were observed in DC incubated with Bl 372 than with Bl 7952 (Fig 1A). Levels of IL-10, TNF-α, IL-6, and IL-12p70 were measured in the supernatants of BM-DC stimulated with both *B. longum* spp. *longum* strains (Fig 1B). The data show that stimulation of BM-DC with Bl 372 resulted in significantly higher levels of secreted cytokines than stimulation with Bl 7952.

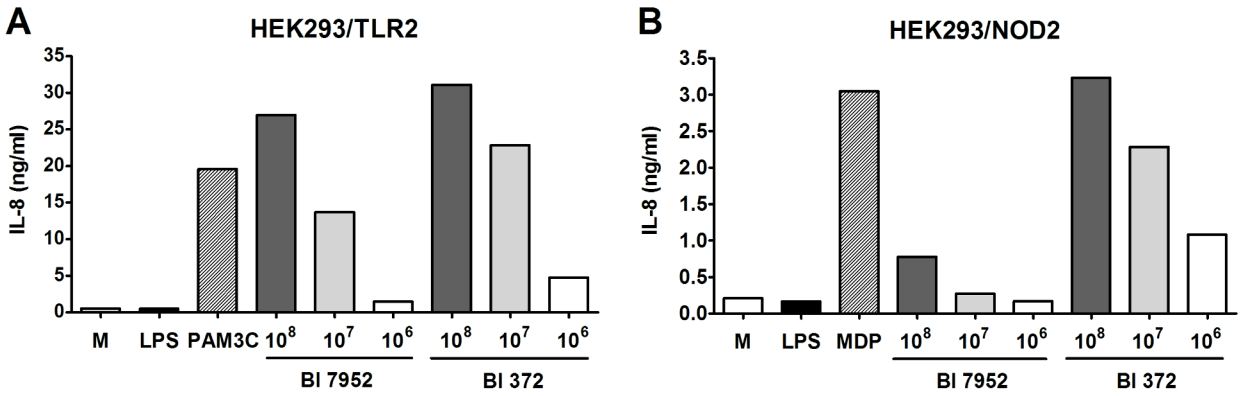

Fig 2. Activation of TLR2 and NOD2 by Bl 7952 and Bl 372. Human embryonic kidney cells (HEK293) stably transfected with an expression vector for human TLR2 (293-hTLR2/CD14) or with NOD2 (293-hNOD2) were stimulated with formalin-inactivated Bl 7952 or Bl 372 for 20 h. Stimulation was performed at concentrations of 10^6, 10^7 or 10^8 CFU/ml. Cells stimulated with ultra-pure lipopolysaccharide from *E. coli* (LPS, 1 μg/ml) or untreated cells (M) were used as negative controls. Cells stimulated with Pam3CSK4 (PAM3C, 1 μg/ml) or muramyl dipeptide (MDP, 1 μg/ml) were used as positive controls for TLR2 or NOD2, respectively. Data are expressed as one representative experiment out of three.

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Both Bl 7952 and Bl 372 signal through TLR2 and NOD2 receptor

To assess the role of TLR2 and NOD2 in recognition of Bl 7952 and Bl 372, HEK293 cells stably transfected with TLR2/CD14 or NOD2 were stimulated with increasing concentrations of both strains. Pam3CSK4 and MDP were used as positive controls for TLR2 and NOD2, respectively. After 20 h of incubation, supernatants were harvested and analysed for IL-8 production. At all three tested concentrations, both Bl 7952 and Bl 372 activated TLR2 in an analogous and dose-dependent manner (Fig 2A). In contrast, stimulation of HEK293/NOD2 with Bl 372 induced markedly higher levels of IL-8 in comparison to stimulation with Bl 7952 (Fig 2B). These results suggest that both Bl 7952 and Bl 372 have similar pattern of usage of TLR2 but distinct patterns of interaction with NOD2. There was no stimulation of HEK293/TLR4 cells with any of *B. longum* strains (data not shown).

Prophylactic application of Bl 7952, but not Bl 372, ameliorates DSS-induced colitis

Here we have shown that two strains of one subspecies *B. longum* ssp. *longum*, Bl 7952 and Bl 372, possess different immunomodulatory properties *in vitro* (Table 1, Figs 1 and 2). To compare their properties *in vivo*, the mouse model of acute ulcerative colitis induced by administration of 2.5% DSS in drinking water was used. Animals received Bl 7952 or Bl 372 on 10 consecutive days prior to colitis induction (Fig 3A). Disease progression was characterized by weight loss, appearance of diarrhoea or loose faeces and visible faecal blood and summarized in disease activity index (DAI) assessed according to the scale (0–4) of Cooper *et al.* [40] (S1 Table). DSS-treatment increased DAI, reduced the colon length, and reduced body weight in control mice (PBS/DSS) in comparison to naïve animals (Fig 3B–3D). Mice pre-treated with Bl 7952 showed improvement of DAI, and reduction of DSS-induced colon shortening and weight loss compare to PBS/DSS mice (Fig 3B–3D). In contrast, pre-treatment with Bl 372 had no impact on any of these parameters and Bl 372-treated mice did not significantly differ from PBS/DSS group (Fig 3B–3D).

Histopathological evaluation of the colonic mucosa after DSS-treatment was performed to establish a score (0–4) as described before [40]. The scoring was based on infiltration of inflammatory cells into lamina propria and submucosa, submucosa thickening, and loss of the entire

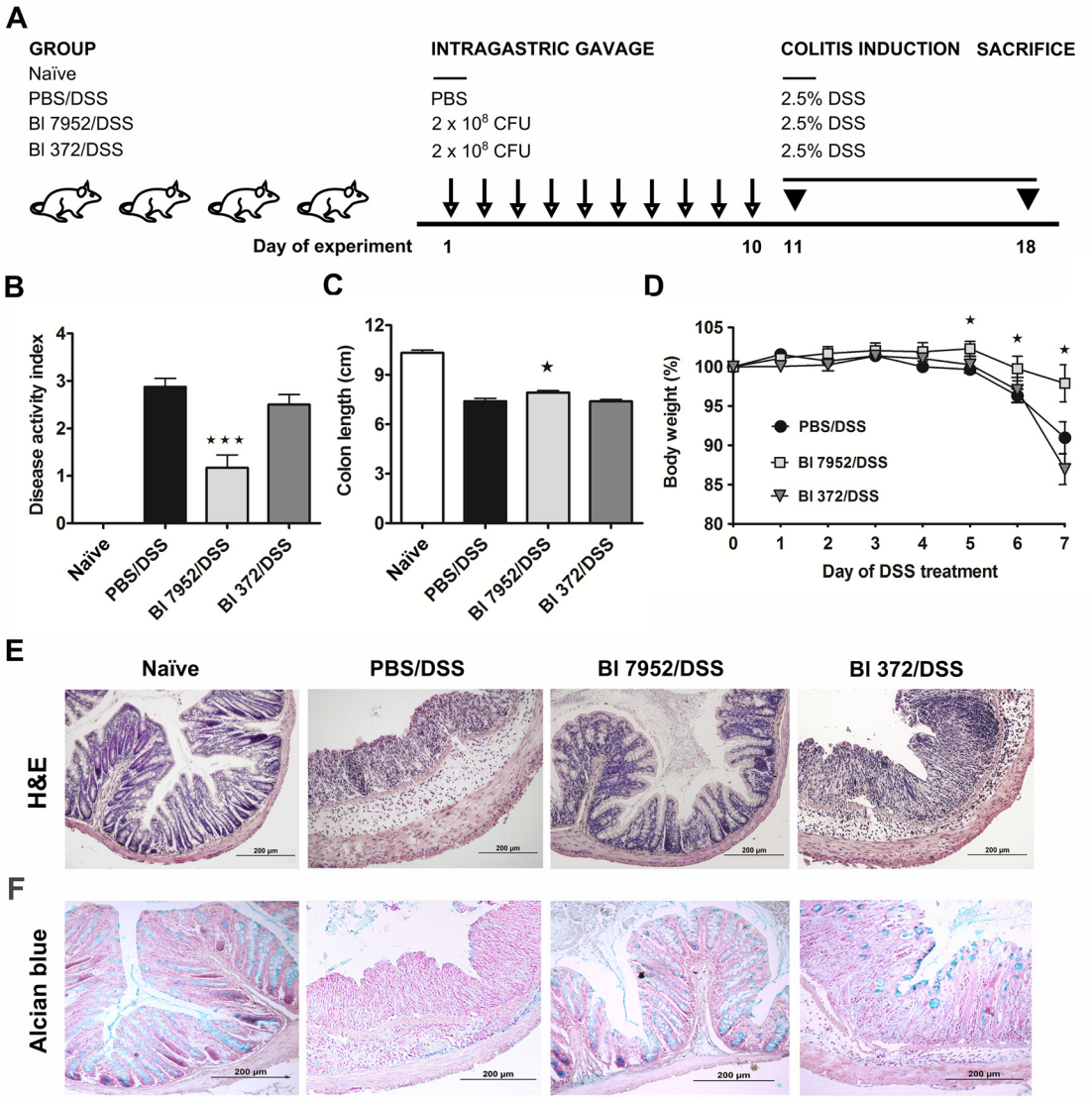

Fig 3. Impact of BI 7952 and BI 372 on DSS-induced colitis. (A) Mice were treated with BI 7952 (n = 10), with BI 372 (n = 8) or with PBS (n = 10) on ten consecutive days. Naïve animals (n = 5) were left untreated. Colonic inflammation was induced by the addition of 2.5% (w/v) DSS in the drinking water for 7 days. (B) Disease activity index and (C) colon length were evaluated at the end of the experiment. (D) Body weight of mice was evaluated throughout the experiment and the values are expressed as percentage of change of the initial value measured before DSS administration. Changes in colonic mucosa after DSS-treatment are shown on representative histological sections of healthy untreated mice (Naïve), mice treated with PBS (PBS/DSS), BI 7952 (BI 7952/DSS) or BI 372 (BI 372/DSS). The sections were stained by H&E (E) to address the degree of inflammation and by alcian blue (F) to show the changes in production of mucus in colonic tissue. Graphs show mean ± SEM and represent one out of two experiments. Unpaired Student's t-test was used for comparison of experimental groups *vs*. control PBS/DSS group (*p < 0.05, ***p < 0.001).

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crypt with retained surface epithelium [40, 41]. On sacrifice, histological finding encompassed infiltration of inflammatory cells into lamina propria, thickening of submucosa, loss of epithelial layer and disappearance of mucosal crypt in colonic wall of DSS-treated controls (PBS/DSS; grade 3.5 ± 0.5) and in BI 372 pre-treated/DSS-treated mice (BI 372/DSS; grade 3.5 ± 0.3) (Fig 4E). In contrast, BI 7952-pre-treated mice displayed inhibitory effect on DSS-induced histological changes (BI 7952/DSS; grade 0.75 ± 0.25) compared to controls. BI 7952 reduced infiltration of inflammatory cells and pathological changes in mucosa or epithelial layer (Fig 3E).

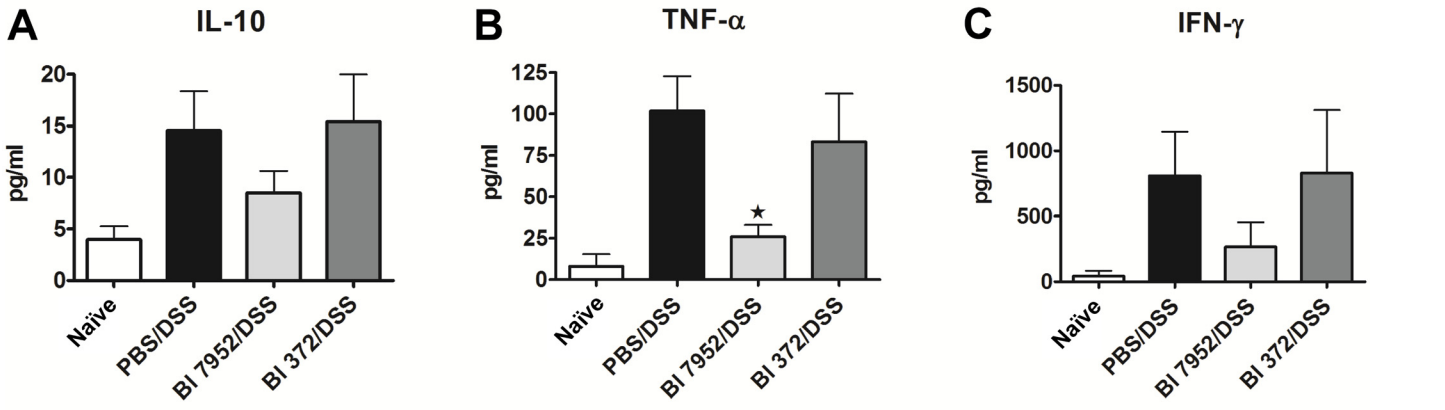

Fig 4. Bl 7952 strain downregulated the secretion of pro-inflammatory cytokines in mesenteric lymph node cells of mice with DSS-induced colitis. Spontaneous production of anti-inflammatory IL-10 (A) or pro-inflammatory cytokines TNF-α (B) and IFN-γ (C) were analysed by multiplex assay in supernatants of mesenteric lymph node cells incubated with media only for 48 h. Data are expressed as mean ± SEM of untreated (n = 5), PBS/DSS (n = 10), Bl 7952/DSS (n = 10) or Bl 372/DSS (n = 8) mice. Unpaired Student's t-test was used for comparison of experimental groups *vs*. PBS/DSS groups (*$p < 0.05$).

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In control mice with colitis (PBS/DSS) and in mice treated with Bl 372 (Bl 372/DSS), colonic mucin production by goblet cells (Alcian Blue staining) was decreased in comparison to naïve mice (Fig 3F). Markedly, application of Bl 7952 preserved the thinning of the mucus layer and goblet cell depletion (Bl 7952/DSS).

Administration of Bl 7952 has an impact on the production of cytokines in the mesenteric lymph node cells

Changes in cytokine microenvironment in the gut associated lymphoid tissue, such as mesenteric lymph nodes (MLN), might impact the development of intestinal inflammation in colitis. Therefore, we investigated whether the protective effect of *B. longum* Bl 7952 on colitis is associated with changes in production of pro- and anti-inflammatory cytokines. MLN cells collected from naïve, PBS/DSS, Bl 7952/DSS or Bl 372/DSS animals were cultivated for 48 h. Level of cytokines in supernatant was measured by ELISA. We found that pre-treatment with Bl 7952 but not with Bl 372 decreased significantly the production of TNF-α (Fig 4B). Although the levels of IL-10 and IFN-γ were reduced in MLN cell cultures by Bl 7952 in comparison to DSS-controls, the difference did not reach significant level (Fig 4A and 4C).

Bl 7952 preserves the expression of zonulin-1 and occludin and decreases colon permeability in DSS-treated mice

Altered intestinal barrier function (breakdown or impairment of the epithelial barrier), which is associated with increased intestinal permeability through decreased expression of tight junction proteins, has been implicated as a critical factor in the development of intestinal inflammation in mouse models of colitis or in human IBD. Occludin and zonulin-1 are proteins involved in the maintenance of the integrity of intact tight junction complexes and barrier function [46]. Here we investigated whether pre-treatment with Bl 7952 interferes with the disruption of these tight junction proteins induced by DSS-treatment. As shown by immunohistochemistry staining (Fig 5A) and Western blotting (Fig 5B), expression of occludin and zonulin-1 was reduced in PBS/DSS mice in comparison to naïve animals. There were no

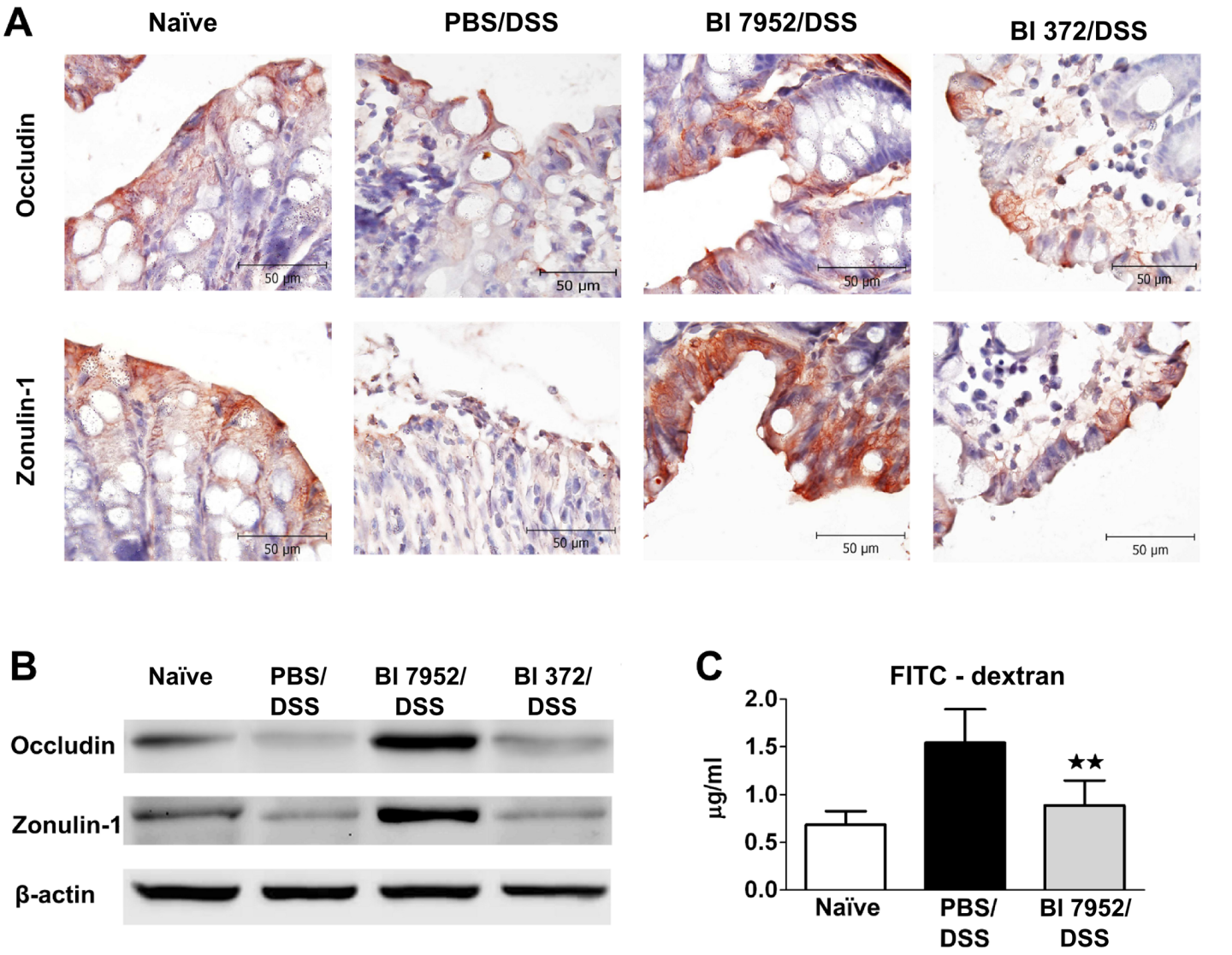

Fig 5. Bl 7952 induces upregulation of zonulin-1 and occludin in colon. Mice were treated with Bl 7952 (n = 10), with Bl 372 (n = 8) or with PBS (n = 10) on ten consecutive days or were left untreated (Naïve; n = 5). Colonic inflammation was induced by addition of 2.5% (w/v) DSS in the drinking water for 7 days. (A) Immunohistochemical detection of occludin and zonulin-1 proteins on representative paraffin-embedded sections of colon. (B) Representative western blotting of occludin and zonulin-1 proteins in the colonic mucosa. Expression of β-actin was used as internal control. (C) Evaluation of intestinal permeability by FITC-dextran. Serum levels of 4.0-kDa FITC-dextran were measured in naïve controls (n = 5), PBS/DSS-treated (n = 5) and Bl 7952/DSS-treated mice (n = 5) 5 hours after its intragastric administration. Data are express as mean ± SEM. Unpaired Student's t-test was used for comparison of Bl 7952/DSS group *vs*. control PBS/DSS group (**p < 0.01).

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differences between levels of these tight junction proteins in PBS/DSS mice and mice pre-treated with Bl 372 (Fig 5A and 5B). In contrast, application of Bl 7952 preserved the loss of expression and alteration of distribution of both proteins (Fig 5A and 5B).

In order to investigate whether preventive application of Bl 7952 could improve the altered gut barrier function in DSS-colitis, single dose of FITC-dextran was administered by gavage and the intensity of fluorescence was measured in serum 5 h later. The data show that pre-treatment with Bl 7952 markedly decreased FITC-dextran serum concentration in comparison to PBS/DSS mice, reaching similar levels found in naïve mice (Fig 5C). Thus, Bl 7952 preserved

the expression of tight junction proteins, which was associated with improved intestinal barrier function in DSS-treated mice.

Discussion

I00t is clearly established that the altered composition of the intestinal microbiota plays a role in initiating and maintaining of IBD [47]. Several studies have reported reduction in potentially beneficial *Bifidobacterium* species in IBD patients [6, 48]. Moreover, recent study has shown that *B. longum* was one of the dominant species decreased in paediatric patients with new-onset Crohn's disease [49]. Therefore, modulation of the gut habitat with probiotics, particularly species from the genera *Bifidobacterium* and *Lactobacillus*, represents a novel and exciting strategy in prevention/treatment of microbial dysbiosis associated with mucosal inflammation [50].

In this study we investigated immunomodulatory properties of nine *Bifidobacterium* strains which were obtained from faeces of healthy breast-fed infants and healthy adults, and which possess probiotic properties, such as resistance to gastric acidity (low pH) and bile toxicity, conditions simulating those of the gut environment (data not shown). These probiotic strains were classified by PCR based methods on the species, subspecies, and strain level as *B. longum* ssp. *longum* and *B. longum* ssp. *infantis*, *B. adolescentis* and *B. animalis*. In order to call a bacterial strain "probiotic", it should be well-characterized, classified and specified for its effect on human health. Probiotic bacteria exert their beneficial effects in different ways, among which the immunomodulation of local and systemic immune responses is an important mechanism [51]. In this respect, we tested all strains for the ability to induce cytokine production in spleen cell cultures derived from naïve mice. Results indicate that all strains possess intrinsic immunostimulatory potential, but their ability to induce cytokine expression varies significantly from one bacterial strain to other. We have shown that some *Bifidobacterium* strains, such as Bad 368, Ban 366 or Bl 7952, are poor inducers of both pro- and anti-inflammatory cytokines, while other strains belonging to the same species or subspecies, such as Bad 370, Ban 218 or Bl 372, can stimulate high levels of all evaluated cytokines. These strain-specific effects are in accordance with previous observations on human immunocompetent cells [52–55]. Nonetheless, comparative studies on the immunomodulatory properties of *Bifidobacterium* strains of the same species or subspecies are limited [54–56].

Regulatory cytokine IL-10, which can be produced by multiple cell types, has been shown to play an important role in the maintenance of intestinal homeostasis [44]. Mice with defects in IL-10 production spontaneously develop severe intestinal inflammation in conventional conditions [57]. Relevant to this point, it has been reported that intragastric administration of IL-10-producing recombinant *Lactococcus lactis* reduced colitis in DSS-treated or IL-10-/- mice [58]. Therefore, the capacity of probiotic strain to induce production of IL-10 may be one factor contributing to their beneficial effects [44]. We observed that the majority of tested *Bifidobacterium* strains, except for Bad 368, induced significant levels of IL-10 from naïve spleen cell cultures. Along these lines, bifidobacteria have been shown to induced IL-10 also in human monocyte-derived DC [53], PBMC [59, 60] or in human colonic lamina propria DC [52].

Innate immune cells, such as DC, play an important role in orchestrating the appropriate responses to the enteric luminal microbiota [61]. Defects in how DC recognize and respond to gut bacteria may contribute to IBD pathogenesis [62]. Here we compared the effects of Bl 7952 and Bl 372 on the maturation pattern of BM-DC, as well as their ability to induce cytokine secretion. We found that the activation potential of the two strains varied significantly, suggesting their different functional roles. These results are well in line with previous studies which show that stimulation of DC with different strains of *B. longum* led to strain-specific production of pro-inflammatory or regulatory cytokines [55].

Although there have been several studies linking immunomodulatory properties of probiotic strain *in vitro* and its ability to prevent experimental colitis in mice, no clear association has been established so far. In an experimental model of TNBS-induced colitis, Foligne *at al.* demonstrated that probiotic strains with high IL-10/IL-12 ratio *in vitro* provided the best protection *in vivo* [63]. Similarly, Kwon *et al.* demonstrated that administration of probiotic mixture with potent anti-inflammatory properties (high levels of the IL-10/IL-12 production ratio) suppressed the progression of experimental colitis in mice [64]. Along these lines, we have shown recently that *B. longum* NCC 3001, a probiotic strain with high IL-10/IFN-γ ratio, offered long term protection in a mouse model of birch pollen allergy [35]. Moreover, we have demonstrated that neonatal colonization of germ-free mice with *B. longum* ssp. *longum* CCM 7952 prevented experimental sensitization in a mouse model of allergy [34].

In this study we employed both *in vitro* culture system and *in vivo* mouse model of DSS-induced colitis to compare the immunomodulatory potential of two probiotic strains of the genus *Bifidobacterium* which belong to the same subspecies *B. longum* ssp. *longum*. We found that the activity of these two strains, Bl 372 and Bl 7952, differs significantly. While Bl 7952, the strain with low stimulatory potential *in vitro* (as demonstrated on stimulated splenocytes and BM-DC), was able to prevent clinical symptoms in a mouse model of DSS-induced colitis, preserved the tight junction proteins expression and protected epithelial barrier function, the strain Bl 372, which induced high levels of cytokines *in vitro*, had no beneficial effect. Our results are consistent with those reported by Mileti *et al.* who tested the effect of application of three different probiotic strains before exposure to DSS, and observed that only one strain, which was characterized by low levels of induced cytokines, reduced severity of DSS-induced colitis [65].

It has recently been shown, that certain probiotic strains exert their immunomodulatory effects through the interaction with TLRs. Administration of *L. plantarum* to healthy subjects increased levels of tight junction-associated zonulin-1 and occludin in the duodenal epithelium and this beneficial effect was shown to be dependent on TLR2-signalling [66]. In our study, both Bl 7952 and Bl 372 have been shown to signal through the TLR2. The fact that only Bl 7952 but not Bl 372 was protective, suggests that TLR2 is not the key player in preserving the gut epithelial barrier in our model.

There is a strong body of evidence suggesting the link between NOD2 and the development of IBD [67, 68]. Within the colonic mucosa, NOD2 can be expresses by various cell populations, such as epithelial cells [69]. In a mouse model, *Nod2* deficiency led to an altered composition of the gut microbiota, predisposing mice to colitis [70]. NOD2 senses many types of peptidoglycan-derived muropeptides, which can vary significantly in their capacity to stimulate NOD2 [71]. Fernandez *et al.* showed that anti-inflammatory capacity of probiotic-derived peptidoglycan was linked to the presence of a NOD2 ligand [72]. In our present study, we demonstrate that although both Bl 7952 and Bl 372 possess ligands which are recognised by NOD2, they potential to stimulate NOD2 differ. Still, the role of NOD2 in recognition of these strains *in vivo* remains to be evaluated.

In humans, IBD is associated with increased intestinal permeability and reduced expression of tight junction proteins, [73] which leads to exposure of luminal antigens to the lamina propria [74, 75]. Therefore, several recently proposed therapeutic approaches to treat IBD are focused on enhancing/restoring of gut barrier integrity [76, 77]. It is of interest, that perfusion of lactic acid bacteria into the small intestine of a healthy subject increased the localization of the scaffold zonula occludens protein zonulin-1 and the transmembrane protein occludin [66]. In an experimental model of colitis, probiotic bacteria have been shown to decrease the intestinal permeability and restore gut barrier integrity by modulation of tight junction proteins [43, 78–80]. In this study we showed that Bl 7952 but not Bl 372 increased expression of zonulin-1

and occludin in the intestinal epithelium which was associated with reduced leakiness of colonic epithelium. Thus, our data show that the choice of *Bifidobacterium* strain for specific benefit, such as maintenance of healthy and functional gut barrier should be considered on a strain-by-strain basis, and interspecies extrapolations are not valid.

In conclusion, our data show strictly strain-specific immune effects of *B. longum* subspecies. Thus, it is getting clear, that the beneficial effects of one probiotic strain cannot be extended to other bacteria of the same genus, species or even subspecies. Our work shows that prophylactic administration of probiotic strain *B. longum* ssp. *longum* CCM 7952 is capable to preserve the disruption of tight junctions proteins associated with ulcerative colitis pathophysiology. Therefore, this bacterial strain plays the role as regulator of the integrity of the intestinal barrier, which might have important implications for understanding of probiotic mechanisms and for the control of intestinal homeostasis.

Supporting Information

S1 Fig. Amplified ribosomal DNA restriction analysis profile. Amplified ribosomal DNA restriction analysis profile of nine studied *Bifidobacterium*strains (*) and six type/collection control strains of corresponding species and subspecies. Dendrogram is generated from restriction of 914 bpamplicon by different enzymes (*Bam*HI, *Nci*I, *Sau*3AI) and based on UPGMA analysis of Pearson correlation coefficients.
(PDF)

S1 Materials and Methods. Identification of *Bifidobacterium* strain by PCR-based methods
(PDF)

S1 Table. Scoring of disease activity index. Scoring of disease activity index is combined score of weight loss, stool consistency and bleeding divided by 3. *Normal stool* = well-formed pellets; *Loose stool* = pasty and semi-formed stool that does not adhere to the anus; *Diarrhoea* = liquid stool that adheres to the anus. Modified according to Cooper *et al.* [1].
(PDF)

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Author Contributions

Conceived and designed the experiments: DS MS TH HK IS. Performed the experiments: DS MS TH ZZ. Analyzed the data: DS MS TH ZZ HK IS. Contributed reagents/materials/analysis tools: VD AS BR. Wrote the paper: DS MS HK IS.

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S1 Materials and Methods

Identification of *Bifidobacterium* strain by PCR-based methods

Nine *Bifidobacterium* strains which have been selected according to their probiotic properties (survival in conditions of gastric acids and bile salt) were classified by molecular-biological methods as described previously [1]. Briefly, DNA was prepared from crude bacterial cell lysates by phenol (pH 7.8) and chloroform: isoamyl alcohol (24 : 1) extraction according to Sambrook and Russell [2]. The purity and concentration of nucleic acids were confirmed by UV spectrophotometry as described previously [3]. The genus *Bifidobacterium* was confirmed by PCR with specific primers Pbi F1/Pbi R2 described by Roy and Sirois [4]. Ten sets of specific primers were used for identification of bifidobacterial species frequently found in human samples: *B. longum* ssp. *longum*, *B. longum* ssp. *infantis*, *B. dentium*, *B. adolescentis*, *B. bifidum*, *B. breve*, *B. catenulatum/pseudocatenulatum*, *B. angulatum*, *B. gallicum* [5] and *B. animalis* [4]. Amplified ribosomal DNA restriction analysis (ARDRA) of genus-specific PCR product using three endonucelases *Bam*HI, *Nci*I (Takara, Shiga, Japan) and *Sau*3AI (Roche Diagnostic, Manheim, Germany) were used for species and subspecies classification as described previously [1, 3]. Four type and two collection strains used as control in ARDRA analysis were obtained from the Czech Collection of Microorganisms (*B. longum* ssp. *longum* CCM 3764, *B. adolescentis* CCM 4987T, *B. animalis* CCM 4988T), the American Type and Culture Collection (*B. longum* ssp. *longum* ATCC 15707T, *B. longum* ssp. *infantis* ATCC 17930), and the Deutsche Sammlung von Mikroorganismen und Zellkulturen (*B. longum* ssp. *infantis* DSM 20088T). The PCR products were visualised using GelRed^{TM} Nucleic Acid Gel Stain (Biotinum, Hayward, CA, USA) and images were obtained by Fluorescent Image Analyser FLA-7000 (Fujifilm Corporation, Tokyo, Japan). The fingerprinting profile was

analysed by the software programme Gel Compare II (2.0 version) and dendrogram were constructed using UPGMA analysis and Pearson correlation coefficient.

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S1 Table

Score	Weight loss	Stool consistency	Occult/gross faecal bleeding
0	None	Normal	Negative
1	1- 5%	Normal	Negative
2	5-10%	Loose	Hemoccult positive
3	10-20%	Loose	Blood in colon
4	>20%	Diarrhoea	Starting bleeding from anus Gross bleeding

Scoring of disease activity index. Scoring of disease activity index is combined score of weight loss, stool consistency and bleeding divided by 3. *Normal stool* = well-formed pellets; *Loose stool* = pasty and semi-formed stool that does not adhere to the anus; *Diarrhoea* = liquid stool that adheres to the anus. Modified according to Cooper *et al.* [1].

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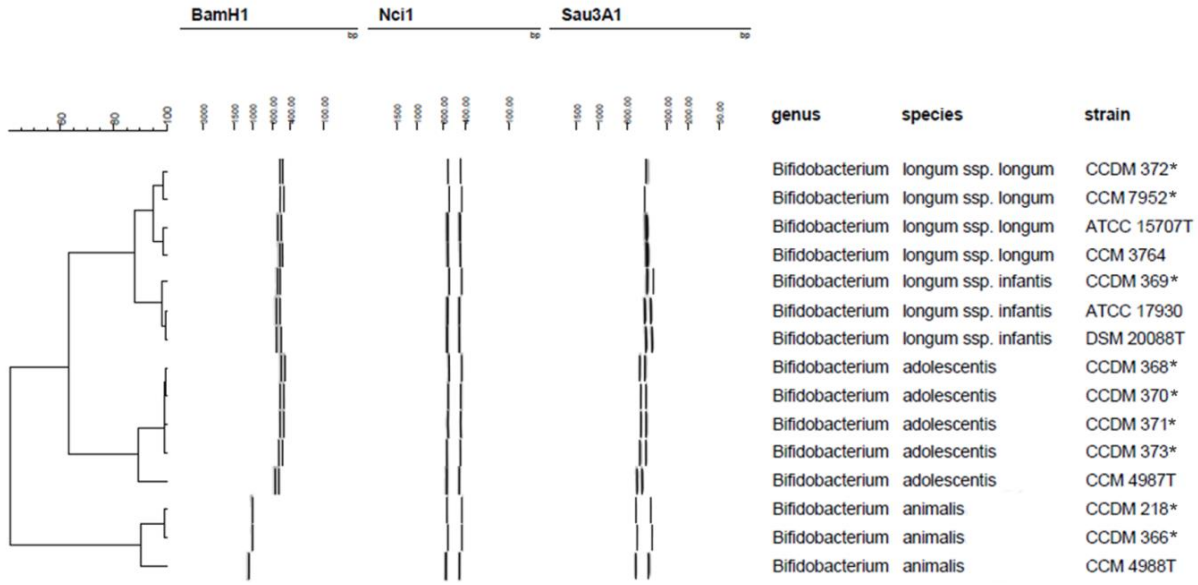
SUPPLEMENTARY FIGURE S1



Amplified ribosomal DNA restriction analysis profile. Amplified ribosomal DNA restriction analysis profile of nine studied *Bifidobacterium* strains (*) and six type/collection control strains of corresponding species and subspecies. Dendrogram is generated from restriction of 914 bp amplicon by different enzymes (*Bam*HI, *Nci*I, *Sau*3AI) and based on UPGMA analysis of Pearson correlation coefficients.

5. PROTECTIVE EFFECT OF *CLOSTRIDIUM TYROBUTYRICUM* IN ACUTE DEXTRAN SODIUM SULPHATE-INDUCED COLITIS: DIFFERENTIAL REGULATION OF TUMOUR NECROSIS FACTOR-α AND INTERLEUKIN-18 IN BALB/C AND SEVERE COMBINED IMMUNODEFICIENCY MICE

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Protective effect of *Clostridium tyrobutyricum* in acute dextran sodium sulphate-induced colitis: differential regulation of tumour necrosis factor-α and interleukin-18 in BALB/c and severe combined immunodeficiency mice

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Summary

One of the promising approaches in the therapy of ulcerative colitis is administration of butyrate, an energy source for colonocytes, into the lumen of the colon. This study investigates the effect of butyrate producing bacterium *Clostridium tyrobutyricum* on dextran sodium sulphate (DSS)-induced colitis in mice. Immunocompetent BALB/c and immunodeficient severe combined immunodeficiency (SCID) mice reared in specific-pathogen-free (SPF) conditions were treated intrarectally with *C. tyrobutyricum* 1 week prior to the induction of DSS colitis and during oral DSS treatment. Administration of DSS without *C. tyrobutyricum* treatment led to an appearance of clinical symptoms – bleeding, rectal prolapses and colitis-induced increase in the antigen CD11b, a marker of infiltrating inflammatory cells in the lamina propria. The severity of colitis was similar in BALB/c and SCID mice as judged by the histological damage score and colon shortening after 7 days of DSS treatment. Both strains of mice also showed a similar reduction in tight junction (TJ) protein zonula occludens (ZO)-1 expression and of MUC-2 mucin depression. Highly elevated levels of cytokine tumour necrosis factor (TNF)-α in the colon of SCID mice and of interleukin (IL)-18 in BALB/c mice were observed. Intrarectal administration of *C. tyrobutyricum* prevented appearance of clinical symptoms of DSS-colitis, restored normal MUC-2 production, unaltered expression of TJ protein ZO-1 and decreased levels of TNF-α and IL-18 in the descending colon of SCID and BALB/c mice, respectively. Some of these features can be ascribed to the increased production of butyrate in the lumen of the colon and its role in protection of barrier functions and regulation of IL-18 expression.

Keywords: butyrate, *Clostridium tyrobutyricum*, confocal fluorimetry, dextran sodium sulphate-induced colitis, SCID mice

Introduction

The enteric mucosa of ulcerative colitis (UC) patients is aberrant due to the abnormal interaction between microbiota and the intestinal mucosal immune system, which leads to mucosal inflammation [1,2]. In experimental dextran sodium sulphate (DSS) models of colitis, interaction of components from both pathogenic and commensal microorganisms with the host mucosal immune system can trigger inflammatory responses and alter the colonic function [3–5]. Under germ-free conditions mice develop markedly limited incidence of colitis [6]. One of the widely used experimental mouse models of UC involves addition of DSS to the drinking water, which causes rapid alterations in the inner colon mucus layer, making it permeable to bacteria [7,8]. Protection of colonic mucosa requires normal production of mucins, such as secretory MUC-2, synthesized by goblet cells in healthy colon of humans, rats and mice [9]. However, in experimental ulcerative colitis the mucus production is impaired [10]. Butyrate ameliorates inflammation in experimental ulcerative colitis by up-regulating the expression of mucin genes [11,12] and by protecting mucosal surfaces against increased mucosal permeability [13]. Several probiotics have been reported to enhance epithelial permeability and/or protect against barrier disruption by pathogens *in vitro* (reviewed recently [14]). Recently a study was performed in human volunteers which showed that perfusion of *Lactobacillus plantarum* into the

duodenum increased the localization (immunofluorescent staining) of occludin and zonula occludens (ZO)-1 in the epithelial tight junctions (TJs) of tissue biopsies [15]. Protection of mice against DSS induced colitis by probiotic *E. coli* Nissle 1917 has been associated with increased expression of TJ protein zonula occludens (ZO-2) expression in epithelial cells [16].

Ulcerative colitis is associated with an elevated production of inflammatory cytokines such as tumour necrosis factor (TNF)-α and interleukin (IL)-1β, leading to increased intestinal permeability and activation of nuclear factor (NF)-κB and c-Jun N-terminal kinase (JNK)/p38 mitogen-activated protein kinase (MAPK) pathways [17]. Decreased production of proinflammatory cytokines constitutes an important mechanism for the partial amelioration of colitis by probiotics [18,19].

Butyrate enemas have been reported to be effective in therapy of UC. Microbially produced butyrate is considered important for colonic health and in the prevention of colorectal cancer, owing to its use as an energy source for colonocytes and as a modulator of oxidative stress and inflammation [20]. Oral administration of *Clostridium butyricum* M 588, characterized by high production of butyrate during fermentative growth, has been shown to protect against DSS colitis in the mouse [21]. Our candidate probiotic *Clostridium tyrobutyricum* (DSM 2637) was isolated from raw cow's milk and is a Gram-positive, rod-shaped, spore-forming obligate anaerobe that can ferment a wide variety of carbohydrates to butyric acid. In a survey of 35 *Clostridium* species and 243 strains, *C. tyrobutyricum* was shown not to produce cytotoxins [22]. To verify further the safety of *C. tyrobutyricum* strain DSM 2637 we assessed its potential to translocate from the gut into the blood and organs of germ-free severe combined immunodeficiency (SCID) mice that were monocolonized for 30 days in a gnotobiotic isolator. All animals colonized with *C. tyrobutyricum* strain DSM 2637 remained healthy and lacked any signs of pathology. As expected, *C. tyrobutyricum* was found in the lumen of the jejunum, ileum, colon ascendens and colon descendens, but could not be detected in the blood, mesenteric lymph nodes (MLNs), liver or spleen.

We have shown previously that immunodeficient SCID (T and B cell-independent) and immunocompetent BALB/c mouse strains reared under specific-pathogen-free (SPF) conditions, but not as germ-free mice, develop colitis after 1 week of DSS treatment [6]. The BALB/c mice survive two rounds of DSS treatment, albeit with clear evidence of colonic damage and T and B cell infiltration in the mucus, whereas SCID mice survive only one round of DSS treatment [6]. The main objectives of this study were to investigate the role of the immune response in regulating colitis in SCID and BALB/c mice and to evaluate the potential protective effect of *C. tyrobutyricum* (DSM 2637) on DSS colitis development. Changes in intestinal mucins, barrier function of TJ and production of the inflammatory cytokines IL-18 and TNF-α were measured during colitis induction. As butyrate enemas and colonic application of faecal bacteria [23] have been shown to be beneficial in clinical studies we also evaluated the therapeutic potential of intrarectal administration of *C. tyrobutyricum* in the DSS colitis model.

Materials and methods

Animals

BALB/c and SCID (background BALB/cJHanHsd-SCID) mice were reared in SPF conditions. The absence of T lymphocytes in SCID mice was proved with fluorescein isothiocyanate (FITC)-labelled monoclonal anti-CD3 antibody (Serotec, Oxford, UK) using fluorescence activated cell sorter (FACS)Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Two-month-old mice were used for these studies and their body weights were measured before and after each experiment.

Bacterial strain and culture conditions

C. tyrobutyricum (DSM 2637) isolated from raw cow's milk was provided from the Food Research Institute, Prague, Czech Republic and cultured in sterile Bryant Burkey bouillon with resazurin and lactate (Merck KGaA, Darmstadt, Germany) at 37°C under anaerobic conditions. Prior to administration to mice a fresh overnight culture of bacteria was adjusted to 10^9 colony-forming units (CFU)/ml in saline.

Intrarectal administration of *C. tyrobutyricum* and induction of acute ulcerative colitis by DSS

The experimental groups of five to 10 mice and their respective treatments are shown in Table 1. Groups 1, 2, 4 and 5 (Table 1) received 2·5% DSS (molecular weight 40 kDa; ICN Biomedicals, Cleveland, OH, USA) in drinking water *ad libitum* for 1 week. The untreated control groups 3 and 6 received only drinking water. The *C. tyrobutyricum*-treated groups 1 and 4 received intrarectally (via tubing) a daily dose of 2×10^8 CFU of strain DSM 2637 in 0·2 ml saline for 7 days prior to DSS exposure and also during the 7 days exposure to DSS in the drinking water. Control groups 3 and 6 received 0·2 ml saline [phosphate-buffered saline (PBS)]. The following clinical symptoms were measured or assessed: firmness of faeces, rectal prolapses, rectal bleeding and colon length after the mice were killed. The colon descendens was divided into two pieces, one being used for TNF-α determination after 48 h culture and the other for histological assessment. Animal experiments were approved by the Ethical Committee of the Institute of Microbiology, Academy of Sciences of the Czech Republic, v.v.i.

Histological evaluation of inflammation

The tissue was fixed in Carnoy's fluid for 30 min, transferred into 96% ethanol and embedded in paraffin. Five-μm

Table 1. Development of DSS-colitis with association of *Clostridium tyrobutyricum* in conventional BALB/c and severe combined immunodeficiency (SCID) mice: clinical and histological gradings and detection of tumour necrosis factor (TNF)-α.

Strain	Treatment	2·5% DSS	Mortality/n	Body weight (g)	Damage score (0–4)	Mucin secretion (4–0)	Length of colon (cm)	TNF-α (pg/10 mg tissue)
1 BALB/c	*C. tyrobutyricum* (i.r.)	+	0/5	18·8 ± 1·2	$1·6 \pm 0·5^{\Delta\Delta\Delta}$	$2·7 \pm 0·4^{\Delta\Delta\Delta}$	$7·6 \pm 0·5^{\#}$	$27·9 \pm 12·7^{\#\#}$
2 BALB/c	saline (i.r.)	+	0/5	17·6 ± 1·9	$3·9 \pm 0·2^{\Delta\Delta\Delta}$	$0·1 \pm 0·2^{\Delta\Delta\Delta}$	$6·9 \pm 0·6^{\#}$	$29·3 \pm 15·3^{\#\#}$
3 BALB/c	saline (i.r.)	–	0/5	19·6 ± 0·5	0	4	9·8 ± 0·7	7·8 ± 5·0
4 SCID	*C. tyrobutyricum* (i.r.)	+	1/10	21·0 ± 2·1	$0·5 \pm 0·4^{\Delta\Delta\Delta}$	$3·5 \pm 0·5^{\Delta\Delta\Delta}$	$10·0 \pm 1·9^{\star}$	$25·2 \pm 5·2^{\star\star,\#\#}$
5 SCID	saline (i.r.)	+	2/10	20·0 ± 2·4	$3·9 \pm 0·1^{\Delta\Delta\Delta}$	$0·1 \pm 0·2^{\Delta\Delta\Delta}$	$7·8 \pm 0·8^{\#}$	$79·3 \pm 8·0^{\#\#}$
6 SCID	saline (i.r.)	–	0/8	21·5 ± 1·2	0	4	10·9 ± 0·3	11·0 ± 2·2

Values are means ± standard deviation, $^{\star}P < 0·05$, $^{\star\star}P < 0·01$, significant difference of group 4 *versus* group 5; $^{\Delta\Delta\Delta}P < 0·0001$, significant difference of group 1 *versus* group 2 and group 4 *versus* group 5 of mice; $^{\#}P < 0·05$, $^{\#\#}P < 0·01$, significant difference of groups 1 and 2 *versus* control group 3 of BALB/c mice and group 4 and 5 *versus* control group 6 of SCID mice; i.r.: intrarectal administration. DSS: dextran sodium sulphate.

paraffin-embedded sections were cut and stained with haematoxylin and eosin (H&E) and Alcian Blue and post-stained with Nuclear Fast Red (all from Vector, Burlingame, CA, USA) for mucin production. The samples were viewed under an Olympus BX 40 microscope equipped with an Olympus Camedia DP 70 digital camera, and the images were analysed using Olympus DP-Soft. The degree of damage to the surface epithelium, crypt distortion and mucin production in individual colon segments were evaluated according to Cooper *et al*. [3].

Expression of CD 11b, ZO-1 and MUC-2

Segments of the colon descendens were frozen in liquid nitrogen. Cryosections (5 μm thick) of acetone-fixed colon were used for immunocytochemistry. The membrane marker CD11b was detected directly by fluorescein-labelled monoclonal antibody anti-CD11b FITC (Serotec, Kidlington, UK). The antigen CD11b is known to be expressed on the surface of polymorphonuclear leucocytes, monocytes and natural killer (NK) cells. Expression of ZO-1 was detected by rabbit anti-mouse polyclonal antibody (Zymed Laboratories, Carlsbad, CA, USA) and secondary antibody Cy3 goat anti-rabbit IgG (Biomeda, Burlingame, CA, USA). Production of MUC-2 was detected by primary, polyclonal rabbit anti-mouse IgG, specific for MUC-2, and secondary antibody Cy3 goat anti-rabbit IgG (all from Biomeda).

Measurement of colonic TNF-α production

Pre-weighed colonic fragments were cultured in RPMI-1640 medium enriched with 10% bovine serum albumin in 5% CO_2 and 95% air at 37°C, in 24-well flat-bottomed plates (Nunc, Roskilde, Denmark) for 48 h. Quantification of TNF-α level was performed by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocols using Infinite 200 apparatus (Tecan Group Ltd, Grödig, Austria).

Measurment of IL-18 in tissues by confocal fluorimetry

Expression of IL-18 in acetone-fixed sections of colon descendens was detected by polyclonal rabbit antibody to mouse IL-18 (Acris Antibodies, Hiddenhausen, Germany) and Cy3-labelled goat anti-rabbit IgG (Biomeda). Relative confocal fluorimetry of IL-18 visualized by Cy3 staining was performed by laser scanning confocal microscopy using Leica SPE and Leica SP-2 microscopes with oil immersion objectives ×20, ×40 and ×63 and an excitation line of 532 nm and emission detection at 550–700 nm; a multiple accumulation of weaker signal was used. Fluorescence intensity of Cy3 in epithelial colonocytes was evaluated (expressed in units on a scale of 0–255) using different regions and sections of at least three mice per group. It was possible to distinguish regions with lower and higher amounts of IL-18 (Table 2) and evaluate them separately, using correct statistical methods.

Measurement of short-chain fatty acids (SCFA)

SCFAs were measured in bacterial cultures and in faecal samples of SCID mice. Concentrations of acetic acid, propionic acid, n-butyric acid, iso-butyric acid, valeric acid, iso-valeric acid and 2-methylbutyric acid were measured using gas chromatography on a HP 5890 GC with flame

Table 2. Evaluation of fluorescence for interleukin (IL)-18 in the colon of mice.

Mouse strain	SCID			BALB/c		
DSS	–	+	+	–	+	+
C. tyrobutyricum	–	–	+	–	–	+
IL-18 (lower intensity)	29·1 ± 7·2	19·2 ± 9·0	77·9 ± 15·8	34·8 ± 11·7	82·7 ± 6·6	50·7 ± 6·3
IL-18 (higher intensity)	76·5 ± 4·8	151 ± 25·4	161·9 ± 32·3	72·2 ± 11·1	146·4 ± 15·0	98·2 ± 11·8

Values are expressed as means ± standard deviation (s.d.) in units of fluorescence intensity on scale 0–255. SCID: severe combined immunodeficiency; DSS: dextran sodium sulphate.

ionization detector (FID) and nitrogen as carrier gas at 1·2 ml/min column flow. The samples were analysed on an Equity-1 column (30 m × 0·32 mm i.d., 1 μm film thickness; Supelco, Prague, Czech Republic). A 1-μl sample was injected into the gas chromatograph. The split ratio was 1:1. The oven temperature was held at 40°C for 1 min, then raised to 230°C at a rate of 10°C/min, and held at 230°C for 15 min; both injector and detector temperatures were 250°C. Peak identification was confirmed by retention times of commercially obtained standards from Sigma-Aldrich.

Statistical analysis

Statistical analyses were performed using Student's *t*-test. Values of $^*P \leq 0{\cdot}05$ were considered significantly different. Levels of Cy3 in colonocytes, expressed in units of fluorescence intensity on a scale of 0–255, were compared in unpaired Student's *t*-test using statistical significance level $^*P = 0{\cdot}001$.

Results

Clinical evaluation of BALB/c and SCID mice under the influence of DSS and *C. tyrobutyricum*

Control BALB/c and SCID mice not exposed to DSS remained healthy during the experiment. In contrast the DSS-treated mice developed clinical signs of colitis, including diarrhoea, rectal prolapses and bleeding. The SCID mice (group 5, Table 1) were more susceptible to DSS than BALB/c mice (group 2, Table 1), resulting in 20% mortality. No significant changes in body weight were observed in BALB/c and SCID mice as a consequence of exposure to DSS. One of the characteristic signs of DSS-induced colitis is shortening of the colon, which was observed in both saline–DSS-treated BALB/c and SCID mice (groups 2 and 5, Table 1) but not in the control mice (groups 3 and 6, Table 1). In SCID mice, treatment with *C. tyrobutyricum* prevented shortening of the colon (group 4, Table 1), while no significant protection was observed in *C. tyrobutyricum*-treated BALB/c mice (group 1, Table 1).

Histological colon damage score in DSS-treated BALB/c and SCID mice

The histological colon damage score after DSS treatment assessed according to the scale (0–4) of Cooper *et al.* [3] is presented in Table 1. The damage score was of grade 0 in control BALB/c and SCID mice (groups 3 and 6). The damage score was of grade 3·9 ± 0·2 in BALB/c and 3·9 ± 0·1 in SCID saline–DSS-treated mice (groups 2 and 5; Fig. 1a). In *C. tyrobutyricum*-treated BALB/c and SCID mice the damage due to DSS exposure was significantly less, grade 1·6 ± 0·5 (group 1) and grade 0·5 ± 0·4 (group 4, Fig. 1b), respectively.

Goblet cell mucins and specific MUC-2 production in the colon

In healthy control mice (groups 3 and 6, Table 1), colonic mucin production (Alcian Blue staining) was grade 4 according to the scale (4–0) of Cooper *et al.* [3]. In both saline–DSS–treated BALB/c and SCID mice (groups 2 and 5, Table 1, Fig. 1c) it was decreased drastically to grade 0·1 ± 0·2. Treatment with *C. tyrobutyricum* attenuated the loss of mucin due to DSS exposure in both BALB/c mice (group 1 grade 2·7 ± 0·4) and SCID mice (group 4 grade

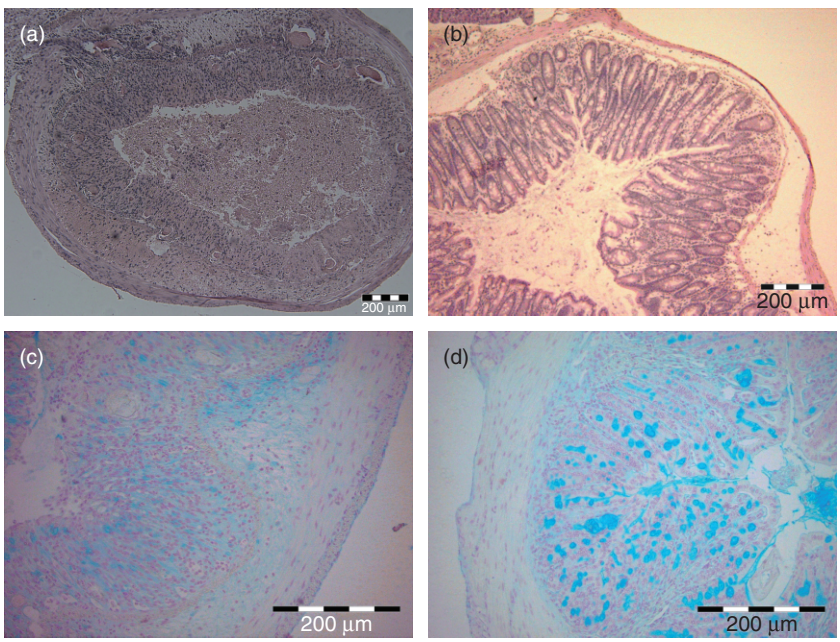

Fig. 1. Histological cross-sectional views of colon descendens of severe combined immunodeficiency (SCID) mice in experimental model of acute ulcerative colitis: (a) saline–dextran sodium sulphate (DSS)-treated mice (damage grade 4); (b) *C. tyrobutyricum*–DSS-treated mice (damage grade 0–1) (haematoxylin and eosin staining); (c) saline–DSS-treated mice, mucin production was decreased to grade 0; (d) *C. tyrobutyricum*–DSS-treated mice, grades 3–4, mucin production was preserved (Alcian blue staining).

3·5 ± 0·5, Table 1, Fig. 1d), with protection being more evident in the SCID mice.

Moreover, changes in the production of the major secreted mucin MUC-2 was verified in SCID mice using immunocytochemistry (Fig. 2). In the saline-treated SCID mice (Fig. 2a) and BALB/c mice (Fig. 2d), the production of MUC-2 was intact. In the saline–DSS-treated SCID (Fig. 2b) and BALB/c mice (Fig. 2e) the production of colonic MUC-2 was depressed, whereas in *C. tyrobutyricum*–DSS-treated SCID (Fig. 2c) and BALB/c mice (Fig. 2f) MUC-2 secretion was preserved.

Expression of TJ protein ZO-1

Expression of TJ protein ZO-1 is presented in Fig. 2. In the saline-treated SCID (Fig. 2g) and BALB/c mice (Fig. 2j) ZO-1 production was intact. In saline–DSS-treated SCID (Fig. 2h) and BALB/c mice (Fig. 2k) the production of ZO-1 was markedly reduced, whereas in *C. tyrobutyricum*–DSS-treated SCID (Fig. 2i) and BALB/c mice (Fig. 2l) the production of ZO-1 was preserved.

Mucosal infiltration of CD11b-positive immune cells

Monoclonal antibody to CD11b, a membrane marker of polymorphonuclear leucocytes, monocytes and natural killer cells, was used (Fig. 3). Saline-treated SCID (Fig. 3a) and BALB/c mice (Fig. 3d) were without infiltration of immune cells. Massive infiltration of inflammatory cells in lamina propria occurred in saline–DSS-treated SCID (Fig. 3b) and BALB/c mice (Fig. 3e), while the colon of *C. tyrobutyricum*–DSS-treated SCID mice (Fig. 3c) and BALB/c mice (Fig. 3f) did not exhibit infiltration of these cells.

Release of proinflammtory cytokine TNF-α in colon organ cultures

Spontaneous release of TNF-α into the medium of cultured sections from colon descendens of control mice was very low (groups 3 and 6, Table 1) after 48 h. Colon segments from saline–DSS-treated mice, and especially the SCID mice, released markedly higher amounts of TNF-α (groups 2 and 5, Table 1) than the controls. Compared to the saline–DSS-treated SCID mice (group 5, Table 1), a significantly lower level of TNF-α was detected in the cultured colon segments from *C. tyrobutyricum*–DSS-treated SCID mice (group 4, Table 1). However, the level of TNF-α released from the colon segments from *C. tyrobutyricum*–DSS-treated BALB/c mice (group 1, Table 1) did not differ from that of saline–DSS-treated BALB/c mice (group 2, Table 1).

Visualization and quantification of proinflammatory IL-18 in the descending colon

Table 2 shows that DSS-induced colitis in BALB/c mice is associated with increased level of cytokine IL-18 in colon epithelium. *C. tyrobutyricum* treatment reduced intracolonic IL-18 content significantly, although not to the level in non-inflamed mucosa. In SCID mice the severity of DSS colitis was not associated with IL-18 production. *C. tyrobutyricum* enhanced significantly the expression of IL-18 in colon epithelium of SCID mice. This might be an effect of butyrate on gene and protein production of IL-18.

C. tyrobutyricum-increased levels of propionic and butyric acids in SCID mice

More than 95% of the SCFA are absorbed and metabolized rapidly by the host [24]. To see any effect of *C. tyrobutyricum* on SCFA production, we determined the percentage of main short-chain fatty acids (acetic, propionic, isobutyric, n-butyric, isovaleric, 2-methylbutyric and valeric) in bacterial culture and in faecal samples obtained from *C.tyrobutyricum*–DSS-treated SCID mice and saline–DSS-treated SCID mice at the end of the experiment using gas chromatography (Table 3). Threefold higher percentage of n-butyric acid and twofold higher percentages of propionic acid were measured in *C. tyrobutyricum*–DSS-treated SCID mice than in the saline–DSS-treated mice. Conversely, a lower percentage of acetic acid was found in the *C. tyrobutyricum*–DSS-treated than in the saline–DSS-treated mice.

Discussion

In the present study, intrarectal administration of *C. tyrobutyricum* prior to the onset of experimental colitis protected both immunocompetent BALB/c and immunodeficient SCID mice from histological damage, shortening of the colon and decreased mucin production.

Both BALB/c and SCID mice display comparable symptoms of DSS-induced colitis as judged by the histological damage score, colon shortening and weight loss. However, 20% mortality occurred in the DSS-treated SCID mice suggesting that parameters such as loss of body weight cannot be used as a measure of the severity of inflammation in these two mouse strains. This conclusion is supported by several previous studies [25–27] not showing an apparent body weight loss before day 6 of acute DSS colitis in BALB/c mice. On the basis of the symptoms, the reduction of TJ protein ZO-1 production, depression of MUC-2 mucin production and infiltration of macrophages during inflammation, it appeared that the two mouse strains behave similarly on the qualitative level.

Decreased numbers of goblet cells and reduced levels of MUC-2 protein were measured in the inflamed colonic mucosa of DSS-treated BALB/c and SCID mice, a result similar to that observed in humans with active UC [28]. This is in agreement with other experimental models of inflammatory bowel diseases that demonstrate the critical role of MUC-2 in colonic protection [29]. Changes in mucins, particularly in MUC-2 mRNA, in experimental

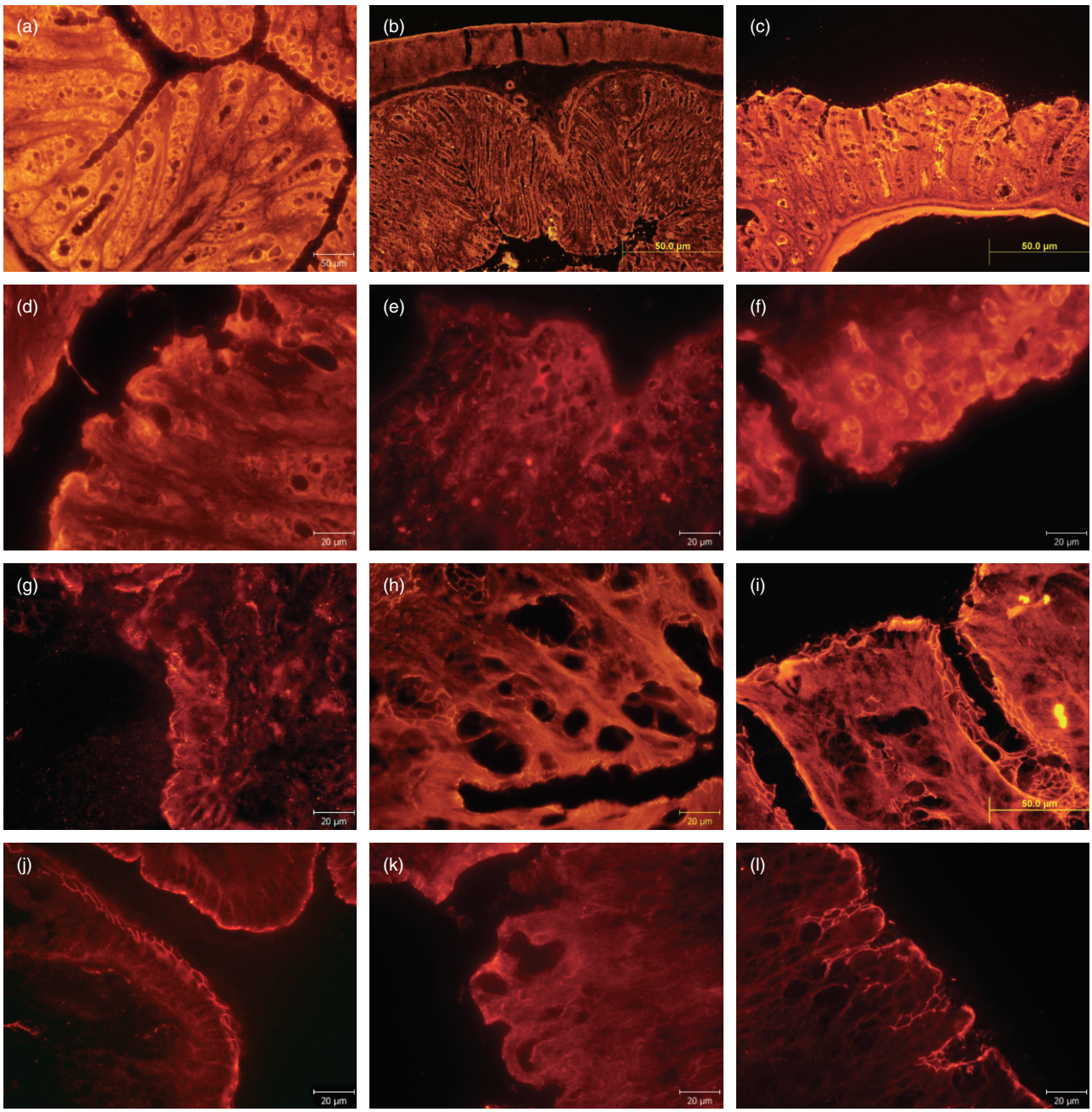

Fig. 2. Immunohistochemical evaluation of mucin (MUC)-2 detected with monoclonal antibody MUC-2/CY3 in colon descendens of severe combined immunodeficiency (SCID) and BALB/c mice in experimental model of acute ulcerative colitis: (a) saline-treated SCID mice with intact production of MUC-2; (b) saline–dextran sodium sulphate (DSS)-treated SCID mice with depressed production of MUC-2; (c) *C. tyrobutyricum*–DSS-treated SCID mice where MUC-2 secretion was preserved; (d) saline-treated BALB/c mice with intact production of MUC-2; (e) saline–DSS-treated BALB/c mice with depressed production of MUC-2; (f) *C. tyrobutyricum*–DSS-treated BALB/c mice MUC-2 secretion was preserved. Immunohistochemical evaluation of tight junction protein zonula occludens (ZO)-1 detected using monoclonal antibody ZO-1/CY3; (g) saline-treated SCID mice with intact production of ZO-1; (h) saline–DSS-treated SCID mice with markedly reduced ZO–1 production; (i) *C. tyrobutyricum*–DSS-treated SCID mice with preserved production of ZO-1; (j) saline-treated BALB/c mice with intact production of ZO-1, (k) saline–DSS-treated BALB/c mice with markedly reduced ZO-1 production; (l) *C. tyrobutyricum*–DSS-treated BALB/c mice with preserved production of ZO-1.

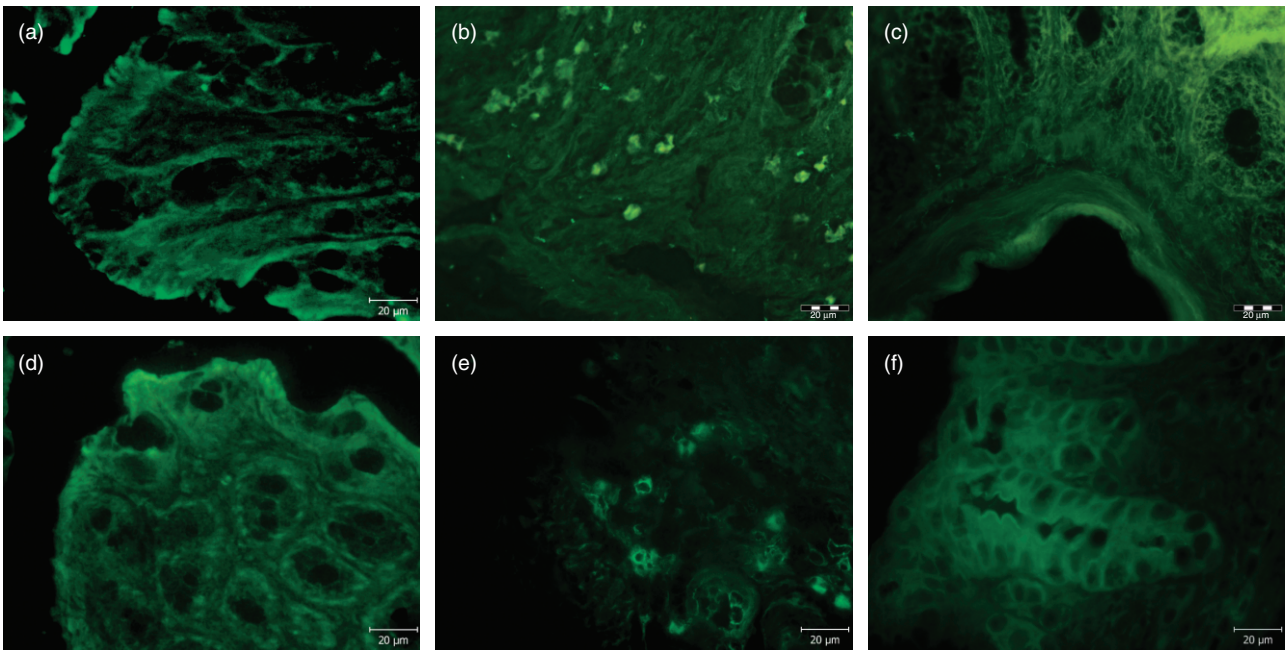

Fig. 3. Immunohistochemical detection of infiltrated proinflammatory cells using fluorescein isothiocyanate (FITC)-labelled monoclonal antibody to CD11b in colon-descendens mucosa of mice: (a) saline-treated severe combined immunodeficiency (SCID) mice without infiltration of immune cells; (b) saline–dextran sodium sulphate (DSS)-treated SCID mice with massive infiltration of proinflammatory cells; (c) *C. tyrobutyricum*–DSS-treated SCID mice, where infiltration of immune cells was not observed; (d) saline-treated BALB/c mice without infiltration of immune cells; (e) saline–DSS-treated BALB/c mice with massive infiltration of proinflammatory cells; (f) *C. tyrobutyricum*–DSS-treated BALB/c mice, where infiltration of immune cells was not observed.

models are considered to contribute to the development of ulcerative colitis [10,30,31]. Recently it has been observed that DSS in drinking water rapidly affects the biophysical structure of the inner mucus layer, making it permeable to bacteria within 12 h [7]. Thus the increased contact of bacteria with the epithelium is probably the trigger for the inflammatory reactions observed in colitis and would explain why DSS does not induce colitis in germ-free mice. Similarly, loss of TJ protein ZO-1 in the intestinal epithelium, as observed in this study, was interpreted as an early event in DSS-induced colitis and is associated with increased permeability and intestinal inflammation [32].

Crucially important in DSS-colitis is an activation of T lymphocytes via macrophages that have been activated directly by DSS [8]. Indeed, direct evidence for the involvement of $CD4^+$ T cells and their proliferation in pathogenesis of DSS-induced colitis has been described previously [33]. Moreover, the introduction of bacterial flagellin-specific $CD4^+$ T cells into naive SCID mice (T and B cell-independent) results in severe colitis [34]. Similarly, transfer of $CD4^+CD45RB^{high}$ T cell subpopulation from conventional mice into SCID mice also induces severe inflammation [5].

A major difference between BALB/c and SCID mice in development of DSS-colitis relates to the expression of the proinflammatory cytokine IL-18. In agreement with other

Table 3. Production of short-chain fatty acids determined as relative concentrations (%) in the bacterial broth of *C. tyrobutyricum* and in the faeces (taken at the end of the experiments). *Clostridium tyrobutyricum* dextran sodium sulphate (DSS)-treated and saline DSS-treated severe combined immunodeficiency (SCID) mice.

Acids	*C. tyrobutyricum* (bacterial culture)	*C. tyrobutyricum* (i.r.) (DSS-treated)	Saline (i.r.) (DSS-treated)
Acetic	62·5	59·2	81·4
Propionic	17·9	18·0	7·8
Isobutyric	10·6	9·2	4·8
n-Butyric	9·0	13·6	2·8
Isovaleric	n.d.	n.d.	2·1
2-Methylbutyric	n.d.	n.d.	0·6
Valeric	n.d.	n.d.	0·5

i.r.: intrarectal administration; n.d.: not detected.

published studies [35], we showed significantly increased IL-18 colon content in DSS-induced colitis in BALB/c mice. In BALB/c and C57BL/6 mice DSS-induced colitis induces increased expression of IL-18 in the colonic mucosa, where it polarizes $CD4^+$ T cells toward T helper cell type 1 (Th1)-mediated immune response [35] and thus increased production of the proinflammatory cytokine interferon (IFN)-γ. IFN-γ stimulated secretion of IL-18 from enterocyte-like IEC-6 cells has been correlated with IFN-γ-increased expression of caspase-1 activity [36], an enzyme required for cleavage of the precursor form of IL-18 into mature biologically active IL-18. Indeed, inhibition of caspase-1 by the specific inhibitor pralnacasan [26] attenuated DSS-induced colitis, this effect being mediated by suppression of proinflamatory IL-18 and IFN-γ.

Recent observations highlight the role of Nod-like receptors (NLRs) when stimulated by inflammatory mediators to form inflammasomes, multi-protein complexes that serve for activation of caspase-1 essential for maturation and secretion of IL-18. The highest levels of IL-18 were localized in intestinal epithelial cells, then macrophages and dendritic cells of the lamina propria. Interestingly, deletion of macrophages or use of neutralizing antibodies to IL-18 or inhibition of caspase-1 [37] result in prevention of the inflammatory cascade leading to sustained infiltration of macrophages, neutrophils and activation of lamina propria effector T cells. Thus, it seems that inflammasome activation [38] would have a proinflammatory effect, with IL-18 inducing inflammation in lamina propria mononuclear cells. Conversely, in intestinal epithelial cells the inflammasome would have a compensatory proliferative response, the secreted IL-18 mediating protection, proliferation and cell integrity [27,39,40]. This study shows for the first time that in SCID mice (a T and B cell-independent model) the severity of colitis was associated with limited production of biologically active form of IL-18. It seems that only constitutively expressed precursor form of IL-18 is associated with normal and inflamed SCID mouse colon. This finding led us to the assumption that SCID mice lack some components of inflammasome, pro-IL-18 could not be activated, and display an increased susceptibility to DSS-induced colitis associated with increased lethality, especially in the chronic phase of inflammation [6]. In SCID mouse colon we detected increased surface antigen CD11b, associated with an increased number of infiltrating immune cells, possibly macrophages [4]. Lack of IL-18 secretion is compensated by increased secretion of inflammatory TNF-α from the colon found in organ cultures. In comparison with UC patients it was suggested that macrophages migrating into the inflamed mucosa [41,42] secrete high levels of TNF-α. We observed only moderate expression of TNF-α in BALB/c mice with induced inflammation which was not commensurate with the marked infiltration of CD11b-positive polymorphonuclear leucocytes and monocytes into the mucosa. These mice represent a model of Th1-mediated immune response that contributes to the production of IL-18 and IFN-γ.

Studies of UC in humans have shown a lower availability and diminished capacity to oxidize butyrate [12,43], an energy source for colonocytes and end-product of the fermentation of undigested fibre and complex carbohydrates by the luminal microbiota. Similarly, a decrease in butyrate oxidation was found in the colonocytes of mice with DSS-induced colitis. Recent studies on faecal microflora of UC and IBS (irritable bowel syndrome) patients showed depletion of members of *Bacteroidetes* and *Firmicutes* (comprising some *Clostridium* groups) [44]. In healthy mice synergistic interactions between specific members of these phyla are linked to butyrate formation [45]. Thus, increased production of butyrate in the lumen of the colon was proposed as a treatment to ameliorate the symptoms of UC [46]. However, butyrate enemas in UC patients in remission were shown recently to have only mild effects on inflammation and anti-oxidant status in the colonic mucosa [47]. In our study we expected a higher faecal concentration of butyrate after intrarectal administration of *C. tyrobutyricum* and protection from DSS-induced colitis. In both BALB/c and SCID mice, intrarectal administration of *C. tyrobutyricum* prevented the reduction of MUC-2 protein observed in DSS-induced colitis and led to an almost normal level of MUC-2 secretion, most probably to the reported stimulation of MUC-2 gene expression in mouse colon [12]. We further showed that *C. tyrobutyricum* protects against impairment of the TJs in the colon of BALB/c and SCID mice by preventing dissolution of ZO-1 from the TJ in DSS-treated mice. Bacterial Toll-like receptor (TLR)-2 ligands have also been reported to increase ZO-1 expression and its localization in the TJs [15,48], and cannot be ruled out as a mechanism for the effects of *C. tyrobutyricum* on ZO-1.

Several *in vitro* and *ex vivo* studies reviewed by Hamer *et al.* [20] assessed the effect of butyrate which, at low concentrations, induces a decrease in permeability associated with increased expression of TJ proteins. However, overproduction of butyrate might be toxic for maturation of the intestine in premature infants and also in newborn rats [49].

TNF-α at concentrations found in inflamed mucosa may reduce oxidation of butyrate and decrease energy supply to colonocytes, as shown *in vitro* in the mucosa of human colonic biopsies [50]. As intrarectal administration of *C. tyrobutyricum* increased production of butyrate in the colon lumen it could overcome any insufficiencies resulting from the increased production of TNF-α. *C. tyrobutyricum* can be considered as a candidate human probiotic due to its beneficial effects in mouse colitis model. In BALB/c mice the expression of TNF-α in inflamed colon was lower in comparison with TNF-α production in the inflamed colon of SCID mice. Additionally, we found that treatment with *C. tyrobutyricum* had no effect on regulation of TNF-α production in BALB/c, but a strongly attenuating effect on TNF-α production in SCID mice.

Our work on IL-18 expression in DSS-induced colitis and its regulation by *C. tyrobutyricum* helped to differentiate between distinct responsiveness of BALB/c and SCID mice. Remarkably, *C. tyrobutyricum* treatment significantly reduced intracolonic IL-18 protein content in the inflamed mucosa of BALB/c mice, although not down to the level in non-inflamed mucosa. In contrast to BALB/c mice, *C. tyrobutyricum* enhanced significantly the expression of IL-18 in colon epithelium of SCID mice which lacked inflammation-associated expression of IL-18. This could be due to the fact that butyrate enhances gene expression and protein production of IL-18 in epithelial cells (HT-29 and Caco-2) *in vitro*, and *in vivo* in butyrate-treated mice [51]. This enhanced effect of butyrate at the transcription level seems to be hidden in BALB/c mice expressing increased IL-18 production in DSS-treatment.

This study demonstrates that in the DSS model, the severity of inflammatory symptoms depends largely but not exclusively on host immune functions. Thus, *C. tyrobutyricum* protection against destruction of mucosal barrier is equally effective in immunodeficient SCID mice and immunocompetent BALB/c mice. Manifestation of cytokines IL-18 and TNF-α in acute DSS-colitis depends largely on immune cell repertoire of the host mouse. As a typical product of macrophages, TNF-α expression increased significantly in the colon epithelium in SCID mice, while mature IL-18, a Th1 cytokine, important for systemic balance between Th1 and Th2 signalling [52], played a key role in immunocompetent BALB/c mice. The combined effect of *C. tyrobutyricum* in suppressing high levels of both cytokines appears promising in treatment of acute experimental colitis.

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Disclosure

The authors declare that they have no conflict of interest related to the publication of this manuscript.

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6. NEONATAL COLONIZATION OF GERM-FREE MICE WITH *BIFIDOBACTERIUM LONGUM* PREVENTS ALLERGIC SENSITIZATION TO MAJOR BIRCH POLLEN ALLERGEN BET V 1

Schwarzer M, Šrůtková D, Schabussová I, Hudcovic T, Akgün J, Wiedermann U, Kozáková H.

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Neonatal colonization of germ-free mice with *Bifidobacterium longum* prevents allergic sensitization to major birch pollen allergen Bet v 1

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ABSTRACT

The main goal in reversing the allergy epidemic is the development of effective prophylactic strategies. We investigated the prophylactic effect of neonatal mother-to-offspring mono-colonization with *Bifidobacterium longum* ssp. *longum* CCM 7952 on subsequent allergic sensitization. Adult male and female germ-free (GF) mice were mono-colonized with *B. longum*, mated and their offspring, as well as age-matched GF controls, were sensitized with the major birch pollen allergen Bet v 1. Furthermore, signaling pathways involved in the recognition of *B. longum* were investigated *in vitro*. Neonatal mono-colonization of GF mice with *B. longum* suppressed Bet v 1-specific IgE-dependent β-hexosaminidase release as well as levels of total IgE and allergen-specific IgG2a in serum compared to sensitized GF controls. Accordingly, Bet v 1-induced production of both Th1- and Th2-associated cytokines in spleen cell cultures was significantly reduced in these mice. The general suppression of Bet v 1-specific immune responses in *B. longum*-colonized mice was associated with increased levels of regulatory cytokines IL-10 and TGF-β in serum. *In vitro*, *B. longum* induced low maturation status of bone marrow-derived dendritic cells and production of IL-10 in TLR2-, MyD88-, and MAPK-dependent manner. Our data demonstrate that neonatal mono-colonization with *B. longum* reduces allergic sensitization, likely by activation of regulatory responses *via* TLR2, MyD88, and MAPK signaling pathways. Thus, *B. longum* might be a promising candidate for perinatal intervention strategies against the onset of allergic diseases in humans.

1. Introduction

There has been a remarkable increase in allergic diseases over the past few decades, especially in Western countries [1]. Accumulating evidence suggests that environmental changes, rather than genetic factors, are driving the epidemic character of allergy, and events acting within a narrow window of opportunity, either prenatally or early in life, might have major physiological effects [2,3].

The germ-free status of fetus changes rapidly after birth and the composition of colonizing microbiota can be influenced by multiple factors such as the mode of delivery, dietary changes, high hygiene or over-use of antibiotics [4]. The exposure to microbial stimuli is crucial for the development, maturation and function of the immune system and association between intestinal dysbiosis and allergic disease has been proposed [5]. Several prospective studies have shown lower neonatal colonization by bifidobacteria and lactobacilli species accompanied by higher counts of *Clostridium difficile* in neonates who developed allergy later in life [6–8].

Due to the ability of bifidobacteria and lactobacilli to interact with the host immune system and to modulate host immune responses, they have been used with some success in prevention or treatment of allergic disease in infants at risk [9]. Several randomized clinical trials have shown that combined prenatal/postnatal probiotic interventions reduced the cumulative incidence of eczema while less beneficial effects were found in trials using exclusively postnatal treatment approaches (recently reviewed in [10]). These data highlight the importance of the correct timing of probiotic interventions, for which the prenatal period seems to be a significant component.

Dendritic cells (DC) are pivotal in early bacterial recognition through engagement of TLR and C-type lectins, which leads to induction of distinct innate responses that shape the type of T helper cell responses [11,12]. In this respect, Konieczna et al. recently showed that preconditioning of DC with probiotic *Bifidobacterium infantis* led to induction of Foxp3 positive regulatory T (Treg) cells with enhanced IL-10 production [13]. Due to the fact

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that allergic diseases have been associated with a deficiency in Treg cells numbers and/or function [14], specific probiotic strains inducing regulatory immune responses might be beneficial in the prevention of allergic disorders. Indeed, we have previously shown that perinatal administration of *Lactobacillus paracasei* to pregnant/lactating mice protected against the development of airway inflammation in offspring by activating regulatory pathways [15]. Similarly, neonatal application of *Lactobacillus rhamnosus* GG or *Bifidobacterium lactis* Bb-12 suppressed allergic sensitization and airway inflammation in mice by induction of Treg cells associated with increased levels of TGF-β [16].

Germ-free (GF) animals represent a powerful model to study the interaction of a single bacterial strain or a defined mixture of microbial strains with the host immune system [17]. Taking the advantage of this model we have previously shown that neonatal mono-colonization of GF mice with the recombinant bacterial strain *Lactobacillus plantarum* producing major birch pollen allergen Bet v 1 reduced the development of allergic responses upon systemic sensitization with the same allergen [18].

Bifidobacterium longum subspecies *longum* CCM 7952 (*B. longum*) is a commensal bacterial strain originally isolated from the feces of a healthy breast fed infant. This strain has been shown to induce regulatory responses *in vitro* and to suppress the inflammatory responses in a mouse model of experimental colitis (Srutkova, unpublished results). In the present study we investigated whether neonatal mother-to-offspring mono-colonization of GF mice with *B. longum* protects the offspring against allergic sensitization to allergen Bet v 1 in a model of type I allergy.

2. Materials and methods

2.1. Animals

Germ-free BALB/c mice were kept under sterile conditions and were supplied with water and sterile pellet diet ST1 (Bergman, Czech Republic) *ad libitum*. Fecal samples were weekly controlled for microbial contamination as previously described [19]. Wild-type (WT) and TLR2$^{-/-}$, TLR4$^{-/-}$, and MyD88$^{-/-}$ mice on a C57BL/6 background, obtained from Prof. M. Müller (Vienna, Austria), were kept under SPF conditions. All animal experiments were approved by the local ethics committee.

2.2. Bacterial strain

Probiotic strain *B. longum* ssp. *longum* CCM 7952 (*B. longum*), originally isolated from the feces of a breast-fed healthy child, was provided by Culture Collection of Dairy Microorganisms (Milcom, Czech Republic). *B. longum* cultures were grown anaerobically in MRS medium (Oxoid, UK) supplemented with L-cysteine hydrochloride (0.5 g/l) for 48 h at 37 °C. For *in vitro* experiments *B. longum* was inactivated with 1% formaldehyde-PBS as described before [20].

2.3. Colonization and experimental design

Eight-week-old GF male and female mice were colonized with a single dose (2×10^8 CFU) of freshly grown *B. longum* in 200 μl of sterile PBS by intragastric administration and mated 20 days later. Stability of colonization was checked by plating of feces on MRS agar supplemented with L-cysteine hydrochloride (0.5 g/l) and CFU were counted after anaerobic cultivation for 48 h at 37 °C. Female offspring, neonatally colonized with *B. longum*, and control GF mice were subcutaneously (s.c.) sensitized on days 1, 14 and 28 with 1 μg of recombinant Bet v 1 (Biomay, Austria) emulsified in 100 μl of Al(OH)$_3$ (Serva, Germany). Seven days after the last immunization, mice were killed by CO_2 asphyxia and samples were taken for further analysis (Fig. 1A).

2.4. Humoral immune responses

Blood samples were taken at sacrifice and serum levels of anti-Bet v 1 IgE, IgG1 and IgG2a were measured by ELISA as previously described [18]. The activity of Bet v 1-specific IgE in serum was measured by rat basophile leukemia cells degranulation assay as described previously [20]. Degranulation was tested by the release of β-hexosaminidase. Levels of total IgE and IgA in serum (dilution 1:10 and 1:400, respectively) were measured by a commercial ELISA kit as recommended by the manufacturers (Bethyl, USA). Levels of IL-10 and TGF-β in serum (final dilution 1:1000) were measured by ELISA Ready-Set-Go! kits (eBioscience, USA) according to manufacturer's instructions. Small intestine was excised and gut lavage was prepared as described previously [15]. Levels of total IgA and TGF-β in gut lavage were measured as described above with final dilution 1:2500 and 1:1000, respectively and reported as absolute units.

2.5. Cellular immune responses

Spleen and mesenteric lymph node (MLN) single cell suspensions from *B. longum*-colonized and control mice were prepared and cultured as previously described [15]. Mononuclear cells (3×10^6 cells/ml) were stimulated with Bet v 1 (20 μg/ml) or media alone in 96-well plates at 37 °C for 60 h in culture medium (RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin). Levels of cytokines in culture supernatants were measured by the MILLIPLEX MAP Mouse Cytokine/Chemokine Panel (Millipore, USA) according to manufacturer's instructions and analyzed with the Bio-Plex System (Bio-Rad Laboratories, USA). Values are expressed as pg/ml after subtraction of baseline levels of unstimulated cultures. Proliferative responses of spleen cell cultures with/without Bet v 1 restimulation were determined by scintillation counting after addition of ^{3}H-Thymidine (0.5 μCi/well; Lacomed, Czech Republic) for the last 16 h of 76 h cultivation. Values are shown as stimulation index.

2.6. Preparation and activation of bone marrow-derived DC

Mouse bone marrow-derived DC (BM-DC) were prepared as previously described [15]. Briefly, the bone marrow precursors were isolated from femurs and tibias of mice. Cells were cultured at 4×10^5/ml in bacteriological Petri dishes in 10 ml culture medium with GM-CSF (20 ng/ml; Sigma–Aldrich, USA). Fresh medium was added at days 3 and 6 and BM-DC were used on day 8 of culture. BM-DC (10^6 cells/ml) were stimulated with 10^6 or 10^7 CFU of inactivated *B. longum* for 18 h. As controls, BM-DC were incubated with Pam3Cys (Pam3CSK4, 1 μg/ml, InvivoGen, USA) or ultrapure LPS (LPS-EB, 1 μg/ml, InvivoGen, USA). Levels of IL-10, TGF-β, and IL-6 in culture supernatants were determined by ELISA Ready-Set-Go! kits (eBioscience, USA) according to manufacturer's instructions. Levels of IL-12p70 were measured with matched antibody pairs (BD Pharmingen, USA). Where indicated, BM-DC were pretreated with mitogen-activated protein kinase (MAPK) specific inhibitors (MEK: PD98059; p38: SB203580; JNK: SP600125) or NF-κB (BAY 11-7082) at 10 μM dissolved in DMSO or DMSO only for 1 h at 37 °C.

2.7. Flow-cytometry analysis

Single cell suspensions of spleens or MLN were stained for regulatory T cells with Foxp3 Staining Buffer Set (eBioscience, USA) as

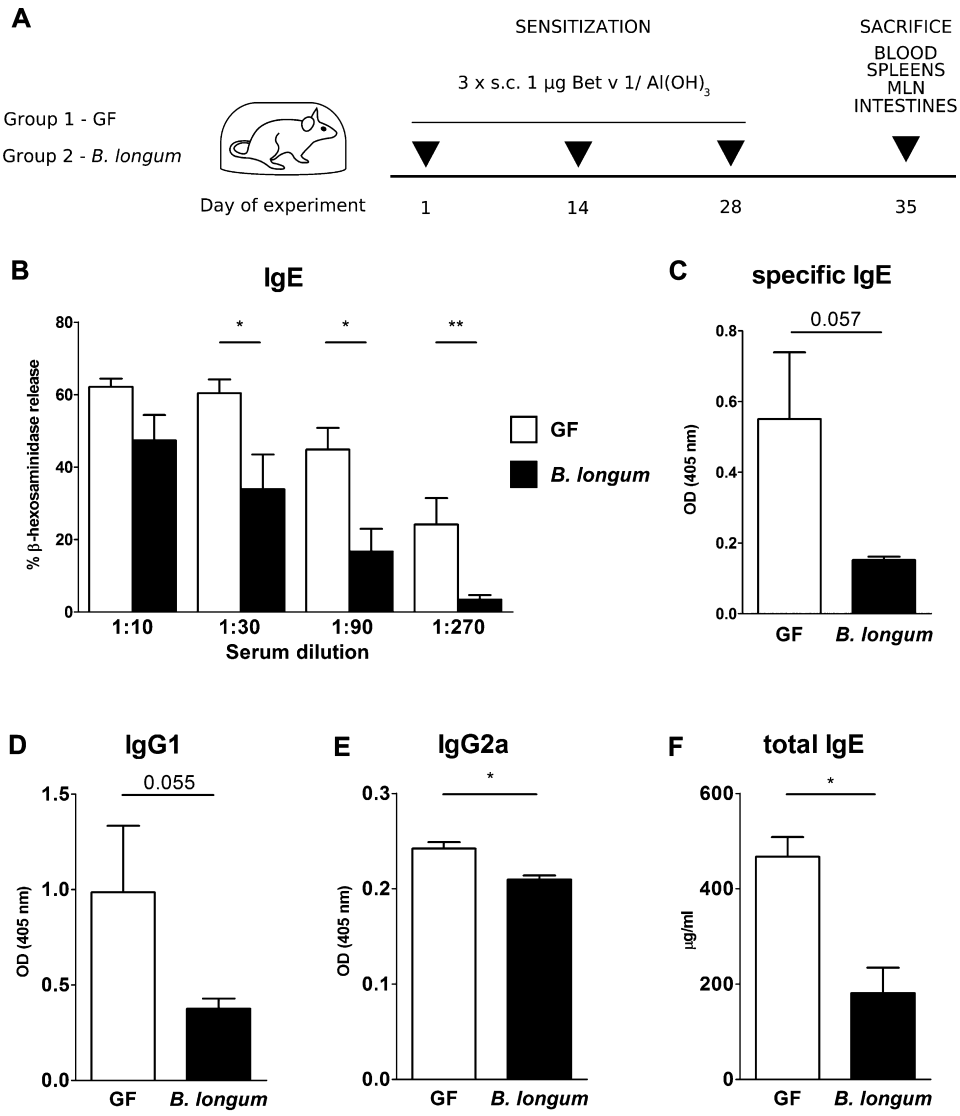

Fig. 1. Systemic sensitization of GF and *B. longum* neonatally colonized mice to Bet v 1. Experimental design: eight-week old germ-free mice (GF; white bars) and age-matched mice neonatally colonized with *B. longum* (*B. longum*; black bars) were subcutaneously (s.c.) sensitized with 1 µg of recombinant Bet v 1 in Alum (Bet v 1/Al(OH)$_3$) on days 1, 14 and 28. Mice were sacrificed one week after the last immunization and blood, spleens, mesenteric lymph nodes and small intestines were collected for further analysis (A). Functional IgE in diluted serum (1:10, 1:30, 1:90, 1:270) was measured by Bet v 1-mediated β-hexosaminidase release from rat basophil leukemia cells (B). Levels of Bet v 1-specific IgE (C), IgG1 (D) and IgG2a (E), as well as levels of total IgE (E) in serum were measured by ELISA. Results are representative of two repeat experiments, each with four to six mice per group, and are expressed as mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$.

described previously [21]. DC were labeled with monoclonal antibodies for CD11c (FITC), MHCII (APC), CD40, CD80 or CD86 (PE) (eBioscience, USA). Appropriate isotype antibodies were used as controls to determine non-specific binding. Cells were analyzed using FACSCalibur flow cytometer (Becton-Dickinson, USA) and obtained data were analyzed with FlowJo 7.6.2 software (TreeStar, USA).

2.8. Stimulation of Human embryonic kidney (HEK) 293 cells stably transfected with TLR

HEK293 cells stably transfected with plasmid carrying human (h)TLR2 gene were kindly provided by M. Yazdanbakhsh (Leiden, Netherlands) and cells transfected with hTLR4/MD2/CD14 were a gift of B. Bohle (Vienna, Austria). Cells were stimulated with formalin-inactivated *B. longum* (10^6, 10^7, or 10^8 CFU/ml). TLR2 ligand Pam3Cys (1 µg/ml) and TLR4 ligand LPS (1 µg/ml) were used as positive controls. After the 20-h incubation period, culture supernatants were harvested and concentration of human IL-8 was analyzed by ELISA (Thermo Scientific, USA) according to the manufacturer's instructions.

2.9. Statistical analysis

Data are expressed as means ± SEM. Statistical analysis was performed by non-parametric Mann–Whitney *U*-test using GraphPad Software (GraphPad Prism 5.04, San Diego, USA); p values < 0.05 were considered significant.

3. Results

3.1. Neonatal mono-colonization with B. longum prevents systemic sensitization to Bet v 1

Colonization of mice with *B. longum* remained stable throughout the experiments and reached the level of approximately 5×10^9 CFU/g of feces (data not shown). Sensitization with Bet v

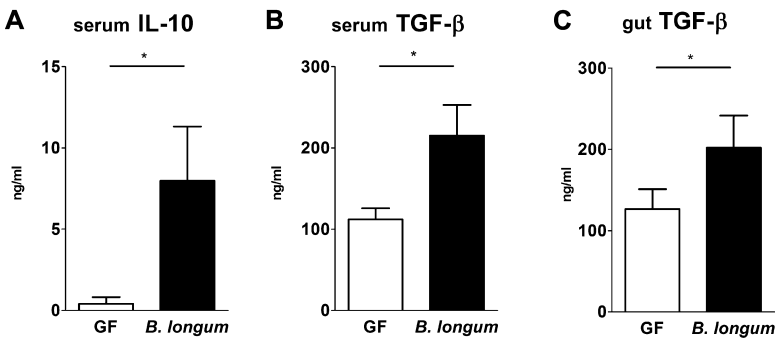

Fig. 2. Influence of *B. longum* neonatal colonization on levels of IL-10 and TGF-β in sera and gut lavage. Levels of IL-10 (A) and TGF-β (B) in sera and TGF-β (C) in gut lavage were measured by ELISA. Germ-free mice (GF, white bars) and *B. longum*-colonized mice (*B. longum*, black bars). Pooled values of two repeat experiments, each with four to six mice per group, are expressed as mean ± SEM. $^{*}p < 0.05$.

1 induced high levels of allergen-specific antibodies in serum of GF mice. Neonatal mono-colonization with *B. longum* prevented the development of allergic sensitization as the production of Bet v 1-specific Th2-related IgE and IgG1 as well as Th1-related IgG2a antibodies in serum were reduced in these mice compared to GF controls (Fig. 1C, D, and E). Furthermore, *B. longum* reduced the IgE-dependent and Bet v 1-driven basophil degranulation (Fig. 1B) as well as levels of total IgE in serum (Fig. 1F). Concomitantly, *B. longum* colonization triggered increased production of IL-10 and TGF-β in serum (Fig. 2A and B). Increased levels of TGF-β were also measured in gut lavage of *B. longum*-colonized mice (Fig. 2C). Surprisingly, *B. longum* did not increase the production of total IgA, as levels of these antibodies in serum and gut lavage (data not shown) were comparable between *B. longum*-colonized mice and GF controls.

3.2. Neonatal mono-colonization with B. longum prevents the development of cellular allergic responses

Neonatal mono-colonization with *B. longum* reduced allergen-specific recall responses in spleen cell cultures *ex vivo*. Production of both Th2-related cytokines IL-5, IL-13 and IL-4 (Fig. 3A–C) and pro-inflammatory cytokines IFN-γ, IL-17 and TNF-α (Fig. 3E–G) as well as proliferative responses (Fig. 3H) were suppressed in *B. longum* mono-colonized mice compared with control GF mice. Interestingly, Bet v 1-induced levels of the regulatory cytokine IL-10 were also decreased by *B. longum* (Fig. 3D). In addition, *B. longum* suppressed Bet v 1-specific responses in MLN cell cultures (data not shown). This was accompanied by increase in population of $CD4^{+}Foxp3^{+}$ cells in spleen and MLN (Fig. 4A and B).

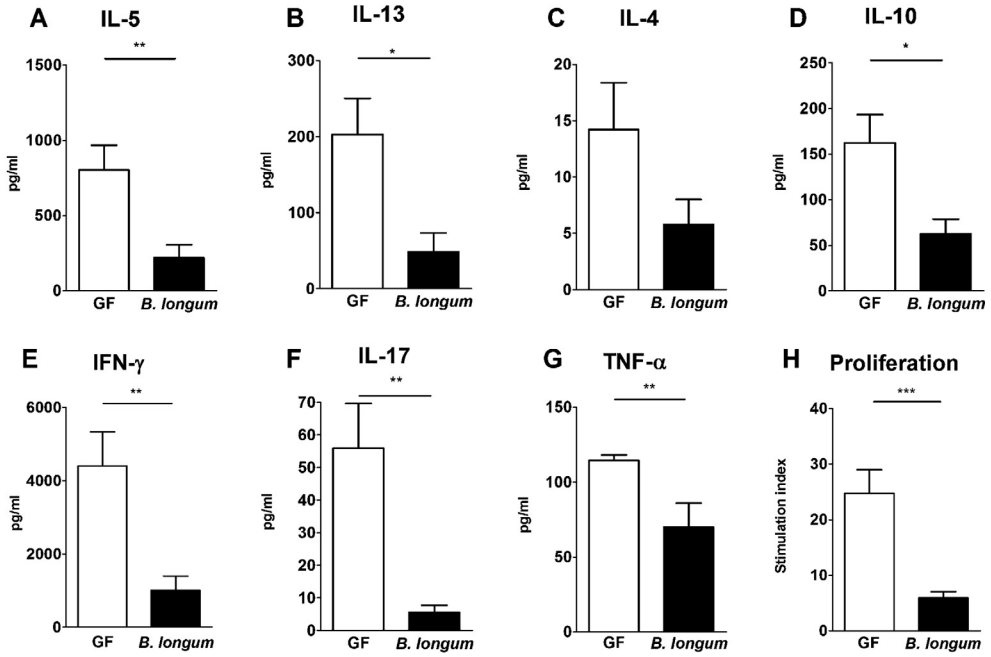

Fig. 3. Influence of *B. longum* neonatal colonization on Bet v 1-induced cytokine production and proliferative response in splenocytes. Spleen cells from germ-free mice (GF, white bars) and *B. longum*-colonized mice (*B. longum*, black bars) were cultured with 20 μg/ml of Bet v 1 for 60 h. Levels of IL-5 (A), IL-13 (B), IL-4 (C), IL-10 (D), IFN-γ (E), IL-17 (F) and TNF-α (G) in culture supernatants were measured by MILLIPLEX Cytokine panel. Cytokine levels are expressed after subtraction of base line levels of unstimulated splenocytes. Proliferative responses of splenic cells were assessed by incorporation of ^{3}H-Thymidine for the last 16 h of 76-h cultivation (H). Values are shown as stimulation index. Pooled values of two repeat experiments, each with four to six mice per group, are expressed as mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$.

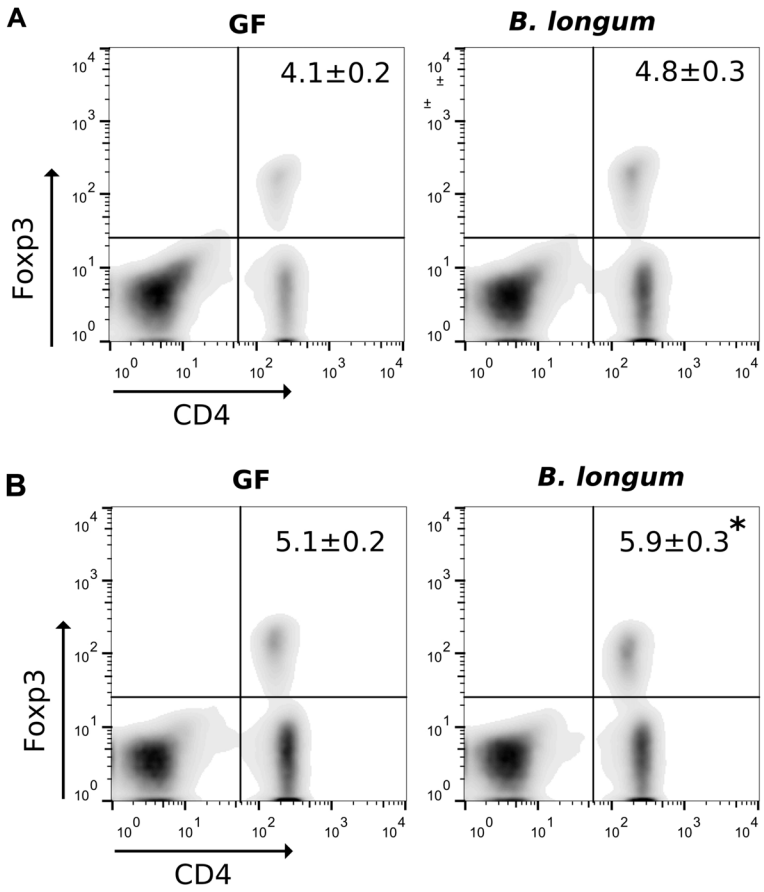

Fig. 4. Numbers of $CD4^+Foxp3^+$ cells in splenocytes and mesenteric lymph nodes cells. Typical plots depicting numbers of Treg cells in suspensions isolated from spleens (A) and mesenteric lymph nodes (B) of germ-free mice (GF) and *B. longum*-colonized mice (*B. longum*). Pooled values of two repeat experiments are shown, each with four to six mice per group. Percentage of $CD4^+Foxp3^+$ cells from all gated lymphocytes are expressed as mean ± SEM. $^*p < 0.05$.

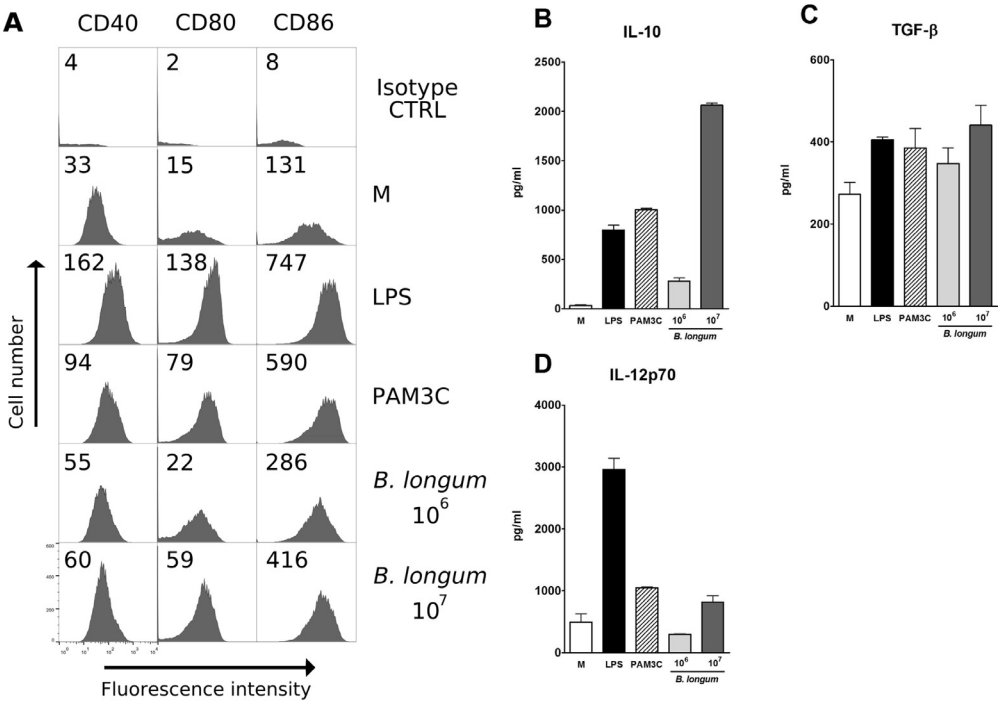

Fig. 5. Maturation of DC and cytokine production induced by *B. longum*. BM-DC cells from naive BALB/c mice were cultured with media alone (M), ultra-pure lipopolysaccharide from *E. coli* (LPS, 1 μg/ml), Pam3Cys (PAM3C, 1 μg/ml) or two different concentrations of formalin-inactivated *B. longum* (10^6 or 10^7 CFU/ml) for 18 h. BM-DC were gated as $MHCII^+CD11c^+$ and analyzed by flow cytometry for CD40, CD80 and CD86 expression. Isotype controls are shown in the first row (A). Numbers shown are median florescence units. One representative out of three experiments is shown. Production of IL-10 (B), TGF-β (C) and IL-12p70 (D) in culture supernatant was determined by ELISA. Mean ± SEM are shown. One representative out of three experiments is shown.

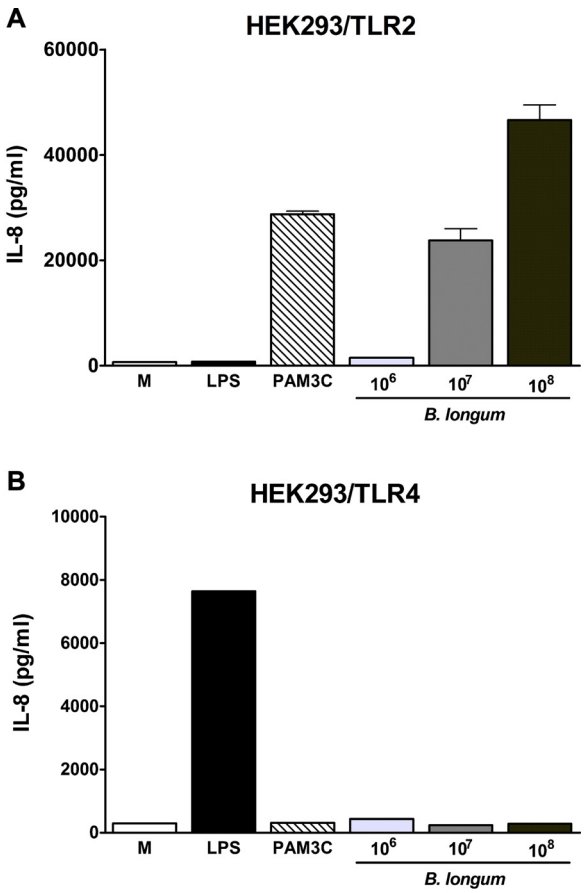

Fig. 6. Activation of TLR receptors by *B. longum*. HEK293 stably transfected with an expression vector for human TLR2 (293-hTLR2) (A) and TLR4 (293-hTLR4/MD2/CD14) (B) were cultured for 20 h with different concentrations of formalin-inactivated *B. longum* (10^6, 10^7, 10^8 CFU/ml) or left unstimulated. Ultrapure lipopolysaccharide from *E. coli* (LPS; 1 μg/ml) and Pam3Cys (PAM3C, 1 μg/ml) were used as positive controls for TLR4 and TLR2, respectively. Unstimulated cells (M) were used as control. Stimulation was evaluated by measurement of IL-8 production; results are expressed as mean ± SEM. One representative experiment out of three is shown.

3.3. *B. longum induces low maturation status of dendritic cells and production of regulatory cytokines*

Stimulation of BM-DC derived from WT BALB/c mice with *B. longum* induced dose dependent but only moderate maturation status (increased levels of CD40, CD80 and CD86) when compared with LPS (TLR4 ligand) or Pam3Cys (TLR2 ligand) (Fig. 5A), but high levels of IL-10 and TGF-β (Fig. 5B and C). High levels of IL-12p70 were detected in cultures stimulated with LPS but only low levels were measured after *B. longum* stimulation (Fig. 5D).

3.4. *TLR2 but not TLR4 is involved in recognition of B. longum*

In order to investigate the role of TLR in *B. longum* recognition, HEK293 stably transfected with TLR2 or TLR4 were stimulated with different concentrations of inactivated *B. longum*. Pam3Cys was used as positive control for TLR2 and LPS for TLR4. Stimulation of HEK293/TLR2 with *B. longum* led to production of IL-8 in dose dependent manner (Fig. 6A). There was no production of IL-8 after stimulation of HEK293/TLR4 with *B. longum* (Fig. 6B).

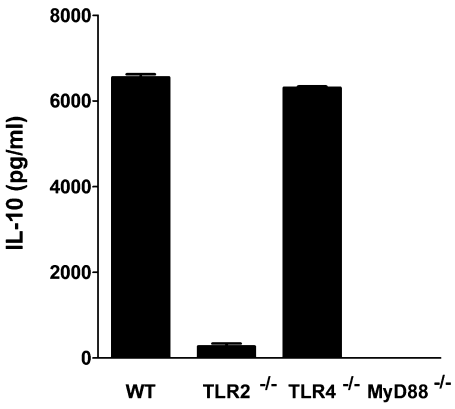

Fig. 7. Involvement of TLR in BM-DC IL-10 cytokine production induced by *B. longum*. BM-DC generated from wild-type (WT), TLR2-, TLR4- and MyD88-deficient mice with C57BL/6 background were cultured with formalin-inactivated *B. longum* (10^7 CFU/ml) for 18 h. Production of IL-10 was measured by ELISA. Mean ± SEM after subtractions of baseline levels from unstimulated cells are shown. One representative out of three experiments yielding similar results is shown.

3.5. *B. longum drives production of IL-10 in DC through TLR2 and MyD88*

Similarly as observed for BM-DC derived from WT BALB/c mice (Fig. 5B), stimulation of BM-DC derived from WT C57BL/6 mice with *B. longum* induced up-regulation of IL-10 production (Fig. 7). This effect was significantly reduced in BM-DC derived from TLR2- or MyD88-deficient mice. The lack of TLR4 had no impact on IL-10 production by *B. longum* (Fig. 7).

3.6. *Activation of MAPK and NF-κB in DC is critical for the production of IL-10 by B. longum*

In order to investigate the functional role of MEK 1, p38, JNK or NF-κB pathways in *B. longum*-triggered production of IL-10, specific inhibitors of the MEK 1 (PD98059), p38 (SB203580), JNK (SP600125) or NF-κB (BAY 11-7082) signaling pathways were used. As shown in Fig. 8, inhibitors of the MEK 1, p38, or NF-κB markedly decreased the production of IL-10 induced by *B. longum* or by TLR2 ligand Pam3Cys in BM-DC derived from WT BALB/c mice. Interestingly, involvement of JNK signaling pathway was important for induction of IL-10 by *B. longum* but not by Pam3Cys (Fig. 8).

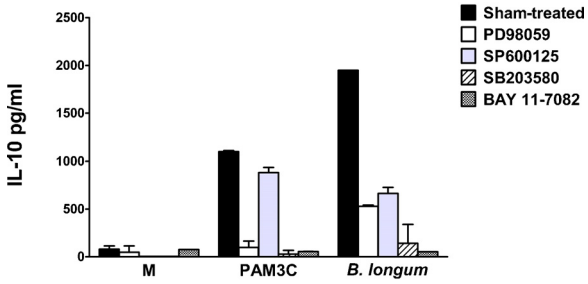

Fig. 8. Involvement of MAPK pathways and NF-κB in *B. longum*-induced production of IL-10. BM-DC from naive BALB/c mice were incubated with DMSO only (Sham-treated, black bars) or with MEK 1 (PD98059, white bars), JNK (SP600125, gray bars), p38 (SB203580, dashed bars) and NF-κB (BAY 11-7082, dotted bars) pathway inhibitors for 1 h prior to stimulation with formalin-inactivated *B. longum* (10^7 CFU/ml) or Pam3Cys (PAM3C, 1 μg/ml). Unstimulated cells (M) were used as controls. Levels of IL-10 were determined by ELISA. Results are expressed as mean ± SEM, one representative experiment out of two is shown.

4. Discussion

In the current study, we demonstrate that neonatal mother-to-offspring mono-colonization with *B. longum* CCM 7952 significantly reduced the development of allergen-specific immune responses in a gnotobiotic mouse model of type I allergy, which was associated with induction of regulatory milieu. Furthermore we show that TLR2, MyD88 and MAPK signaling pathways are crucial for the recognition of *B. longum* and induction of IL-10.

Clinical and experimental studies differ greatly regarding the outcome of probiotic interventions in prevention/treatment of allergic diseases (reviewed in [22]). However, most of them claim that the probiotic application in prenatal/perinatal period appears to be crucial for manifestation of beneficial effects, confirming the existence of a 'window of opportunity' in the programming of the well balanced immune system in the neonatal period [15,16,23].

In order to investigate the interaction between a single bacterial strain and the host immune system, as well as the impact of the specific strain on the 'reprograming' and maturation of the immune system, we established a mouse model of neonatal mother-to-offspring mono-colonization using GF mice. By that we previously showed that constitutive delivery of Bet v 1 by recombinant *L. plantarum*, but not the wild-type bacteria alone, was able to modulate subsequent systemic sensitization to Bet v 1 [18]. Here we show that mother-to-offspring mono-colonization of GF mice with *B. longum* decreased levels of Th2- as well as Th1-associated Bet v 1-specific antibodies in serum. These data suggest that the inhibition of sensitization was not achieved simply by immune shift toward the Th1 mode, but rather that general reduction of immune responses took place.

Consistent with the results observed in serum, *B. longum* also inhibited antigen-specific recall responses in spleen cells. The Bet v 1-induced production of both Th2 and Th1 cytokines, as well as antigen specific proliferation was dramatically reduced in mice colonized with *B. longum* in comparison to controls. These data are in agreement with our previous study where prophylactic mucosal application of *B. longum* NCC 3001 induced general suppression of allergen-specific cytokine production [24]. Similarly, *Bifidobacterium* has been shown to inhibit T-cell proliferation [25] and antigen-specific cytokine production [26] in OVA food allergy model. It is necessary to stress that although the reduction of humoral and cellular immune responses observed with *B. longum* is feasible in the context of sensitization to allergens, it remains important to evaluate whether the perinatal application of *B. longum* or other strains with immunosuppressive properties could interfere with immune responses against concomitant infections, vaccines or anti-tumor immunity.

We have shown that *B. longum* neonatally-colonized mice exhibited increased levels of $Foxp3^+$ cells in MLN and high levels of regulatory cytokines IL-10 and TGF-β in serum. This is in agreement with recent studies both in animals and in humans [13,25], where probiotic bacteria have been shown to drive the generation of Treg cells. These $Foxp3^+$ Treg cells have been shown to be protective in mouse allergy models [26] and they are indispensable for successful immunotherapy in humans [14]. Interestingly, it has been recently shown that bifidobacteria are able to induce another subset of $Foxp3^-$ type 1 regulatory cells (Tr1) that exert their immunoregulatory properties by production of IL-10 [27]. However, IL-10 and TGF-β are produced by a broad variety of cells [11], thus the precise source and role of these cytokines as well as Tregs in prevention of allergic sensitization by *B. longum* in our model remains to be investigated.

Next we have shown that stimulation of BM-DC with *B. longum* triggered production of IL-10 and TGF-β; however, the expression of surface markers was rather low. DC are viewed as the key players in routing the immune responses and inducing different T cell subsets [13,27]. In this respect immature DC and/or IL-10-producing DC have been regarded as tolerogenic [28] and they were shown to be able to induce Treg cells [13,25].

Our data demonstrate that TLR2 is important for recognition of *B. longum* and signaling, through this receptor leads to induction of IL-10. Interestingly, stimulation of antigen-presenting cells with TLR2 ligand inhibited allergen-specific Th2 responses in sensitized patients [29] and stimulation of TLR2 on DC was shown to enhance their ability to induce Treg cells [30]. These data suggest that TLR2 ligands derived from *B. longum* might have beneficial effects on allergen-specific responses, providing an alternative to viable probiotics. Along these lines, IL-10 inducing properties have been suggested for *B. longum* BCRC 14634 derived exopolysaccharides (EPS) [31]. Recently, we have shown that *B. longum* CCM 7952 also produces EPS which lead to production of IL-10 in TLR2-dependent manner (Górska-Frączek, Schwarzer, unpublished results) and we are currently investigating the role of *B. longum*-derived EPS in allergy prevention/therapy.

Since TLR2 has been shown to activate MAPK and NF-κB [32], we further investigated the role of these signaling pathways in IL-10 production induced by *B. longum*. Similar to the synthetic TLR2 ligand Pam3Cys, *B. longum*-induced IL-10 is critically dependent on NF-κB activation and involves p38 and MEK pathways. In contrast, JNK signaling pathway was indispensable only for *B. longum*-induced, but not for Pam3Cys-induced IL-10 production.

Taken together, our data clearly show that mono-colonization with *B. longum* prevents the development of allergic sensitization to Bet v 1 in a mouse model of clinically relevant birch pollen allergy. This probiotic strain likely induces regulatory responses through TLR2/MyD88 signaling pathway. Identification and characterization of *B. longum* derived TLR2 ligands may provide a tool to investigate the molecular interaction between components of innate immunity and allergic-effector cells, leading to novel strategies to prevent/treat allergic diseases in humans.

Acknowledgements

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7. COLONIZATION OF GERM-FREE MICE WITH THE MIXTURE OF THREE *LACTOBACILLUS* STRAINS ENHANCES THE INTEGRITY OF GUT MUCOSA AND AMELIORATES ALLERGIC SENSITIZATION

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RESEARCH ARTICLE

Colonization of germ-free mice with a mixture of three *lactobacillus* strains enhances the integrity of gut mucosa and ameliorates allergic sensitization

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Increasing numbers of clinical trials and animal experiments have shown that probiotic bacteria are promising tools for allergy prevention. Here, we analyzed the immunomodulatory properties of three selected lactobacillus strains and the impact of their mixture on allergic sensitization to Bet v 1 using a gnotobiotic mouse model. We showed that *Lactobacillus* (*L.*) *rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908 and *L. casei* LOCK0919 are recognized *via* Toll-like receptor 2 (TLR2) and nucleotide-binding oligomerization domain-containing protein 2 (NOD2) receptors and stimulate bone marrow-derived dendritic cells to produce cytokines in species- and strain-dependent manners. Colonization of germ-free (GF) mice with a mixture of all three strains (Lmix) improved the intestinal barrier by strengthening the apical junctional complexes of enterocytes and restoring the structures of microfilaments extending into the terminal web. Mice colonized with Lmix and sensitized to the Bet v 1 allergen showed significantly lower levels of allergen-specific IgE, IgG1 and IgG2a and an elevated total IgA level in the sera and intestinal lavages as well as an increased transforming growth factor (TGF)-β level compared with the sensitized GF mice. Splenocytes and mesenteric lymph node cells from the Lmix-colonized mice showed the significant upregulation of TGF-β after *in vitro* stimulation with Bet v 1. Our results show that Lmix colonization improved the gut epithelial barrier and reduced allergic sensitization to Bet v 1. Furthermore, these findings were accompanied by the increased production of circulating and secretory IgA and the regulatory cytokine TGF-β. Thus, this mixture of three lactobacillus strains shows potential for use in the prevention of increased gut permeability and the onset of allergies in humans.
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Keywords: allergic sensitization; germ-free; intestinal barrier; *Lactobacillus*; probiotics

INTRODUCTION

Humans, like all vertebrates, are essentially born germ-free (GF). This GF status changes rapidly during and after delivery, and subsequent interactions between the host and colonizing microbiota plays crucial roles in the development and function of the immune system as well as the maintenance of intestinal homeostasis.[1,2] Perturbations in colonizing microbiota lead to the breakdown of the equilibrium between commensal and pathogenic microbes. This dysbiosis has been linked to the increased permeability of the epithelium[3,4] and the development of chronic inflammatory diseases, such as allergies and inflammatory bowel disease.[5–7]

Allergies have become a serious health burden in developed countries. In accordance with the general hypothesis of Strachan[8] that the rapid increase in allergic diseases in humans is dependent on microbial deprivation early in life, reduced bacterial diversity and lower counts of lactobacilli and bifidobacteria have been detected in the gut of allergic children.[9,10]

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This finding has been the rationale for the administration of probiotic bacteria for the prevention and/or treatment of allergies.[11–13]

Probiotic lactobacilli and bifidobacteria are non-invasive and non-pathogenic Gram-positive bacteria possessing immunomodulatory properties that are strictly strain-dependent.[14] They have been documented to compete with pathogens and toxins for adherence to the intestinal epithelium and to promote intestinal epithelial cell survival, enhance barrier function and directly interact with cells of the immune system, such as dendritic cells (DCs).[15] Through the engagement of innate receptors, such as Toll-like receptors (TLRs), nucleotide-binding oligomerization domains (NODs) or C-type lectin receptors, probiotic lactobacilli and bifidobacteria induce distinct innate responses and cytokine profiles that subsequently shape T-helper cell responses.[16–18] There is accumulating evidence that certain strains possess intrinsic Th1-type immunomodulatory properties,[18,19] while others are able to induce regulatory responses.[17,20,21]

Transforming growth factor (TGF)-β is present at high concentrations in the intestine and has a crucial involvement in modulating the immune response.[22] It has been shown to inhibit the proliferation and differentiation of both B and T cells,[23] and altered TGF-β signaling has been linked to the development of allergic disease.[24] Furthermore, TGF-β is an initial trigger for the production of mucosal IgA, which has a role in regulating mucosal integrity.[25] Along these lines, we have previously shown that *Lactobacillus paracasei* stimulates the production of the regulatory cytokine TGF-β from bone marrow-derived DCs in a TLR2/4-dependent manner.[21]

Among the inhalant allergens, the pollen of the white birch (*Betula verrucosa*) is one of the most important sources responsible for eliciting allergic symptoms.[26] In an experimental model, we have shown that the oral application of *L. paracasei* to pregnant mothers prevents the development of allergies in their offspring in a mouse model of birch pollen allergy.[21] Similarly, intranasal application of probiotic bacteria reduces allergic poly-sensitization in adult mice.[27] Although the majority of studies use single strains, supplementation with probiotic mixtures might have a greater efficacy.[28]

Germ-free animals represent a unique tool to study the interactions of hosts with specific probiotic strains or with defined probiotic mixtures and to investigate their impacts on the development of the immune system.[6,29] Using a mouse model of allergic sensitization to the major birch pollen allergen Bet v 1, we have previously shown that neonatal colonization of GF mice with *Bifidobacterium longum* is able to prevent allergic sensitization,[20] but the underlying mechanism of the host–bacteria interaction in gnotobiotic models is still far from being elucidated.

Recently, we have selected three lactobacillus strains, *L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908 and *L. casei* LOCK0919, out of 24 strains isolated from stool samples obtained from healthy infants.[30] These selected strains showed properties required for probiotic bacteria, e.g., resistance to gastric acids and bile salts and inhibitory activities against bacterial pathogens.[30] Moreover, the mixture of these strains (Lmix) showed synergistic effects in the induction of anti-allergic Th1-type cytokines and regulatory cytokine TGF-β in human whole blood cell cultures compared with the levels induced by each single strain alone.[31] Our pilot study showed that the supplementation of children presenting the first symptoms of allergy (atopic dermatitis) with the Lmix reduced serum levels of IgE and IL-5 and diminished the severity of the disease (Cukrowska, unpublished data).

Based on these observed effects, the aims of this study were to further characterize the immunomodulatory properties of the individual lactobacillus strains *L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908 and *L. casei* LOCK0919, as well as their mixture Lmix, *in vitro* and to investigate the effects of Lmix on the development of allergic sensitization to the allergen Bet v 1 in a gnotobiotic mouse model.

MATERIALS AND METHODS

Bacterial strains

L. rhamnosus LOCK0900,[32] *L. rhamnosus* LOCK0908[33] and *L. casei* LOCK0919[34] were obtained from the Pure Culture Collection of the Technical University of Lodz, Poland (LOCK). Overnight cultures in MRS broth (Oxoid, Basingstoke, UK) were centrifuged and washed in sterile phosphate-buffered saline (PBS), and their concentrations were adjusted to 10^9 CFU/ml. For the *in vitro* experiments, single bacterial strains were inactivated with 1% formaldehyde-PBS for 3 h at room temperature, washed twice with sterile saline (PBS) and stored at −40 °C.

Stimulation of HEK293 cells stably transfected with TLR2, NOD2 and TLR4

The human embryonic kidney cell line HEK293 stably transfected with a plasmid carrying the human (h)TLR2/CD14 gene was kindly provided by M. Yazdanbakhsh (Leiden, The Netherlands), cells transfected with hTLR4/MD2/CD14 were a gift from B. Bohle (Vienna, Austria), and cells transfected with hNOD2 were purchased from InvivoGen (InvivoGen, Toulouse, France). The cells were stimulated with the formalin-inactivated *L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908, *L. casei* LOCK0919 or their equal-part mixture (Lmix) at a concentration of 10^7 CFU/ml. TLR2 ligand Pam3CSK4 (PAM3; 1 μg/ml; InvivoGen), NOD2 ligand muramyl dipeptide (100 ng/ml; InvivoGen) and TLR4 ligand ultrapure LPS-EB (LPS; 1 μg/ml, InvivoGen, Toulouse, France) were used as positive controls. After a 20-h incubation period, culture supernatants were harvested, and human IL-8 concentrations were analyzed by Enzyme-Linked Immunosorbent Assay (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Preparation and activation of bone marrow-derived DCs (BM-DCs)

Mouse BM-DCs were prepared as previously described.[21] Briefly, bone marrow precursors were isolated from the femurs and tibias of conventional (CV) BALB/c mice. Cells were cultured

at 4×10^5/ml in bacteriological Petri dishes in 10 ml of culture medium with GM-CSF (20 ng/ml; Sigma-Aldrich, Saint-Louis, MO, USA). Fresh medium was added on days 3 and 6, and the BM-DCs were used on day 8 of culture. The BM-DCs (10^6 cells/ml) were stimulated with 10^7 CFU/ml of inactivated *L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908, *L. casei* LOCK0919 or their equal-part mixture (Lmix) for 18 h. BM-DCs incubated with Pam3CSK4 (PAM3; 1 μg/ml) or ultrapure LPS-EB (LPS, 1 μg/ml) were used as controls. Levels of IL-10, TGF-β and TNF-α in the culture supernatants were determined by ELISA Ready-Set-Go! Kits (eBioscience, San Diego, CA, USA) according to manufacturer's instructions. IL-12p70 levels were measured with matched antibody pairs (BD Biosciences, San Jose, CA, USA).

Animals

GF inbred BALB/c mice were born and housed under sterile conditions and fed a sterile standard pellet diet (ST1; Bergman, Kocanda, Czech Republic; 59 kGy irradiated for 30 min) and were provided sterile water *ad libitum*. The animals were kept in a room with a 12 h light–dark cycle at 22 °C. Fecal samples were evaluated weekly for the presence of aerobic and anerobic bacteria, molds and yeast by standard microbiological methodologies. CV BALB/c mice ($n=5$) were fed the same sterile diet as their GF counterparts. The animal experiments were approved by the Committee for the Protection and Use of Experimental Animals of the Institute of Microbiology v.v.i., Academy of Sciences of the Czech Republic (approval ID: 50/2013).

Experimental design

Eight-week-old GF mice ($n=12$) were divided into two groups. The mice were colonized by intragastric tubing with 2×10^8 CFU of equal parts of overnight cultures of *L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908 and *L. casei* LOCK0919 in 0.2 ml of sterile PBS (group 1). The second group served as a GF control. Three weeks after colonization, the *Lactobacillus*-colonized mice and GF controls were intraperitoneally immunized three times with 1 μg of the recombinant birch pollen allergen Bet v 1 (Biomay, Vienna, Austria) adsorbed to 2 mg aluminium hydroxide (Serva, Heidelberg, Germany) at 10-day intervals, as previously described.[35] The mice were euthanized at seven days after the last immunization by cervical dislocation (Figure 4a). Blood was collected, and serum samples were stored at −40 °C until analysis. Terminal ileum samples were removed for immunohistochemistry, Western blot and electron microscopy analysis, and the rest of the small intestine was excised for the determination of total IgA, with lavages performed as previously described.[36] Mesenteric lymph nodes (MLNs, pooled per group) and the spleen were aseptically removed and prepared for *in vitro* cytokine assays. Briefly, after gentle crushing, straining through a 70-μm cell strainer (BD Biosciences, San Jose, CA, USA) and the lysis of red blood cells (180 mM NH_4Cl and 17 mM Na_2EDTA, pH 7.3; Sigma-Aldrich, Saint-Louis, MO, USA), mononuclear cells were resuspended in complete RPMI 1640 medium (Sigma-Aldrich, Saint-Louis, MO, USA) containing 10% fetal calf serum, 2 mM glutamine, 100 U penicillin and 100 μg/ml streptomycin.

Bacterial colonization

The bacterial colonization of the mice was evaluated on the first 2 days and then at weekly intervals throughout the experiment. The fecal samples were pooled for each group, diluted (1 : 9, w/v) in sterile PBS and exhaustively vortexed with sterile glass beads. Volumes of 1 ml at the appropriate 10-fold dilution were plated onto MRS agar (Oxoid, Basingstoke, UK) and cultivated in triplicate at 37 °C for 48 h. At the species level, bacteria were distinguished on the basis of colony morphology. The strain *L. casei* LOCK0919 formed small, white, non-mucosal colonies, whereas the strains *L. rhamnosus* LOCK0900 and LOCK0908 formed larger white-gray-colored mucosal colonies. To distinguish between *L. rhamnosus* strains, we isolated DNA from the feces of colonized mice and performed strain-specific qPCR (Supplementary Information).

Immunohistochemical detection of IgA-producing cells

Segments of the terminal ileum were embedded in Tissue-Tek (Sakura Finetec Europe B.V., Netherlands) and frozen in liquid nitrogen. Cryosections (5 μm thick) of acetone-fixed colon were used for immunocytochemistry. Immunostaining was performed with a goat anti-mouse IgA-FITC antibody (Thermo Fisher Scientific, Waltham, MA, USA). Samples were viewed under an Olympus BX 40 microscope equipped with an Olympus DP 70 digital camera. Photographs were taken with a Camedia Master 2.5 and DP-Soft (Olympus Corporation, Tokyo, Japan).

Transmission electron microscopy

The ileum tissues were cut into small pieces (1×1 mm) and immediately fixed in 2.5% glutaraldehyde in PBS for 90 min. After fixation in 1% osmium tetroxide (Sigma-Aldrich, Saint-Louis, MO, USA) for 1 h and washing in 0.1 M cacodylate buffer, the samples were successively dehydrated in 35%, 70%, 96% and 100% ethanol and propylene oxide (EMS, Hatfield, PA, USA). Subsequently, they were embedded in Epon resin (EMS, Hatfield, PA, USA). Selected semi-thin sections of ileum were cut into 65 nm ultra-thin sections by Leica Ultracut Uct52 (Leica Microsystems, Wetzlar, Germany), stained with uranyl acetate and lead citrate, and examined by electron microscopy (Jem 1011; Jeol, Peabody, MA, USA). Images of the ultrastructural features of the ileal structures and junctions were visualized at magnifications ranging from ×3000 to ×100 000. Specimens were obtained from five mice from each group. The widths and lengths of the intracellular junctions were measured using the morphometric iTEM program (Olympus Corporation, Tokyo, Japan) at a magnification of ×100 000. For each specimen, 10–15 measurements were performed, and the results are presented in nm.

Western blot analysis of ZO-1 and occludin

The terminal ileum was homogenized on ice in protein extract buffer with a protease inhibitor cocktail (Thermo Fisher

Scientific, Waltham, MA, USA) for 10 min and sonicated. Samples were centrifuged at 10,000 rpm for 10 min at 4 °C and stored at −80 °C until use. Protein concentrations were measured using a BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Western blotting was performed as previously described.[37] The membranes were blocked with 2% (w/v) dry milk in 0.05% PBS-Tween-20 for 1 h at room temperature and incubated overnight at 4 °C with antibodies against occludin (1 : 1000) ZO-1 (1 : 1000) (Thermo Fisher Scientific, Waltham, MA, USA) and β-actin (1 : 5000) (Abcam, Cambridge, UK). After incubation with the respective primary antibodies, secondary staining was conducted using horseradish peroxidase-conjugated species-specific antibodies (1 : 1000) (Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at room temperature. The reactions were developed using a SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA, USA), and the signal intensities were measured with a G : BOX (Syngene, Cambridge, UK) and processed with the ImageJ program.[38]

Allergen-specific antibody responses: ELISA and basophil release assay

Allergen-specific serum IgG1, IgG2a and IgA levels were determined by ELISA as previously described.[39] Briefly, 96-well microtiter plates were coated with Bet v 1 (2 μg/ml). Serum samples were diluted 1 : 10000 for IgG1, 1 : 100 for IgG2a and 1 : 10 for IgA. Rat anti-mouse IgG1, IgG2a and IgA antibodies (1 μg/ml; BD Biosciences, San Jose, CA, USA) were applied, followed by peroxidase-conjugated mouse anti-rat IgG antibodies (1 : 1000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for detection. Antibody levels were reported as optical densities. Allergen-specific IgE levels in the sera were quantified by the degranulation of rat basophil leukemia (RBL-2H3) cells as previously described.[40] RBL-2H3 cells were plated in 96-well tissue culture plates (4×10^4 cells per well) and passively sensitized by incubation with mouse sera at a final dilution of 1 : 30 for 2 h. After washing, Bet v 1 (0.3 μg/ml) was added for 30 min at 37 °C to induce degranulation. Supernatants were incubated with 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide (Sigma-Aldrich, Saint-Louis, MO, USA) for analysis of β-hexosaminidase using an Infinite M200 (Tecan Group, Männedorf, Switzerland) fluorescence microplate reader (λ_{ex}: 360 nm/λ_{em}: 465 nm). The results are reported as the percentage of total β-hexosaminidase release from the cells after disruption with 1% Triton X-100.

Total IgA and IgE responses

Total IgA and IgE were measured in the sera and gut lavages (IgA only) with a mouse IgA and IgE ELISA quantification kit (Bethyl, Montgomery, TX, USA) according to manufacturer's instructions. The serum samples were diluted 1 : 400 for the IgA and 1 : 10 for the IgE measurements, and for IgA determination in the gut lavages, a 1 : 2500 dilution was used. Antibody levels are reported as μg/ml for the sera and as μg/g for the gut lavages.

Cytokine production

Spleen cells and pooled MLN cell suspensions were cultured in 48-well flat-bottom plates at a concentration of 5×10^6 cells in 500 μl of complete RPMI 1640 medium. Cells were cultivated with/without Bet v 1 (10 μg/well) restimulation at 37 °C under 5% CO_2 for 48 h. After cultivation, supernatants were collected and stored at −40 °C until analysis. IL-4, IL-5, IL-10 and interferon (IFN)-γ levels were determined by a Mouse Cytokine/Chemokine Multiplex Immunoassay (Millipore, Billerica, Ma, USA) according to the manufacturer's instructions and analyzed with a Luminex 200 System (Bio-Rad Laboratories, Hercules, CA, USA) at sensitivities of <0.3 pg/ml for IL-4, <0.3 pg/ml for IL-5, <10.3 pg/ml for IL-10 and <0.7 pg/ml for IFN-γ. TGF-β was measured in the culture supernatants and in 1 : 10 diluted serum samples with an ELISA kit (R&D Duoset Systems, Minneapolis, MN, USA) according to the manufacturer's instructions, with a detection limit of <4 pg/ml.

Statistical analyses

The non-parametric Mann–Whitney test was used for comparisons between two groups, and for comparisons between multiple groups, ANOVA with Tukey's multiple comparison test was performed with the GraphPad Prism 5.02 software. Values of $P<0.05$ were considered significantly different. All data are expressed as the mean±standard error of the mean (s.e.m.) unless stated otherwise.

RESULTS

TLR2 and NOD2 but not TLR4 are involved in the recognition of all three investigated *Lactobacillus* strains

To specify pattern recognition receptors involved in *Lactobacillus* signaling pathways, the single strains *L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908, *L. casei* LOCK0919 or their equal-part mixture (Lmix) were incubated with HEK293 cells transfected either with TLR2, TLR4 or NOD2. The cytokine IL-8 level was measured as an indicator of cell stimulation *via* a specific receptor, and it was found to be significantly increased in the supernatants of the HEK293/TLR2 cells incubated with *L. rhamnosus* LOCK0900 and in the HEK/NOD2 cells exposed to *L. casei* LOCK0919 and Lmix (Figure 1a and b). There was no IL-8 stimulation detected in the HEK293/TLR4 cells incubated with any single lactobacillus strain or Lmix (Figure 1c).

Strain-specific profile of cytokines produced by stimulated BM-DCs

Activation of BM-DCs with the single strains *L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908 and *L. casei* LOCK0919 or Lmix showed a trend toward the increased induction of the regulatory cytokine TGF-β independent of the applied bacterial strain. However, the production of IL-10, IL-12p70 and TNF-α was strictly species- and strain-dependent, and the stimulation of cytokine production by Lmix corresponded with the average of the cytokine concentrations induced by individually applied bacterial strains (Figure 2).

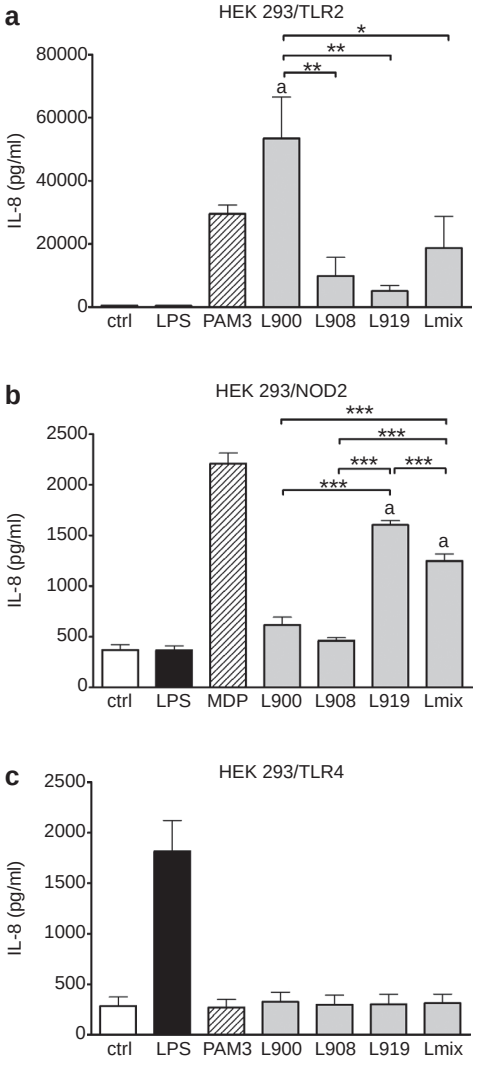

Figure 1 Stimulation of HEK293 TLR2-, NOD2- and TLR4-transfected cells with *Lactobacillus* strains. Human embryonic kidney cells (HEK293) stably transfected with an expression vector for human TLR2 (293-hTLR2) (**a**), NOD2 (pUNO-hNOD2) (**b**) and TLR4 (293-hTLR4/MD2/CD14) (**c**) were cultured for 20 h with 10^7 CFU/ml of formalin-inactivated *L. rhamnosus* LOCK0900 (L900), *L. rhamnosus* LOCK0908 (L908), *L. casei* LOCK0919 (L919) or an equal-part mixture of these strains (Lmix). PAM3 (1 μg/ml), MDP (10 μg/ml) and ultrapure lipopolysaccharide from *E. coli* (LPS; 1 μg/ml) were used as positive controls for TLR2, NOD2 and TLR4, respectively. Unstimulated cells (ctrl) were used as negative controls. Stimulation was evaluated by the measurement of IL-8 production. The results are expressed as the mean±s.e.m. Pooled values of at least three experiments are shown. PAM3, MDP and LPS served as positive or negative stimulated controls and were not included in statistical analysis. [a]Significantly different from unstimulated control; *$P<0.05$, **$P<0.01$ and ***$P<0.001$. Ctrl, unstimulated cells; HEK293, human embryonic kidney cell line 293; MDP, muramyl dipeptide; NOD2, nucleotide-binding oligomerization domain-containing protein 2; PAM3, Pam3CSK4; TLR, Toll-like receptor.

Colonization with Lmix improves the intestinal barrier

To evaluate the effect of Lmix colonization on the intestinal barrier, ultrastructural analyses of the apical portion of ileal enterocytes were performed. In mice reared under conventional conditions, the brush borders were regular, straight and contained microfilaments extending into the terminal web (TW) (Figure 3a). The apical junctional complex, including the tight junction, adherens junction (AJ) and desmosome, were well organized. In contrast, the enterocyte brush borders of the GF mice were irregularly arranged and exhibited decreased numbers of cytoskeletal microfilaments and a lack of elongation into the TW. As shown in Table 1, the AJ region was significantly broader and shorter in the GF mice compared with the CV and Lmix-colonized mice. Interestingly, incomplete apical junctional complexes lacking desmosomes (DE) were observed in approximately 30% of the enterocytes of the GF mice (Figure 3b). Lmix colonization of the GF mice led to a more organized arrangement of enterocyte microvilli with cytoskeletal microfilaments anchored in the TW, similar to the CV mice (Figure 3c). In these mice, DEs were detected in each apical junctional complex in contrast with the GF mice. Moreover, the AJs in the Lmix-colonized mice were significantly elongated and narrow compared with the GF mice, resembling those found in the CV mice (Table 1). Western blot analysis of the terminal ileum further confirmed the electron microscopic findings. The levels of ZO-1 (Figure 3g) were significantly increased in the CV and Lmix-colonized mice compared with the GF controls. Concomitantly, the occludin level was significantly higher in the CV mice, and there was a trend toward its increase in the Lmix-colonized mice (Figure 3h).

Colonization of GF mice with Lmix

The stability of colonization with Lmix was evaluated throughout the experiment. By plating the fecal samples on MRS agar, we were able to distinguish the bacteria at the species level. As shown in Figure 4b, starting from the second day after colonization, the concentration of *L. casei* reached $3.3–5.0\times10^9$ CFU/g of feces, while *L. rhamnosus* strains were detected at concentrations ranging from $0.2–8.0\times10^8$ CFU/g. To distinguish between the two *L. rhamnosus* strains, we isolated the DNA from the stool samples and showed that the LOCK0908 strain was more abundant compared with the LOCK0900 strain by qPCR (Figure 4c).

Colonization by Lmix suppresses Bet v 1-specific antibody production

To analyze the effect of Lmix colonization on allergic sensitization, our recently published mouse model[20,41] was applied, and the production of specific antibodies and cytokines were evaluated. Lmix-colonized and GF mice were immunized intraperitoneally with the recombinant birch pollen allergen Bet v 1 at 10-day intervals starting at 3 weeks after bacterial colonization (Figure 4a). Colonization with Lmix significantly reduced Bet v 1-specific IgE ($P<0.03$), IgG1 ($P<0.03$) and IgG2a ($P<0.03$) serum antibodies compared with the age-matched Bet v 1-sensitized GF controls (Figure 4d–f). No differences were found in Bet v 1-specific IgA antibodies between both groups (GF: 0.187±0.44 OD and Lmix: 0.167±0.027 OD; $P=0.857$).

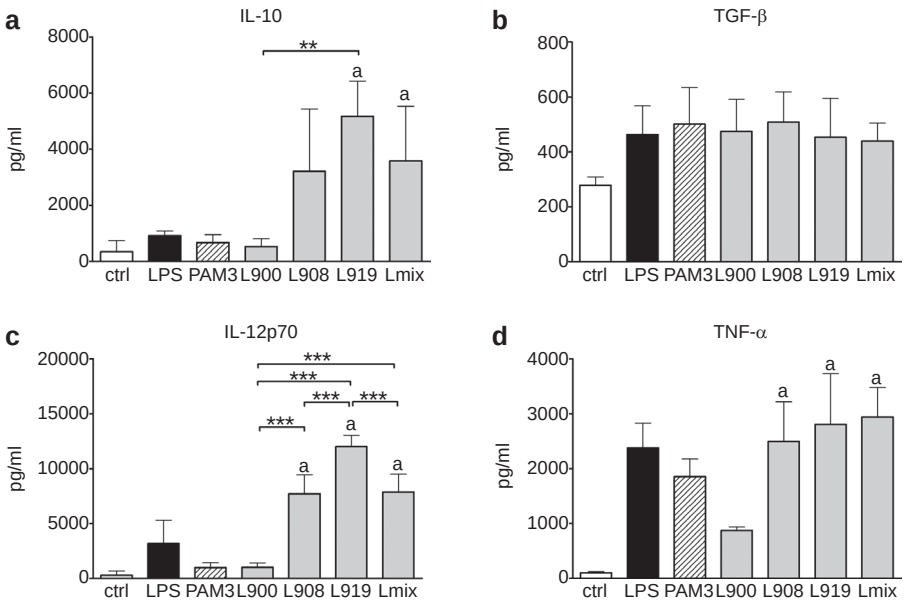

Figure 2 Stimulation of bone marrow-derived dendritic cells with *Lactobacillus* strains. BM-DCs were cultured with 10^7 CFU/ml of formalin-inactivated *L. rhamnosus* LOCK0900 (L900), *L. rhamnosus* LOCK0908 (L908), *L. casei* LOCK0919 (L919) strains or an equal-part mixture of these strains (Lmix) for 18 h. As positive controls, PAM3 (1 μg/ml) or ultrapure lipopolysaccharide from *E. coli* (LPS; 1 μg/ml) were applied. Ctrl served as negative controls. The levels of IL-10, TGF-β, IL-12p70 and TNF-α in the culture supernatants were determined by ELISA and expressed as the mean±s.e.m. Pooled values of at least three experiments are shown. PAM3 and LPS served as stimulated controls and were not included in statistical analysis. [a]Significantly different from unstimulated control; *$P<0.05$, **$P<0.01$ and ***$P<0.001$. BM-DC, bone marrow-derived dendritic cells; ctrl, unstimulated cell; Lmix, *Lactobacillus* mixture; PAM3, Pam3CSK4; TGF, transforming growth factor; TNF, tumor necrosis factor.

Colonization with Lmix reduces systemic IgE and induces systemic and local IgA production

Colonization of the GF mice with Lmix induced a decreasing trend in the level of total IgE in the serum samples (Figure 5a), while the total levels of IgA in the serum samples ($P<0.013$) and small intestinal lavages were significantly increased ($P<0.04$) in comparison with the Bet v 1-senzitized GF controls (Figure 5b and c). In the Lmix-colonized group, the induction of activated IgA-secreting plasma cells in the lamina propria of the terminal ileum was confirmed by immunofluorescence staining (Figure 5d). However, no IgA-producing cells were found in the age-matched GF controls (Figure 5d).

Lmix colonization reduces Bet v 1-specific IL-4 and IL-5 cytokine production

To investigate the impact of Lmix on Th1 and Th2 cytokine production, splenocytes and pooled MLN cells from Bet v 1-sensitized mice were cocultured with Bet v 1 *in vitro*. We observed the significantly reduced secretion of the Th2 cytokine IL-4, a trend toward a reduction in IL-5 and a slight increase in the level of the Th1-type cytokine IFN-γ in spleen cell supernatants from the Lmix-colonized mice compared with the GF controls (Figure 6a–c). No IL-4 production was detected in the pooled MLN cell cultures, and the levels of both IL-5 (Figure 6d) and IFN-γ (GF: 5.56 pg/ml and Lmix: 1.30 pg/ml) were lower in the supernatants from the Lmix-colonized mice compared with the GF controls.

Colonization with Lmix stimulates TGF-β production

To evaluate the effects of Lmix colonization on the regulatory cytokine response, the level of TGF-β was determined in serum samples and supernatants from spleen or MLN cells co-cultured with Bet v 1 *in vitro*. A significant upregulation of TGF-β in the sera was detected in the mice colonized with Lmix compared with the GF controls ($P<0.009$) (Figure 7a). We observed a significant increase in the TGF-β level in supernatants of the Bet v 1-stimulated splenocyte cultures of the Lmix-colonized mice compared with the GF controls (Figure 7b). A similar tendency was detected in supernatants of the MLN cells isolated from the Lmix-colonized mice (Figure 7c). There was no difference between the Lmix-colonized and GF control groups in IL-10 production in any of the cell culture supernatants (data not shown).

DISCUSSION

In the present study, we aimed to investigate the ability of Lmix, a mixture of the three *lactobacillus* strains *L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908 and *L. casei* LOCK0919, to modulate allergic sensitization in a gnotobiotic mouse model. We showed that colonization with Lmix ameliorated Bet v 1-specific allergic responses at both the humoral and cellular levels. Furthermore, Lmix colonization improved the barrier structure of the gut, which was immature in the GF mice.

The modulation of immune responses by single bacterial strains or by mixtures of different probiotic strains has been

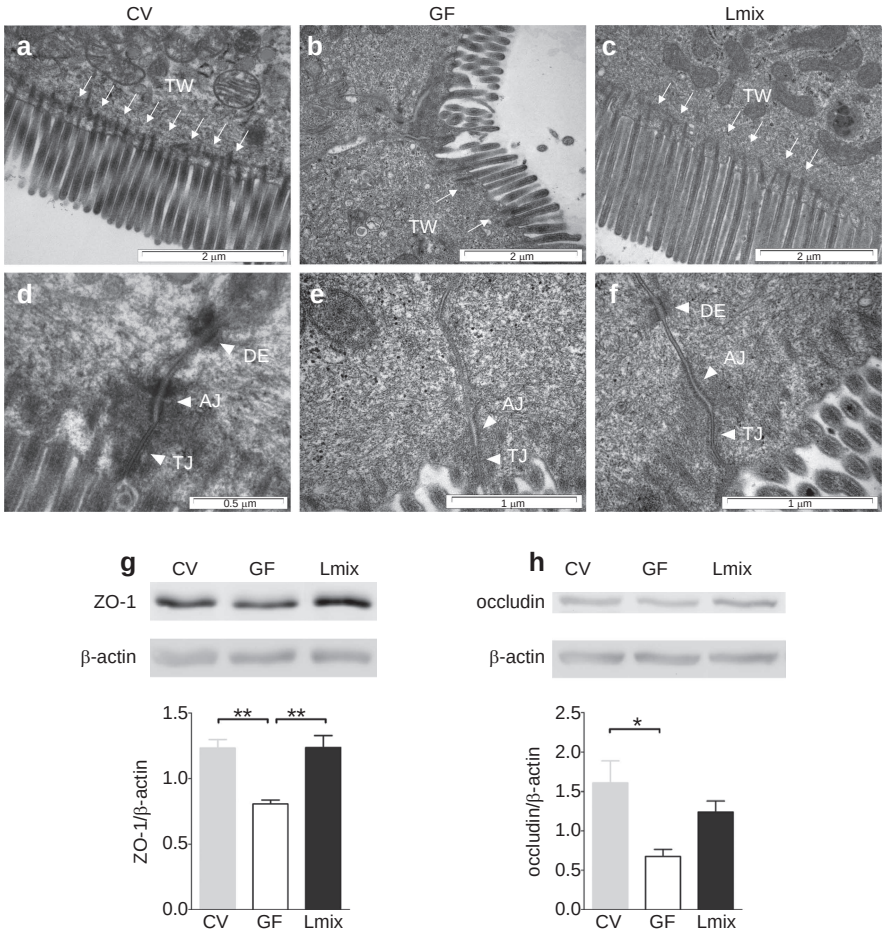

Figure 3 The effects of Lmix colonization on the architecture of the apical junctional complex of enterocytes and the production of ZO-1 and occludin. Electron microscopy micrographs of the apical surfaces of ileal enterocytes in CV, GF and Lmix-colonized mice (Lmix). The epithelial surface is covered by microvilli. Microfilaments extend from the microvilli into the apical cytoplasm and filamentous TW, which was deficient in the GF animals and was restored in the Lmix-colonized mice (**a**–**c**). The epithelial cell junctional complex contains the TJs, AJs and DEs. DEs were absent in 30% of the junctional complexes in the GF mice (**d**–**f**). Representative micrographs were obtained from 10–15 measurements per sample; $n=5$ samples per group. Western blot analysis of ZO-1 (**g**) and occludin (**h**) in the ileum. A representative mouse from each group is shown (3–4 mice per group were analyzed). Quantification of the signals was performed using ImageJ. The data are expressed as the mean±s.e.m. of 3–4 mice per group. *$P<0.05$ and **$P<0.01$. AJ, adherens junction; DE, desmosome; GF, germ-free; Lmix, *Lactobacillus* mixture; TJ, tight junction; TW, TW, terminal web; ZO-1, zonulin-1.

Table 1 Effects of bacterial colonization on the width (*W*) and length (*L*) of the apical intracellular junction in the ileum of CV, GF and Lmix-colonized mice

	Tight junctions (nm)		*Adherens junctions (nm)*	
Group	*W*	*L*	*W*	*L*
CV	10±1	336±40	30±2	226±50
GF	10±1	203±50*	40±10*	181±40*
Lmix	11±3	236±80	30±7	234±70

Abbreviations: CV, conventional; GF, germ-free; Lmix, *Lactobacillus* mixture.
The values are expressed as the mean±s.e.m. (nm) and were obtained from 10–15 measurements per sample, and $n=5$ samples were assessed per group.
* $P<0.05$, significant difference of the GF group *versus* the Lmix and CV groups.

documented in mouse models as well as in human trials.[42,43] This modulation occurs either by the promotion of Th1-type responses[44] or by the induction of regulatory cells and cytokines.[20,45] Using a gnotobiotic mouse model, we showed that colonization with Lmix reduced the serum levels of the Th2-related Bet v 1-specific IgE and IgG1 antibodies as well as the Th1-related IgG2a antibody, implicating the involvement of regulatory mechanisms. These findings were further supported by the significantly higher serum level of TGF-β. After the *in vitro* restimulation of splenocytes or MLN cells with Bet v 1, we observed an alteration in Th2/T regulatory (Treg) cytokine production. We detected the downregulation of the Th2-associated cytokines IL-4 and IL-5 and the upregulation of TGF-β production in the Lmix-colonized group, suggesting that Lmix colonization induced immunoregulatory mechanisms. Previously, Feleszko *et al.*[45] have demonstrated that the oral delivery of

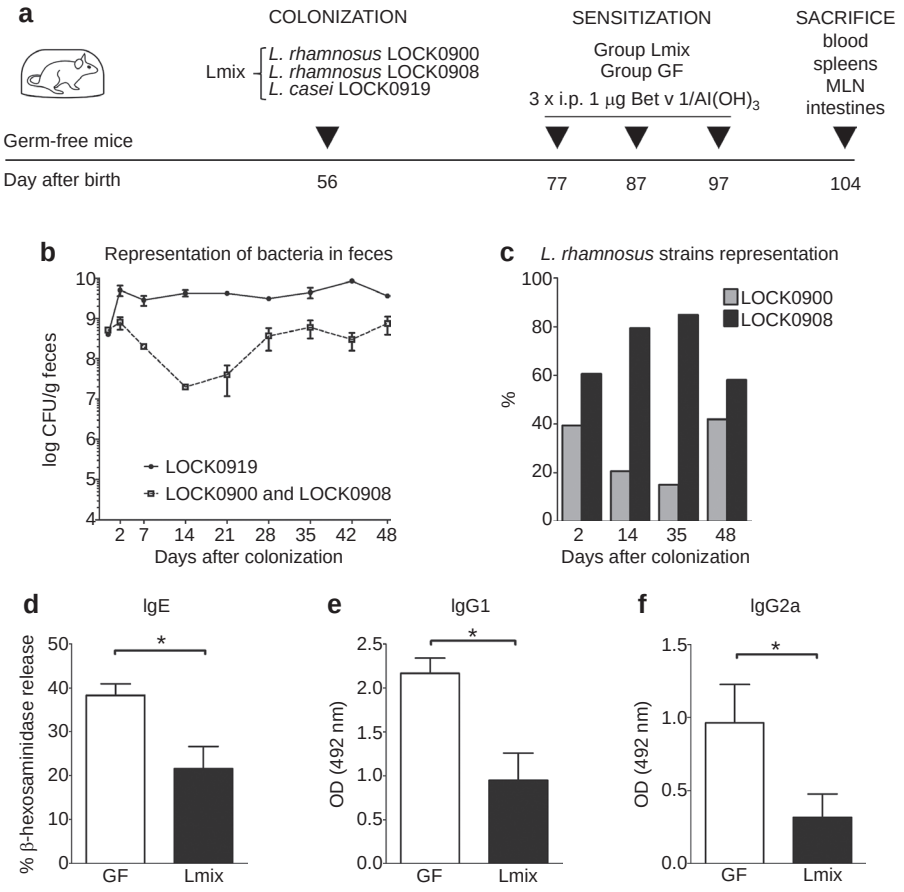

Figure 4 Sensitization of GF and Lmix-colonized mice with the major birch pollen allergen Bet v 1. (**a**) The experimental design was as follows: 8-week-old GF mice (n=12) were divided into two groups. The first group (Lmix) received an equal-part mixture (2×10^8 CFU/ml) of *L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908 and *L. casei* LOCK0919 by intragastric tubing. The second group was kept GF. Mice were sensitized three times intraperitoneally with recombinant Bet v 1 (1 μg in alum) on days 77, 87 and 97. One week after the last immunization (day 104), tissue samples were collected for further analyses. Bacterial colonization of the Lmix-colonized mice was evaluated on the first 2 days and then at weekly intervals throughout the experiment. At the species level, bacteria were distinguished based on colony morphology by the cultivation of appropriate serial dilutions of the fecal samples. (**b**) *L. casei* LOCK0919 (full circles, solid line), *L. rhamnosus* LOCK0900 and LOCK0908 (open squares, dotted line). *L. rhamnosus* strain-specific discrimination was performed by qPCR using DNA isolated from the fecal samples at the indicated time points (**c**) *L. rhamnosus* LOCK0900 (gray bars) and *L. rhamnosus* LOCK0908 (black bars). The data are shown as a percentage of each strain out of all detected *L. rhamnosus* bacteria on the indicated day after colonization. Bet v 1-specific antibodies were measured in the sera of GF (white bars) and Lmix-colonized mice (black bars). IgE was measured by Bet v 1-mediated β-hexosaminidase release from rat basophil leukemia cells (**d**). Levels of IgG1 (**e**) and IgG2a (**f**) were evaluated by ELISA and expressed as OD units. The data are shown as the mean±s.e.m. One representative out of two experiments is shown; n=6/group. *P<0.05 and **P<0.01. GF, germ-free; Ig, immunoglobulin; i.p., intraperitoneally; Lmix, *Lactobacillus* mixture; OD, optical density.

probiotic bacteria leads to the suppression of allergic sensitization and airway inflammation by TGF-β-producing Treg cells, which can be found in MLNs. It has also been shown that the peripheral conversion of $CD4^+$ T cells to Treg cells occurs primarily in gut-associated lymphoid tissue in the presence of TGF-β and retinoic acid.[46] In accordance with these findings, we suggest that colonization with a *lactobacillus* mixture induces the upregulation of TGF-β production in the intestine and the generation of Treg cells.

In correlation with the increased production of TGF-β, we found a significant increase in the gut and serum IgA levels in the Lmix-colonized mice. Secretory IgA has been shown to play a crucial role in maintaining bacterial homeostasis in the gut (reviewed in Ref. 47). These results are in accordance with previous findings that the colonization of GF mice with probiotic bacteria induces the activation of IgA production and that a mixture of probiotic strains is more effective in the development of plasmablasts in the gut compared with single strains.[48]

The intestinal barrier is immature in GF mice,[49] and Lmix significantly improves this condition. We found that the enterocyte brush borders of the GF mice were irregularly arranged and exhibited a decreased number of cytoskeletal microfilaments and a lack of elongation into the terminal web. The adherens junctions in the Lmix-colonized mice were significantly elongated and narrow compared with those in the GF mice and resembled those found in CV mice. This fortification of the intestinal barrier was further evident from the increased levels of the ZO-1 and occludin proteins in the Lmix-colonized and CV mice. To our knowledge, this is the first report

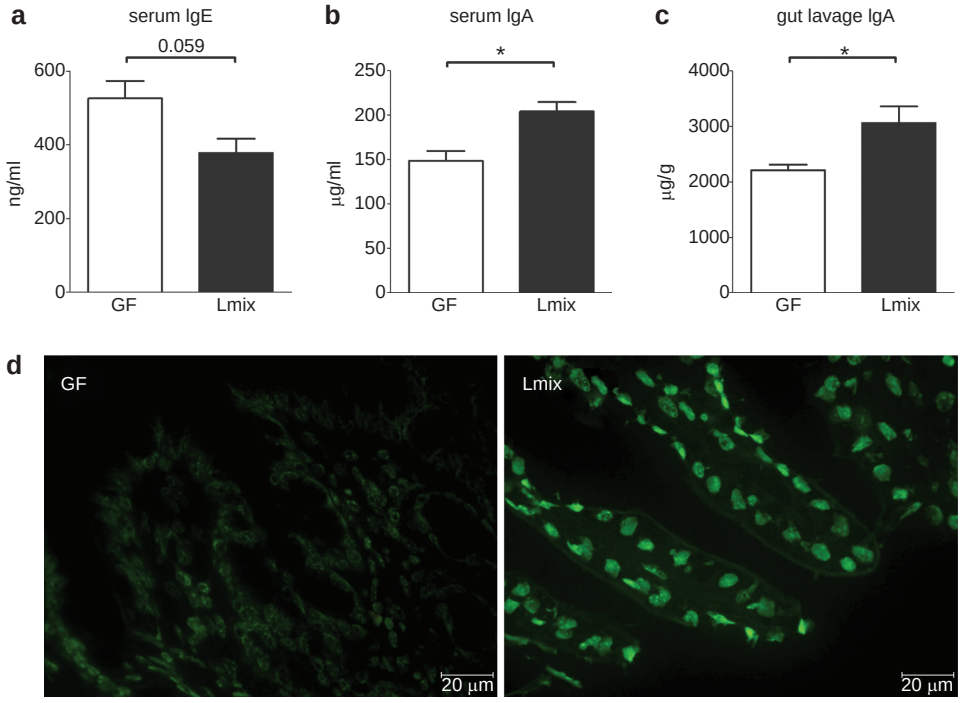

Figure 5 Local and systemic humoral responses in sensitized GF and Lmix-colonized mice. Levels of total IgE (**a**) and total IgA (**b**) in the sera and total IgA (**c**) in the gut lavage were measured by ELISA. Germ-free mice (GF, white bars) and Lmix-colonized mice (Lmix, black bars). The data are shown as the mean±s.e.m. One representative out of two experiments is shown; $n=6$/group. *$P<0.05$ and **$P<0.01$. IgA-positive plasmocytes in the lamina propria of the terminal ileum were visualized with an FITC-labeled anti-IgA antibody (**d**). GF, germ-free; Ig, immunoglobulin; Lmix, *Lactobacillus* mixture.

documenting the effect of lactobacillus colonization on the ultrastructure of brush border and apical junctional complexes of enterocytes in gnotobiotic mice. Along these lines, increased gut permeability has been found in children with food allergies[50] and it has also been recently detected in asthmatic patients.[51] The homeostasis of the intestinal epithelium is maintained by a complex interplay of multiple regulatory mechanisms.[52] *In vitro* studies have indicated that the pro-allergic cytokine IL-4 contributes to barrier impairment in contrast with TGF-β, which enhances the barrier function and activates the expression of proteins comprising intercellular junctions.[53] In our study, the improvement of the gut barrier in the Lmix-colonized mice was accompanied by the reduced secretion of pro-allergic cytokines and the significant enhancement of TGF-β.

There is increasing evidence that probiotic bacteria can exert their functions by directly interacting with pattern recognition receptors. In this study, we showed that TLR2 played an important role in the recognition of *L. rhamnosus* LOCK0900 and that NOD2 participated in the recognition of *L. casei* LOCK0919. In contrast, *L. rhamnosus* LOCK0908 was poorly recognized by both of these receptors. Interestingly, a significant feature of the *L. rhamnosus* LOCK0908 strain is its high level of exopolysaccharide (EPS) production.[33] Fanning *et al.*[54]

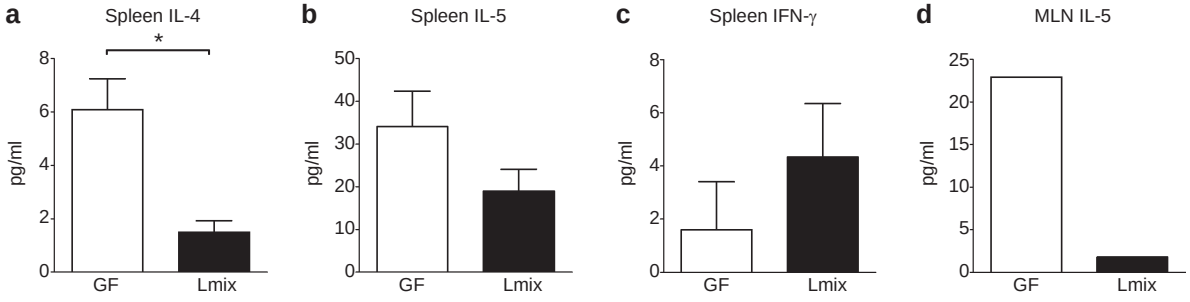

Figure 6 The effect of Lmix colonization on cytokine production *in vitro*. Spleen and pooled MLN cells from Bet v 1-sensitized GF (white bars) and Lmix-colonized mice (black bars) were restimulated with Bet v 1 (10 µg/well) for 48 h. The levels of IL-4 (**a**), IL-5 (**b**) and IFN-γ (**c**) in the spleen cell cultures and IL-5 (**d**) in the pooled MLN cells were determined by ELISA. The results are the values obtained following the subtraction of the cytokine levels measured in the supernatants of the non-stimulated cell cultures. One representative out of two experiments is shown; $n=6$/group. *$P<0.05$. GF, germ-free; MLN, mesenteric lymph node.

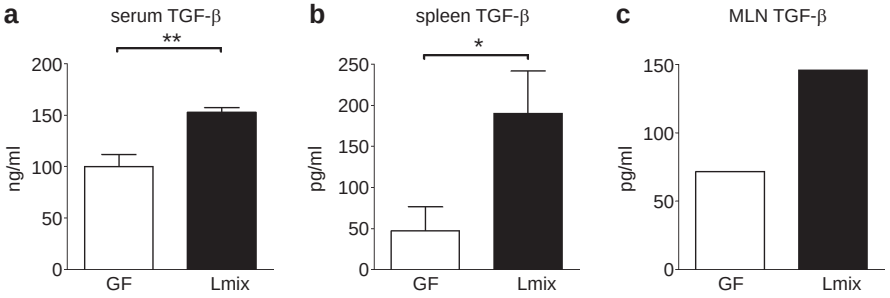

Figure 7 The effect of Lmix colonization on systemic and local TGF-β production. The level of TGF-β in Bet v 1-sensitized GF (white bars) and Lmix-colonized mice (black bars) in the sera (**a**) and supernatants from Bet v 1-restimulated spleen cell (**b**) or pooled MLN cell (**c**) cultures was determined by ELISA. The results are expressed after the subtraction of the cytokines measured in the supernatants of the non-stimulated cell cultures. One representative out of two experiments is shown; $n=6$/group. $^{**}P<0.01$ and $^{*}P<0.05$. GF, germ-free; MLN, mesenteric lymph node; TGF, transforming growth factor.

have recently shown that bifidobacterial strain-producing surface EPS fail to elicit a strong immune response compared with EPS-deficient variants. Thus, it is tempting to speculate that the lack of TLR2, TLR4 and NOD2 activation by *L. rhamnosus* LOCK0908 may be caused by EPS covering the bacterial surface and masking bioactive components, which play a role in binding to pattern recognition receptors.

We have previously shown in human blood cell cultures that the application of *L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908 and *L. casei* LOCK0919 strains together as a mixture has synergistic effects on the induction of anti-allergic Th1-type cytokines compared with the levels induced by each single strain alone.[31] We were not able to confirm these findings using mouse BM-DCs, and we did not observe any synergistic effects on cytokine production. This discrepancy may be explained by the different manners of bacterial inactivation (heating *vs.* formalin inactivation)[55] and also by the different donor species and cell types used.

By evaluating bacterial colonization, we were able to show that all three bacterial strains were detectable in the fecal samples until the end of the experiment. Two days after colonization, *L. casei* LOCK0919 became the dominant strain in the feces of the colonized mice. This finding can be related to a recent analysis of the complete genomic sequence of *L. casei* LOCK0919, which has revealed the presence of factors relevant to its colonization and persistence in the human gut, including proteins with roles in adhesion to host cell structures.[34] However, further experiments are needed to determine whether the effects observed *in vivo* can be achieved by the colonization of mice by *L. casei* LOCK0919 alone. Although the *L. rhamnosus* strains represented a minority of the strains present in the feces of the colonized mice, we cannot exclude that they may play an important role in the immunomodulatory activity of the mixture and that they are necessary for the successful reduction of allergic sensitization. This argument is supported by our recent finding that EPS produced by the *L. rhamnosus* LOCK0900 strain can modulate the cytokine production of BM-DCs induced by another bacteria.[56]

In conclusion, we have shown that the three *lactobacillus* strains in Lmix, *L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908 and *L. casei* LOCK0919, were able to reduce sensitization to Bet v 1. The specific serum IgE and IgG levels as well as the production of the pro-allergic cytokines IL-4 and IL-5 by splenocytes and MLN cells were also reduced. This suppression was accompanied by the upregulation of the regulatory cytokine TGF-β and the improvement of the epithelial gut barrier. These results clearly demonstrate the beneficial roles of the selected lactobacillus strains in the process of allergic sensitization and support their uses in the early prevention of allergies.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

HK, MS and BC designed the experiments. HK, MS, DS, IS and PH performed the experiments and analyzed the data. EC, IR and BC performed and analyzed the electron microscopy micrographs. ZZ performed and analyzed the western blot experiments. TA-P and AK-B performed and analyzed the qPCR experiments. TH performed and analyzed the immunohistochemistry experiments. HK, MS, LT, IS, HT-H and BC wrote the manuscript.

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8. GENERAL DISCUSSION

Intestinal commensal microflora plays a crucial role in human health and disease. It contributes to nearly every aspect of the host's physiological and immunological development; therefore many of the human diseases and dysfunctions are associated with an imbalance in bacterial composition, number or habitats of the intestinal microflora [1]. Several studies have illustrated that microbial dysbiosis is associated with a number of diseases particularly prevalent in the 21st century. These so called "civilization" or "life-style diseases" comprise IBD, allergy, obesity, hypercholesterolemia, cancer and others [1-3]. One of the possibilities to favourably alter the intestinal microbiota composition and to contribute to the maintenance of homeostasis is the administration of probiotics. Supplementation of probiotics has shown promising results in a large number of clinical studies involving IBD, antibiotic-associated diarrhoea, colon cancer and allergy reviewed in [1].

The studies discussed in this thesis provide new insight into how selected strains of commensal microflora contribute to the development, maturation and regulation of the host immune system and protect the host against development of disease in experimental models of IBD and allergic sensitization. We focused on two main objectives: 1) selection of the probiotic strains or their mixture, their characterization and determination of immunomodulatory properties *in vitro* and 2) evaluation of the *in vivo* activity of selected probiotic strains in experimental models of ulcerative colitis and allergic sensitization. Moreover we investigate, whether the immunomodulatory properties of probiotics achieved in the *in vitro* assays could predict their effect *in vivo*.

8.1 Characterization and identification of probiotic bacteria

Although commensals in the gut are often source of probiotic strains, until these strains are isolated, characterized and their health effects are verified, they cannot be called as probiotics [4]. Therefore, there has been growing interest in the exact characterization and identification of newly isolated strains with probiotic properties. Molecular-genetic techniques based on polymerase chain reaction (PCR) have become methods of choice for prompt and reliable bacterial identification and discrimination at both subspecies and strain levels. In **Chapter 3** we investigated four PCR-based methods (species- and subspecies-specific PCRs, random amplified polymorphic DNA (RAPD) method using 5 primers, repetitive extragenic palindromic (rep)-PCR with BOXA1R and $(GTG)_5$ primers and

amplified ribosomal DNA restriction analysis (ARDRA) and biochemical analysis for the subspecies classification of twenty-eight *B. longum* isolates from the faeces of healthy breast-fed infants and adults. Nevertheless, only ARDRA analysis succeeded in differentiation of analyzed strains into the *B. longum/infantis* subspecies using the cleavage analysis of genus-specific amplicon with just one enzyme, *Sau*3AI. Therefore we suggest this technique as the first method of choice for distinguishing between *B. longum* ssp. *longum* and *B. longum* ssp. *infantis*. Further we used species-specific PCR and restriction analysis (ARDRA) for identification of nine selected potentially probiotic bifidobacterial strains isolated from healthy humans in the study described in **Chapter 4**. We have shown that the strains, which could survive in milieu of GIT (low pH and bile salt acid), belong to the species *B. animalis*, *B. adolescentis*, *B. longum* ssp. *longum* and *B. longum* ssp. *infantis*. These PCR-based methods using 16S rRNA sequence offer rapid, reliable and powerful tools for identification of lactic acid bacteria and bifidobacteria without the necessity of time/money consuming sequencing [5].

8.2 Immunomodulation by potentially probiotic bacteria

Although some mechanisms underlying probiotic effect such as the production of useful metabolites or enzymes are widespread across the taxonomic groups, other effects, such as immunomodulatory potential, are more likely to be strain-specific [4]. Nevertheless, it is necessary to understand the mechanism involved in modulation of immune responses by probiotic strains, which may represent an important factor for their therapeutic or preventive application. In **Chapter 4, 5, 6** and **7** we focused on *in vitro* and *in vivo* studies of immunomodulatory properties of selected strains of the genera *Bifidobacterium*, *Lactobacillus* and *Clostridium*.

In **Chapter 4** we have tested nine selected bifidobacterial strains for their immunomodulatory properties assessed by stimulation of spleen cell cultures *in vitro*. We have found that cytokine production of spleen cells stimulated with different bifidobacterial strains is strain-specific and differ even among the strains belonging to the same species or subspecies. We have shown that two *B. longum* ssp. *longum* strains have completely different capacity to drive the response of immune cells *in vitro*. *B. longum* strain CCDM 372 induced high levels of pro- and anti-inflammatory cytokines in comparison to the strain CCM 7952 and seems to be strong stimulatory agent of immune response in both splenocytes and BM-DCs. On the other hand, the strain CCM 7952 induces only elevated level of anti-inflammatory cytokine IL-10 in spleen cell cultures. This strain also did not trigger the high

expression of co-stimulatory molecules (CD40, CD80 and CD86) in BM-DCs which indicates the small effect on maturation of these cells. Therefore we suppose that the strain CCM 7952 could induced the differentiation into tolerogenic DCs which are associated with a partial or failed maturation of these cells [6]. This is supported by recent study [7] showing that immature DCs and/or IL-10-producing DCs are considered tolerogenic and able to induce Treg cells. Moreover, in **Chapter 6** we have shown, that the strain *B. longum* CCM 7952 was able to induce the production of regulatory cytokines (IL-10, TGF-β) from BM-DCs and their production is dependent on TLR2/MyD88 signaling pathway.

The effect of formalin-inactivated strains *L. rhamnosus* LOCK0900 and LOCK0908 and *L. casei* LOCK0919 and their mixture on cytokine production in BM-DC cultures was assessed in **Chapter 7**. We have demonstrated that the production of IL-10, IL-12p70 and TNF-α was strictly lactobacilli-strain dependent and the cytokine production by Lmix-stimulated BM-DCs corresponded with the average value of all applied bacterial strains. In this respect, recent studies [8, 9] have shown that forty-two *L. plantarum* strains differed considerably in their ability to induce pro- and anti-inflammatory cytokines. Authors have suggested that the diversity in immunomodulatory properties of different strains of the same species correlate with the strain-specific genomic composition. Interestingly in our study, the strain *L. casei* LOCK0919, which was the most robust inducer of cytokines in BM-DC analysis, became the dominant strain in faeces of Lmix-colonized GF mice in *in vivo* experiment. Recent analysis of complete genome sequence of the LOCK0919 strain revealed the presence of factors relevant to the adhesion to host cell structures, which might have effect on immunomodulatory and the colonization capacity of this strain [10]. Furthermore, the strains, which were found to produce high amount of exopolysaccharides (EPS) (*L. rhamnosus* LOCK0900 and LOCK0908, *B. longum* ssp. *longum* CCM 7952) were poor inducers of cytokines in *in vitro* analyses. This is in agreement with recent findings of Fanning *et al*. [11] showing that bifidobacterial strain producing surface EPS failed to elicit a strong immune response compared with EPS-deficient variant. This is also supported by our recent finding that EPS produced by *L. rhamnosus* LOCK0900 strain can down-regulate cytokine production of BM-DCs induced by other bacteria [12].

8.3 Recognition of probiotic bacteria by pattern recognition receptors

The potential of the various types of cells to recognize, capture and process antigens is mediated by pattern recognition receptors (e.g. TLR and NOD families) [13]. They generally recognize molecular structures shared by a variety of bacterial species, which are referred to

as microbe-associated molecular patterns (MAMPs) [14]. Recognition of commensal bacteria by TLRs plays an important role in maintaining intestinal homeostasis and contributes to the prevention of intestinal injury. Deregulated interaction between molecular pattern of bacteria and TLRs may promote chronic inflammation and tissue damage [15]. Differential activation of TLRs by multiple MAMPs could induce complex intracellular signal cascades leading to different modulation of cytokine production [16, 17]. To investigate the involvement of TLRs and NOD2 receptors in sensing of analyzed *B. longum* and *Lactobacillus* strains, we co-cultivated these strains together with human embryonic kidney cells (HEK) 293 stably transfected with TLR2, TLR4 or NOD2. Our data in **Chapter 4, 6** and **7** demonstrate that TLR2 is an important receptor for recognition of both *B. longum* strains CCDM 372 and CCM 7952, *L. rhamnosus* strains LOCK0900 and LOCK0908 and *L. casei* strain LOCK0919. Signaling through this receptor is dose dependent. In addition, the strains *B. longum* CCDM 372 and *L. casei* LOCK0919, which stimulated the production of high amount of cytokines *in vitro* in splenocytes or BM-DC cultures, were able to strongly activate NOD2 receptor on HEK293 cells. On the other hand, the strains *B. longum* CCM 7952 and *L. rhamnosus* LOCK0900, which induced low level of cytokine response in splenocytes or BM-DCs, also stimulate moderate response in HEK 293 cells transfected by NOD2. Recent studies illustrated that NOD2 receptor detects muramyl dipeptides presented in all peptidoglycan of all bacteria, and even species possessing the same pentapeptide bridge can induce different NOD2-dependent immune response [18, 19]. NOD2 is one of the critical factors in regulation of the bacterial load in the intestine. One way is through the activation of NF-κB and mitogen–activated protein kinase signaling cascades leading to induction of immune response of genes encoding pro-inflammatory cytokines and chemokines. A balanced relationship between microbiota and the mucosal immune system with the signalization through TLRs and NOD receptors is one of the key factors to sustain gastrointestinal homeostasis [20, 21].

8.4 Probiotic bacteria improved the epithelial barrier dysfunction

In humans, microbial colonization of intestinal mucosa occurs during the first days after the birth and is connected to the so called “gut closure” — the establishment of a mature epithelial barrier that prevents excessive influx of macromolecules across the intestinal epithelium [22]. In **Chapter 7** we have demonstrated that in GF mice the brush border and apical junctional complexes of enterocytes were irregularly arranged and exhibited the deficit of cytoskeletal microfilaments without elongation into the terminal web compared to conventional mice. Colonization of GF mice by mixture of *L. rhamnosus* LOCK0900 and

LOCK0908 and *L. casei* LOCK0919 directly promoted the maturation of intestinal barrier and brush border structures to the similar level found in conventional mice.

A breakdown or impairment of epithelial barrier has been associated with chronic immune diseases, including IBD, food allergy and celiac disease. Although altered intestinal barrier function can be a consequence of disease exacerbation, clinical evidence suggests that it might be a primary causative factor predisposing to disease development [23]. Understanding of the interactions between innate and adaptive immunity and intestinal barrier function will provide important insight into the pathogenesis of inflammatory and autoimmune diseases and will have important clinical implications for the development of preventive or therapeutic agents targeted at modulating intestinal barrier function.

Recent data indicates that some probiotics may initiate repair of the barrier function after damage. *E. coli* Nissle 1917 is able to prevent disruption of the mucosal barrier by enteropathogenic *E. coli* and restore the mucosal integrity due to the enhanced expression and redistribution of the TJ proteins [24]. Likewise, *L. casei* DN-114001 [25] and probiotic mixture VSL#3 [26] are able to sustain the intestinal barrier function. Recent study in human volunteers showed that administration of *L. plantarum* is able to increase the localization of occludin and zonula occludens (ZO)-1 in the epithelial TJs of tissue biopsies from IBD patients [27]. Concomitantly, the epithelial TLR2 activation has been described to protect against barrier disruption by enhancing ZO-1 expression in IEC in a protein kinase C-dependent manner [28]. Moreover, Cario *et al*. [29] showed that administration of TLR2 ligands protect from induction of experimental colitis and thus enhance the homeostasis in the intestinal epithelium. In contrast, Li *et al*. [30] have shown that activation of TLR4 increases intestinal permeability and results in enhanced bacterial translocation. In **Chapter 4** we have shown that, although both analyzed *B. longum* strains have ligands for TLR2 signaling pathway, only CCM 7952 strain, but not CCDM 372 is able to preserved the expression of TJs protein (ZO-1 and occludin) in the colon of mice and improved the gut epithelial barrier in the mouse model of DSS-induced colitis. Therefore we suggest that TLR2 activation is not the critical factor for protection against disruption of epithelial barrier in the intestine.

8.5 Prophylactic effect of probiotic bacteria in model of DSS-induced ulcerative colitis

In our studies we used experimental model of acute colitis induced by solution of DSS in drinking water. DSS solution cause rapid alterations of the epithelial layer within the first day of administration with the downregulation and/or reorganization of barrier proteins and

increased permeability to the intestinal microbiota [31-33]. Subsequent translocation of commensal bacteria through lamina propria elicits a strong inflammatory response, due to basolateral TLR stimulation, activation of the NF-κB pathway and following induction of pro-inflammatory chemokine and cytokine secretion [34]. The extensive inflammatory response to the microbiota triggers the changes in structure and function of intestinal mucosa leading to specific IBD-associated symptoms. Nevertheless, modulation of IBD-associated dysbiosis has been demonstrated by probiotic, prebiotic and synbiotic supplementation. Treatment of CD or UC patients with the probiotic strain *E. coli* Nissle 1917, *Sacchromyces boulardii* or probiotic mixture VSL#3 has been shown to induce remission more rapidly compared to untreated control patients, and the maintenance of remission was higher after probiotic supplementation [35-37]. In the **Chapter 4** and **5** we have shown that the administration of some potentially probiotic strains ameliorate the symptoms of acute DSS-induced colitis in mice. In both studies we have shown the down-regulation of pro-inflammatory cytokines, mainly TNF-α, which was described to be involved in mechanism of epithelial barrier disruption.

In **Chapter 4** we investigated two *B. longum* ssp. *longum* strains for their ability to prevent against development of experimental colitis. We demonstrated that administration of the strain *B. longum* CCDM 372, which induced high expression of pro- and anti-inflammatory cytokines *in vitro*, failed to protect against DSS-induced colitis. On the other hand, administration of the strain CCM 7952, which induced in *in vitro* analysis low level of pro-inflammatory and moderate level of anti-inflammatory cytokines, prevents against development of severe intestinal inflammation in experimental mice through the improved expression of TJs protein (ZO-1 and occludin) and preserved epithelial barrier function.

In **Chapter 5** we have demonstrated that intrarectal administration of butyrate producing *C. tyrobutyricum* strain prevented appearance of the symptoms of DSS-colitis in mice, protected against impairment of the TJs caused by DSS, restored normal MUC-2 production and decreased level of pro-inflammatory cytokines TNF-α and IL-18 in colon. It has been widely documented that microbially produced butyrate acts as an energy source for colonocytes, regulates mucosal immunity and modulates an oxidative stress and inflammation in colon, and therefore plays role in a prevention of intestinal inflammation and colorectal cancer [38]. Several studies have described that butyrate in low concentration is capable to decrease the intestinal permeability associated with increased expression of TJ proteins [39, 40]. Recently has been shown that SCFA can induce regulatory T cells in the colon and thus maintain homeostasis in the intestine [41-43].

In this chapter we have further demonstrated that the severity of colitis in SCID mice has been associated with limited production of biologically active form of IL-18. The lack of IL-18 secretion is compensated by increased secretion of inflammatory TNF-α. On the other hand, this cytokine was upregulated in the colon of DSS-treated BALB/c mice. The important role of IL-18 in modulation of immune response has been described by Takagi *et al*. [44]. It has been shown that overproduction of IL-18 or deletion of TNF-α exacerbate DSS colitis, while mice deficient for IL-18 develop severe colitis associated with high lethality [44, 45]. It has been documented that the mucosa-associated immune system is shaped by microbial dysbiosis in two distinct ways: the outgrowth of opportunistic bacteria which drives the enhancement of inflammation; and the loss of symbiotic commensal bacteria that are able to produce metabolites such as butyrate resulting in reduced immunoregulation [46].

8.6 Effect of selected probiotic bacteria on mouse model of birch pollen allergic sensitization

Several clinical studies demonstrated that the role of probiotic interventions in preventive strategies of allergic diseases have different outcomes [reviewed in 47]. The most of the studies claim that the probiotic intervention in prenatal/perinatal period appears to be crucial for manifestation of beneficial effects, confirming the existence of "window of opportunity" in the programming of the immune system in early life [48, 49]. In **Chapter 6** we used previously established [50] mouse model of neonatal mother-to-offspring monocolonization of originally germ-free mice. We investigated the impact of the strain *B. longum* CCM 7952 on the reprograming and the maturation of offspring's immune system. We have shown that mono-colonization with *B. longum* down-regulated specific allergic responses both on humoral and cellular levels, and thus prevented the development of allergen-specific immune responses in mouse model of allergic sensitization by birch pollen allergen Bet v 1. Our results suggest that the inhibition of sensitization was not achieved simply by immune shift toward the Th1 mode, but rather the general reduction of immune responses might take a place. Moreover, we have demonstrated that *B. longum* is a strain with the ability to induce regulatory T cell response. This is in agreement with recent studies showing that bifidobacteria are able to induce specific subsets of FoxP3+Treg cells [51] but also FoxP3- subset IL-10-producing type 1 regulatory cells (Tr1) [52] which interfere with the Th2 effector cells.

The gnotobiotic mouse model was also utilized in the **Chapter 7**, where we investigate the ability of the *Lactobacillus* mixture (Lmix) of the strains *L. rhamnosus*

LOCK0900, *L. rhamnosus* LOCK0908 and *L. casei* LOCK0919 to modulate allergic sensitization. We showed that colonization of GF mice with Lmix ameliorates Bet v 1-specific allergic responses both on humoral and cellular levels. Lmix colonization was able to reduce serum levels of both Th2-related Bet v 1-specific IgE and IgG1 antibodies as well as Th1-related IgG2a antibody, implicating the involvement of regulatory mechanisms. This was supported by significantly elevated level of regulatory cytokine TGF-β in serum. In accordance with our data, other authors showed that application of certain probiotic strains, such as *L. rhamnosus* LGG, *B. bifidum* G9-1 or *L. paracasei* NCC 2461 and *B. longum* NCC 3001, are able to induce a general immunosuppression of T cell responses rather than a shift of the allergen-specific Th2 responses towards the Th1 phenotype in mice model of allergy [48, 53-55]. In contrast, clinical study failed to show evidence for protective effect of LGG on atopic dermatitis or airway symptoms in young children [56]. Likewise, other authors did not find any improvement in birch pollen allergic patients treated orally with some probiotic lactobacilli strains [57]. Abrahamsson *et al*. [58] showed preventive effect of *L. reuteri* ATCC 55730 on infant IgE-associated eczema at 2 years of age. Nevertheless, this effect did not lead to a lower prevalence of respiratory allergic disease in school age in a seven-year follow-up of a randomized controlled trial [59]. These studies indicate that the beneficial effects of probiotics are dependent on the use of specific strains and on the timing of treatment. Recent meta-analyses have shown that the evidence for efficacy of probiotics is more convincing in prevention than in treatment [60-62].

8.7 Linking of *in vitro* assays and *in vivo* immune effects of probiotic bacteria

Although there have been several studies linking immunomodulatory properties of probiotic strain *in vitro* and its ability to prevent experimental colitis or allergic reaction in mice, no clear association has been established so far. *In vitro* co-culture experiments with different types of immune cells are generally considered as useful tools for selection of probiotic strains with specific immunomodulatory potential on cytokine production [63]. Production of IL-10 by probiotic strains may direct T cells towards Th2 or regulatory T cell response and is associated with protective effect of probiotics on inflammatory or allergic diseases [64]. Foligne *et al*. [65] demonstrated that probiotic strains with high IL-10/IL-12 ratio *in vitro* provided the best protection in an experimental model of TNBS-induced colitis. Similarly, Kwon *et al*. [66] demonstrated that administration of probiotic mixture with high levels of the IL-10/IL-12 suppressed the progression of experimental colitis in mice. Along

these lines, it has recently been shown that *B. longum* NCC 3001 (a probiotic strain with high IL-10/IFN-γ ratio) offered long term protection in a mouse model of birch pollen allergy [67].

In **Chapter 4** we employed both *in vitro* culture system and *in vivo* mouse model of DSS-induced colitis to compare the immunomodulatory potential of two probiotic strains which belong to the same subspecies *B. longum* ssp. *longum*. We found that the activity of these two strains, CCDM 372 and CCM 7952, differs significantly. The strain CCM 7952 with low stimulatory potential *in vitro* was able to prevent clinical symptoms in a mouse model of DSS-induced colitis. On the other hand, the strain CCDM 372 induced high level of pro- and anti-inflammatory cytokines *in vitro* and had no beneficial effect on DSS-induced colitis. These data are in agreement with the study by Mileti *et al*. [68] showing that the probiotic strain which is poor inducer of both pro- and anti-inflammatory cytokines from human monocyte-derived DC, but not strain with strong immunostimulatory properties, is protective against experimental DSS-induced colitis in mice.

In **Chapter 6** we have shown that stimulation of BM-DC with *B. longum* ssp. *longum* CCM 7952 triggered production of cytokines IL-10 and TGF-β. Moreover, we have demonstrated that neonatal colonization of germ-free mice with this probiotic strain increased levels of Foxp3+cells in MLN and regulatory cytokines IL-10 and TGF-β in serum, and prevented experimental sensitization in a mouse model of allergy. This evidence suggest, that the beneficial effect of the *B. longum* strain which is mediated by induction of Treg cells *in vivo*, could be predicted from *in vitro* co-culture assay using BM-DCs.

Nevertheless, most of the studies used a variety of "simplified" *in vitro* systems in which many potential players of the mucosal regulatory response were lacking. Therefore, the selection of beneficial probiotic strains only on the basis of *in vitro* immunomodulatory properties is insufficient and must be taken into correlation with *in vivo* outcomes.

8.8 Conclusion and future perspectives

Among the most important health benefits of probiotic bacteria belongs their capacity to interact with the immune system of the host. In this regard, this thesis helps to elucidate the effect of probiotic bacteria on mucosal immune system of the host in states of health or inflammatory and allergic disease. We identified and characterized newly isolated strains with potentially probiotic properties. The results from *in vitro* and *in vivo* studies strongly suggest that the effect of probiotics on immune system is highly dependent on the used bacterial strain. Their immunomodulatory properties vary considerably among the strains and they could not be extrapolated to other strains even within the same species or subspecies.

Therefore, we suggest that probiotic strains should be selected in comparative studies and the determination of the precise mechanism of their effects comes from the correlation of *in vitro* data with the outcomes *in vivo*.

Two experimental mouse models of civilization diseases (acute ulcerative colitis induced by DSS and allergic sensitization to birch pollen allergen Bet v 1) were utilized in this thesis to study the effect of selected probiotic strains on immune system. Modulation of mucosal immune responses, improvement of intestinal barrier function and correction of microbial dysbiosis are among the most anticipated mechanisms of probiotic effect. In **Chapters 3** and **4** we have investigated the effect of *B. longum* ssp. *longum* CCM 7952 and *C. thyrobutyricum* DSM 2637 on DSS-induced colitis. We have shown that not only selected probiotic strain, but also the route of probiotic application (intragastric vs. intrarectal), the dose of bacteria and the timing of their administration (prophylactic vs. therapeutic) must be taken in consideration. In both studies we have shown that the prophylactic administration of these selected probiotic strains preserves the expression of tight junction proteins, improve barrier function and prevented appearance of clinical symptoms of inflammation. **Chapters 5** and **6** clearly show that colonization with probiotic *B. longum* CCM 7952 or mixture of three lactobacilli strains (*L. rhamnosus* LOCK0900, LOCK0908 and *L. casei* LOCK0919) prevents the development of allergic sensitization to Bet v 1 in a mouse model of clinically relevant birch pollen allergy. Moreover, we suggest that these probiotic strains are able to induced regulatory immune response and inhibit allergic sensitization by general immunosuppression of T cell responses.

This thesis points out the beneficial role of selected probiotic strains *B. longum* ssp. *longum* CCM 7952, *C. tyrobutyricum* DSM 2637 or mixture of *L. rhamnosus* LOCK0900, LOCK0908 and *L. casei* LOCK0919 in the processes of acute ulcerative colitis and allergic sensitization, and thus suggests their use as novel strategies in prevention of IBD and allergic diseases in humans. Therefore, our further effort will be focused on identification and characterization of effector molecules from this probiotic bacteria which help to elucidate the molecular mechanism of interaction between probiotics and host immune system, and contribute to fully understanding of their mode of effect.

8.9 References

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APPENDIX A - ABREVITATIONS

AIM2	Absent in melanoma 2 receptor
AJs	Adherens junctions
Alum	Aluminium hydroxide
AMP	Antimicrobial proteins
APC	Antigen-presenting cells
Ara h 1	Seed storage protein from Arachis hypogaea (peanuts)
ARDRA	Amplified ribosomal DNA restriction analysis
BCR	B cell receptor
Bet v 1	Major birch pollen allergen
B-l	B lymphocyte
BM-DC	Bone marrow-derived dendritic cells
CCDM	Czech collection of dairy microorganisms
CCL20	Chemokine (C-C motif) ligand 20
CCM	Czech collection of microorganisms
CD 4	Cluster of differentiation 4
CD	Crohn's disease
CX3CR1	Chemokine (C-X3-C motif) receptor 1
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
DSS	Dextran sodium sulphate
EPS	Exopolysaccharide
ERK	Extracellular signal-regulated kinase
FAE	Folicle-associated epithelium
Fc	Antibody fragment, crystalizable
FcεRI	IgE high affinity receptor
FDC	Follicular dendritic cells
FoxP3	Forkhead box P3
GALT	Gut-associated lymphoid tissue
GATA3	Transcription factor specific for Th2 lineage
GF	Germ-free
GIT	Gastrointestinal tract
HEK	Human embryonic kidney cells
HEV	High endothelial venules
HSP	Heat shock protein
IBD	Inflammatory bowel disease
IDO	Indoleamine 2,3-dioxygenase
IEC	Intestinal epithelial cell
IFN	Interferon
Ig	Immunoglobulin
IKB	Inhibitor of NF-κB
IL	Interleukin
ILC	Inate lymphoid cells
iNOS	Inducible nitric oxid synthase
IRAK	Interleukin-1 receptor-associated kinase
JAM	Junctional adhesion molecules

JNK	One of the mitogen-activated protein kinases
kDa	Kilo Dalton
LAB	Lactic acid bacteria
LBP	LPS-binding protein
LOCK	Pure culture collection of the Technical university of Lodz, Poland
LP	Lamina propria
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
MAL	MyD88 adaptor-like
MALT	Mucosa-associated lymphoid tissue
MAMP	Microbe associated molecular pattern
MAPK	Mitogen-activated protein kinase
MC	Mast cell
M cells	Microfold cells
MF	Macrophage
MHC	Major histocompatibility complex
MLN	Mesenteric lymph nodes
MO	Monocyte
MyD88	Myeloid differentiation primary response gene 88
MyDC	Myeloid DC
N	Neutrophil
NF-κB	Transcription factor Nuclear factor kappa B
NK cells	Natural-killer cells
NLR	Nucleotide oligomerization domain-like receptor
NOD	Nucleotide oligomerization domain
p38	One of the mitogen-activated protein kinases
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
pDC	Plasmacytoid DC
PFGE	Pulse-field gel electrophoresis
Phl p 1	Major allergen from timothy grass (Phleum pretense)
PKC-ζ	Isoform of protein kinase C
PP	Peyer's patch
PPR	Pattern recognition receptor
RA	Retinoic acid
RAG2	Recombination activating gene 2
RAPD	Randomly amplified polymorphic DNA
Rep-PCR	Repetitive extragenic palindromic PCR
RFLP	Restriction fragment length polymorphism
RIG-I	Retinoic acid inducible gene I
RLR	Retinoic acid inducible gene I-like receptor
RORγt	Retinoic acid-related orphan receptor
rRNA	Ribosomal Ribonucleic acid
*Sau*3AI	DNA restriction enzyme from *Staphylococcus aureus*
SCFA	Short chain fatty acids
SCID	Severe combined immunodeficiency
SIgA	Secretory IgA
SIgM	Secretory IgM
STAT	Signal transducer and activator of transcription
T-bet	T-box transkription factor specific for Th1 lineage

TCR	T cell receptor
Tfh	Follicular helper T cell
TGF	Transforming growth factor
Th	T helper
TIR	Toll-interleukin 1 receptor
TJs	Tight juctions
T-l	T lymphocyte
TLR	Toll-like receptor
TNBS	2,4,6-Trinitrobenzene sulfonic acid
TNF	Tumor necrosis factor
TRAF	TNF receptor associated factors
TRAM	TRIF-related adaptor molecule
Treg	T regulatory lymphocytes
TRIF	TIR-domain-containing adapter-inducing interferon-β
TSLP	Thymic stromal lymphopoetin
UC	Ulcerative colitis
VSL#3	Probiotic mixture
ZO	Zonula occludens

APPENDIX B – CURRICULUM VITAE

Born: August 7, 1981, Přerov, Czech Republic

Education and Employment:

2001-2004: Masaryk University in Brno, Faculty of Science, Department of Experimental Biology, specialization Cell and Molecular Diagnostic – Bachelor of Science

2004-2005: Laboratory assistant, Clinical microbiology, Regional hospital Náchod

2005-2007: Masaryk University in Brno, Faculty of Science, Department of Experimental Biology, specialization Microbiology – Master of Science

2007-now: Institute of Microbiology of the CAS, v. v. i., Laboratory of Gnotobiology, Nový Hrádek, PhD project – Characterization and utilization of faecal microflora components in experimental models of human civilization diseases. Supervisor: RNDr. Hana Kozáková, CSc.

Memberships:

Member of Czech Immunological Society

Member of Czech Society of Allergology and Clinical Immunology

Stay:

2008 Department of Pathology, the Children's Memorial Health Institute, Warsaw, Poland

Patent:

2013 Patent number 304791 "Kmen bakterie *Bifidobacterium longum* CCM 7952 a jeho využití v lidské výživě"

Publications:
Total: 11
Sum of citations: 161
H-index (WoS): 4

APPENDIX C – LIST OF PUBLICATIONS

Srutkova D, Schwarzer M, Hudcovic T, Zakostelska Z, Drab V, Spanova A, Rittich B, Kozakova H, Schabussova I. *Bifidobacterium longum CCM 7952 promotes epithelial barrier function and prevents DSS-induced intestinal inflammation in strictly strain-specific manner*. PLoS ONE, 2015. 10(7):e0134050. doi:10.1371/ journal.pone.0134050. Epub July 28, 2015.

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of Clostridium tyrobutyricum in acute dextran sodium sulphate-induced colitis: differential regulation of tumour necrosis factor-alpha and interleukin-18 in BALB/c and severe combined immunodeficiency mice. Clin Exp Immunol, 2012. **167:**356-365.

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