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

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ABBREVIATIONS

ANOVA	One-Way Analysis of Variance
ARDRA	Amplified Ribosomal DNA Restriction Analysis
Bet v 1	Birch pollen allergen v1
BM-DC	Bone marrow-derived dendritic cells
CCDM	Czech Collection of Dairy Microorganisms
CCM	Czech Collection of Microorganisms
CD	Cluster of differentiation
CFU	Colony forming units
DAI	Disease activity index
DNA	Deoxyribonucleic acid
DSM	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DSS	Dextran sodium sulphate
ELISA	Enzyme-linked immunosorbent assay
EPS	Exopolysaccharide
FACS	Flow cytometry
FoxP3	Forkhead box P3
GF	Germ-free
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HEK	Human embryonic kidney
IBD	Inflammatory bowel disease
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
LOCK	Pure culture collection of the Technical University of Lodz, Poland
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MLN	Mesenteric lymph nodes
MRS	De Man, Rogosa and Sharpe medium
MUC-2	Mucus-2 protein
MyD88	Myeloid differentiation primary response gene 88
NOD	Nucleotide oligomerization domain
PCR	Polymerase chain reaction
RAPD	Randomly Amplified Polymorphic DNA
Rep-PCR	Repetitive extragenic palindromic PCR
rRNA	Ribosomal ribonucleic acid
SCFA	Short chain fatty acids
SCID	Severe combined immunodeficiency
TGF-β	Transforming growth factor-beta
Th/Treg	Helper T cells/natural regulatory T cells
TJ	Tight junction
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
ZO	Zonula occludens

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

Commensal microflora of the intestine represents open ecosystem formed by resident and transiently presented microbes, which interact with its host on mucosal surfaces [1]. The colonization 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 [2]. Several factors, such as life-style, diet, host genotype, use of antibiotics, co-infection and disease may lead to disturbed balance of beneficial and detrimental bacteria (dysbiosis) [3]. Dysbiosis leads to delayed maturation of the immune system and disruption in the mechanism of immunological tolerance, which could result in number of inflammatory, allergic or autoimmune diseases [4, 5]. Their increasing incidence in Western countries in last few decades has become a significant health burden, suggesting that environmental and lifestyle changes are a major factor in the development and progression of these diseases [6, 7].

According to the „ hygiene“ or „microflora“ hypothesis, 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 [8-10]. Absence of microbial stimulation as a consequence of increased hygiene or antibiotic use results in the reduction of mucosal immunity accompanied by more frequent sensitization to allergens, which are tolerated by healthy population [1]. Insufficient microbial stimulation of the immune system and subsequent changes in Th1/Treg cytokine profile can cause a shift towards Th2 response, which is responsible for development of allergies [6].

Inflammatory bowel disease (IBD), such as ulcerative colitis and Crohn's disease are idiopathic chronic relapsing disorders associated with uncontrolled inflammation within the gastrointestinal tract [11]. They are caused by failure of intestinal homeostasis and dysregulated immune responses to the resident microbiota as a consequence of host genetic defects in mucosal barrier function, innate bacterial killing, or immunoregulation [12, 13]. Increased epithelial permeability for luminal bacteria due to under-expression and/or reorganization of certain tight junction proteins (e.g. occludin, ZO-1) have been shown to precede the development of intestinal inflammation [14]. Decreased microbial diversity or changes in microbial composition, such as reduction of mucosa-associated *Bifidobacterium* and *Lactobacillus* species, along with an increased relative abundance of pathogenic bacteria has been widely documented in IBD patients [15-17].

Since IBD and allergies are clearly multifactorial and result from complex host-microbiota interactions, preventive strategies targeting the aberrant composition of the

intestinal microbiota may have the potential to tackle these diseases. In this regard, certain probiotic bacteria are able to improve imbalance of gut microbiota, restore mucosal barrier function and stimulate immune system, and thus they might be valuable tools for prophylaxis or therapy of these disorders [18]. Probiotics are defined as live microorganisms which when administered in adequate amounts, confer a health benefit to the host [19]. The selected probiotic strains of *Bifidobacterium* and *Lactobacillus* genera, *Escherichia coli* Nissle, *Saccharomyces boulardii* or probiotic mixture VSL#3 have been successfully used for the prevention and/or treatment of gastrointestinal inflammatory diseases [20, 21] or allergy [22-24]. However, it is getting clear that not only particular disease and the immunological status of the host, but also the selection of probiotic strain, time and mode of application are important factors to be taken into consideration [25].

Members of human commensal microbiota are often sources from which probiotics are isolated. In order to call bacterial strain probiotic, it has to be identified, characterized and beneficial effects must be verified [26, 27]. Progress in basic knowledge of probiotic strains and in understanding their mechanisms of action is required for the design of novel efficacious preventive/therapeutic approach. For these reasons, experimental animal models mimicking human diseases represent indispensable tools that enable a better understanding of IBD or allergic disease development and help to search their new preventive/therapeutic strategies.

2. HYPOTHESIS AND AIMS

This thesis provides insight into how newly 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 potentially 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.

Specific aims of the thesis:

- 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.

- Determination of the immunomodulatory capacity of nine bifidobacterial strains of human origin with potential probiotic properties; selection of two strains of *Bifidobacterium longum* subspecies with completely different immunomodulatory pattern and determination of their potential in prevention of experimentally induced ulcerative colitis.

- Investigation of the effect of butyrate-producing *Clostridium tyrobutyricum* on dextran sodium sulphate (DSS)-induced colitis in immunocompetent BALB/c and immunodeficient SCID mice

- Evaluation of effect of the neonatal mother-to-offspring colonization of germ-free mice with *Bifidobacterium longum* strain in prevention of the allergic sensitization to the birch pollen allergen.

- Characterization of the immunomodulatory properties of three individual *Lactobacillus* strains and their mixture (Lmix) in *in vitro* study and determination of the effects of Lmix on the development of allergic sensitization in a gnotobiotic mouse model.

3. MATERIAL AND METHODS

3.1 Bacterial strains and culture conditions

Bifidobacterium strains were cultivated anaerobically in MRS broth supplemented with L-cysteine-hydrochloride (0.5 g/l) at 37 °C. *Clostridium tyrobutyricum* strain was cultured in Bryant Burkey bouillon with resazurin and lactate at 37°C under anaerobic conditions. *Lactobacillus* strains were cultured in MRS broth at 37°C under aerobic conditions. List of analysed bacterial strains is shown in Table 1.

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 and stored at -40°C or +4 °C. For the administration to mice in *in vivo* experiments, fresh overnight cultures were washed in sterile saline and their concentrations were adjusted to 10^9 CFU/ml.

Table 1: List of analysed bacterial strains

Strain ID	Classification	Isolation source	Articles in the thesis
RB 17, RB20A V6A6, RB45P, RB46P, RB47, RB50P, RB52P RB4V, RB25, RB47, RB50, RB52, RB27, RB37, RB21, RB23, RB12MPP, RB14P, RB17P, RB18P, RB18P V6A6, RB19A, RB19B, RB34P, RB38AP, RB38BP, RB65 6A, RB65 6B	*B. longum* strains	Faeces of breast-fed infants and adults	**Article I** Šrůková *et al*. 2011
CCM 7952, CCDM 218, CCDM 366, CCDM 368, CCDM 369, CCDM 370, CCDM 371, CCDM 372, CCDM 373	*Bifidobacterium* strains	Faeces of breast-fed infants and adults	**Article II** Šrůková *et al*. 2015
DSM 2637	*C. tyrobutyricum*	Raw cow's milk	**Article III** Hudcovic *et al*. 2012
CCM 7952	*B. longum* ssp. *longum*	Faeces of breast-fed infant	**Article IV** Schwarzer *et al*. 2013
LOCK0900	*L. rhamnosus*	Faeces of infants	**Article V** Kozáková *et al*. 2015
LOCK0908	*L. rhamnosus*		
LOCK0919	*L. casei*		

3.2 Identification of *Bifidobacterium* strains

Bifidobacterial DNA was isolated from crude bacterial cell lysates by phenol (pH 7.8) and chloroform: isoamyl alcohol (24:1) extraction according to Sambrook and Russell [28]. The genus *Bifidobacterium* was confirmed by PCR with specific primers Pbi F1/Pbi R2 described by Roy and Sirois [29]. The species and subspecies identification was carried out by biochemical test using API 50CHL kit and Apiweb software (bioMérieux, France), and four PCR-based methods: 1) Species- and subspecies-specific PCR using primers developed from 16S rRNA gene sequences [29, 30]; 2) Amplified ribosomal DNA restriction analysis (ARDRA) of genus-specific PCR product using endonucelases *Alu*I, *Bam*HI, *Nci*I, *Sau*3AI, *Sau*96I and *Taq*$^{\alpha}$I [31, 32]; 3) Random amplified polymorphic DNA (RAPD) using five random decamer primers [33-35]; and 4) Repetitive sequence-based (rep)-PCR with primers $(GTG)_5$ and BOXA1R [36, 37]. The PCR products were separated by agarose gel electrophoresis. The fingerprinting profiles were analysed by the programme Gel Compare II and dendrograms were constructed using UPGMA analysis and Pearson or Jaccard correlation coefficient. (**Articles I, II**)

3.3 Determination of immunomodulatory properties by *in vitro* experiments

Immunomodulatory potential of nine selected *Bifidobacterium* strains was tested *in vitro* on splenocytes isolated from naïve BALB/c mice. Spleen cells were stimulated with

formalin-inactivated bifidobacteria (1:10), Pam3CSK4 or media alone 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, USA) and analysed with Bio-Plex System (Bio-Rad Laboratories, USA). **(Article II)**

Mouse bone marrow-derived dendritic cells (BM-DC) derived from naïve BALB/c mice were prepared from precursors isolated from femurs and tibias and cultured in medium containing RPMI 1640, FCS, gentamycin and mouse GM-CSF (Sigma-Aldrich) for 8 days. BM-DC were stimulated with formalin-inactivated bifidobacteria or lactobacilli strains (10^6 or 10^7 CFU/well), Pam3CSK4, ultrapure LPS or left untreated for 18 h. The levels of cytokines were analysed in supernatants of stimulated cells by ELISA using Ready-Set-Go! kits (eBioscience, USA). For cell surface marker analysis, BM-DC were labelled with anti-mouse FITC-conjugated CD11c, APC-conjugated MHC II and PE-conjugated CD40, CD80 or CD86 monoclonal antibodies (eBioscience). The data were acquired on a BD FACSAria III flow cytometer (BD Biosciences, USA) and analysed with FlowJo software 7.6.2 (TreeStar, USA). **(Articles II, IV, V)**

3.4 Stimulation of HEK 293 cells stably transfected with TLRs and NOD2

HEK 293 cells stably transfected with plasmid carrying human genes hTLR2/CD14, hTLR4/MD2/CD14 or hNOD2 were stimulated for 20 h with Pam3CSK4, LPS, muramyl dipeptide and formalin-inactivated bifidobacteria or lactobacilli strains at concentrations of 10^6, 10^7, or 10^8 CFU/ml. Concentrations of IL-8 were determined in cell supernatants by ELISA (Thermo Scientific, USA). (**Articles II, IV, V**)

3.5 Mice

All experimental mice (8-10 weeks of age; BALB/c or SCID) were housed under conventional, SPF or germ-free (GF) conditions, exposed to 12 : 12-h light-dark cycles, fed with standard pellet diet (ST1, Kocanda, Czech Republic) and tap water *ad libitum*. Stool samples of GF mice were checked every two weeks, to control bacterial contamination. All experiments were approved according to the Animal Experimentation Ethics Committee of the Institute of Microbiology of the Academy of Sciences of the Czech Republic.

3.6 Experimental model of acute DSS-induced colitis

In order to analyse the anti-inflammatory effect of *B. longum* strains, we administered intragastrically $2x10^8$ CFUs of *B. longum*, spp. *longum* CCM 7952 or CCDM 372 strains in saline for ten consecutive days prior to colitis was induced. The *C. tyrobutyricum*-treated mice received intrarectally a daily dose of $2x10^8$ CFU of strain DSM 2637 in saline for 7 days prior to DSS exposure and during the 7 days exposure to DSS solution. Controls received saline only. Experimental acute colitis was induced by drinking of 2.5% DSS in water (MW 40 kDa; ICN Biomedicals, USA) for 7 days. Clinical symptoms of inflammation (firmness of faeces, rectal prolapses, rectal bleeding and body weight decrease) were evaluated daily, degree of colitis was determined as disease activity index (DAI) according to Cooper *et al*. [38]. Length of colon was measured and segments of colon descendens were fixed in 4% buffered paraformaldehyde or Carnoy's fluid or frozen for histological and immunohistochemical analysis. **(Articles II, III)**

3.7 Experimental model of allergic sensitization

Neonatally *B. longum*-colonized female mice and GF controls were subcutaneously sensitized on days 1, 14 and 28 of experiment with 1 μg of recombinant birch pollen allergen Bet v 1 (Biomay, Austria) emulsified in $Al(OH)_3$ (Serva, Germany). Eight-week-old GF mice were colonized by intragastric tubing with $2x10^8$ CFU of equal parts of overnight cultures of *L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908 and *L. casei* LOCK0919 in 0.2 ml of sterile saline. The lactobacilli-colonization was checked in regular intervals by plating of the mice stool on MRS agar (Oxoid, UK) and strain-specific qPCR. Three weeks after colonization, the *Lactobacillus*–colonized mice and GF controls were intraperitoneally immunized three times with 1 mg of the Bet v 1 adsorbed to $Al(OH)_3$ at 10-day intervals. Seven days after the last immunization, mice were killed by CO_2 asphyxia and samples were taken for further analysis. **(Articles IV, V)**

3.8 Histological and immunohistochemical evaluation

Paraffin-embedded sections of colon descendens were cut and stained with haematoxylin and eosin or Alcian Blue and post-stained with Nuclear FastRed (Vector, USA) for mucin production. The degree of damage of the surface epithelium, crypt distortion and mucin production were evaluated according to Cooper *et al*. [38]. **(Articles II, III)**

Cryosections of acetone-fixed colon were used for immunohistochemical determination of expression of CD 11b, ZO-1 and MUC-2. **(Article III)**

3.9 Evaluation of intestinal barrier function

For immunohistochemical determination of ZO-1 and occludin in colon of *B. longum* treated mice, deparaffined colon sections were stained with polyclonal rabbit anti-ZO-1 or anti-occludin (ZYMED Laboratories Inc., USA), incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (Jackson ImmunoLabs., USA) and stained by AEC chromogen (Dako, USA). **(Article II)**

To evaluate the effect of Lmix colonization on the intestinal barrier, ultrastructural analyses of the apical portion of ileal enterocytes were performed by transmission electron microscopy. The widths and lengths of the intracellular junctions were measured using the morphometric iTEM program (Olympus Corporation, Japan). **(Article V)**

Western blot analysis of ZO-1 and occludin was performed in the segments of colon descendens or terminal ileum. Briefly, segments of intestine were homogenized in protein extract buffer with a protease inhibitor cocktail (Thermo Fisher Scientific, USA). Protein concentrations were measured using a BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Western blotting was performed as previously described [39] with antibodies against occludin, ZO-1 (Thermo Fiher Scientific, USA) and β-actin (Abcam, UK). The reactions were developed using a SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, USA), and the signal intensities were measured with a G:BOX (Syngene, UK) and processed with the ImageJ program. **(Articles II, V)**

3.10 Evaluation of the intestinal permeability *in vivo*

The intestinal permeability was measured by spectrophotofluorometrical determination of the amount of FITC-dextran in blood. Briefly, mice were intragastrically gavaged by *B. longum* ssp. *longum* CCM 7952 or saline for ten consecutive days prior to induction of DSS-colitis. On the last day of DSS administration, each mouse was intragastrically gavaged by FITC-dextran (MW 4.0 kDa; Sigma-Aldrich). Serum levels of FITC-dextran were determined 5 hours after administration and quantified by serially diluted FITC-dextran as standard. **(Article II)**

3.11 Cellular immune responses

Spleen and MLN cell suspensions were prepared and cultivated in RPMI medium at 37 °C under 5% CO_2 for 48 h. Where indicated, the spleen and MLN cells were restimulated with Bet v 1. Levels of cytokines in culture supernatants were measured by the MILLIPLEX

MAP Mouse Cytokine/Chemokine Panel and analysed with the Bio-Plex System (Bio-Rad Laboratories, USA). **(Articles II, IV, V)**

Pre-weighed colonic fragments were cultured in RPMI medium. Quantification of TNF-α level was performed by ELISA (R&D Systems, USA). Evaluation of IL-18 production in colon descendens was carried out by confocal fluorimetry. **(Article III)**

TGF-β was measured in culture supernatants by ELISA (R&D Duoset Systems, USA). Regulatory FoxP3+ T cells were determined in single cell suspensions of spleens or MLN by flow-cytometry analysis using FACS Calibur flow cytometer (Becton-Dickinson, USA) and FlowJo 7.6.2 software (TreeStar, USA) as described previously [40]. **(Articles IV, V)**

3.12 Humoral immune responses

Serum levels of anti-Bet v 1 IgE, IgG1, IgG2a and IgA were measured by ELISA (Pharmingen, USA) and the activity of Bet v 1-specific IgE in serum was measured by rat basophile leukemia cells degranulation assay as previously described [41]. Levels of total IgE and IgA in serum were measured by ELISA (Bethyl, USA). Levels of IL-10 and TGF-β in serum were measured by ELISA Ready-Set-Go! kits (eBioscience, USA). Small intestine was excised, gut lavage was prepared as described previously [42] and levels of total IgA and TGF-β were measured by ELISA. IgA-producing cells in terminal ileum of Lmix-treated mice were determined by immunohistochemical staining with a goat anti-mouse IgA-FITC antibody (Thermo Fisher Scientific, USA). (**Articles IV, V**)

3.13 Statistical analysis

Differences between more experimental groups were assessed by ANOVA with Tukey's comparison test. Differences between two groups were evaluated using an unpaired two-tailed Student's t-test or using nonparametric Mann-Whitney test. The statistical evaluation was used GraphPad Prism software (version 5.03, GraphPad Software, USA).

4. RESULTS

This thesis is based on five following articles:

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

Šrůtková D, Španová A, Špano M, Dráb V, Schwarzer M, Kozáková H, Rittich B. *Journal of Microbiological Methods*, **2011**. 87: p. 10–16.

In this study 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.

- **Species- and subspecies-specific PCRs and rep-PCR showed a high degree of genetic homogeneity of analysed strains and did not show any discrimination potential for their resolution into investigated subspecies.**
- **By RAPD using 5 primers, the strains formed profile with many separate clusters without any potential for subspecies discrimination.**
- **ARDRA succeeded in differentiating of analysed strains into the *B. longum/infantis* subspecies using the cleavage analysis of genus-specific amplicon with just one enzyme - *Sau*3AI.**
- **According to our results the majority of the *B. longum* isolates from breast-fed infants belong to the *B. longum* ssp. *infantis* (75%).**

We showed that ARDRA is easy-to-perform molecular technique with 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 *Sau*3AI restriction enzyme as the first method of choice for distinguishing between *B. longum* ssp. *longum* and *B. longum* ssp. *infantis*.

Article II ***Bifidobacterium longum* CCM 7952 promotes epithelial barrier function and prevents acute DSS-induced colitis in strictly strain-specific manner.**

Šrůtková D, Schwarzer M, Hudcovic T, Zákostelská Z, Dráb V, Španová A, Rittich B, Kozáková H, Schabussová I. *PLoS ONE*, **2015**, 10(7): e0134050. doi:10.1371/ journal.pone.0134050. Epub July 28, 2015.

In this study we addressed the question how specific immunomodulatory properties of probiotic strains could correlate to their potential in prevention of inflammatory disorder. In co-culture experiment we determined the immunomodulatory capacity of nine bifidobacterial strains of human origin with potential probiotic properties (resistance to low pH and resistance to bile salt). Based on the cytokine profiles, we selected two strains of *B. longum* ssp. *longum* (CCDM 7952 and CCM 372) with completely different immunomodulatory pattern and analysed their potential to prevent the experimentally induced ulcerative colitis.

- **Strains of the genus *Bifidobacterium* were classified into *B. longum* ssp. *longum, B. longum* ssp. *infantis*, *B. animalis* and *B. adolescentis* by PCR-based methods.**
- **The *in vitro* stimulation of spleen cells of BALB/c mice with nine *Bifidobacterium* strains revealed distinct and strain-specific pattern of cytokine production.**
- **Strains *B. longum* ssp. *longum* CCDM 7952 and CCM 372 have differential ability to activate dendritic cells *in vitro*.**
- **Both CCDM 7952 and CCM 372 signal through TLR2 and NOD2 receptor.**
- **Prophylactic application of CCM 7952, but not CCDM 372 strain, alleviates acute DSS-induced colitis which was demonstrated by amelioration of DAI, reduction of DSS-induced colon shortening, weight loss and histopathological changes in mucosa or epithelial layer and downregulation of TNF-α in the mesenteric lymph node cells.**
- **CCM 7952 preserves the expression of ZO-1 and occludin and decreases colon permeability in DSS-treated mice.**

This study showed that immunostimulatory properties of probiotic strains of the genus *Bifidobacterium* are strictly strain-specific. Further we demonstrated that prophylactic administration of probiotic strain *B. longum* ssp. *longum* CCM 7952 is capable to preserve the disruption of tight junction 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.

Article III 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.

Hudcovic T, Kolínská J, Klepetář J, Štěpánková R, Řezanka T, **Šrůtková D**, Schwarzer M, Erban V, Du Z, Wells JM, Hrnčíř T, Tlaskalová-Hogenová H, Kozáková H. *Clinical and Experimental Immunology*, **2012**, 167: p. 356–365.

In this study we evaluated the protective effect of butyrate-producing bacterium *Clostridium tyrobutyricum* DSM 2637 on experimentally induced colitis and we investigated the role of the immune response in regulation of colitis in SCID and BALB/c mice.

- **Intrarectal administration of *C. tyrobutyricum* prior to the onset of acute DSS-induced colitis protected both immunocompetent BALB/c and immunodeficient SCID mice from histological damage, shortening of the colon and decreased mucin production.**
- **In both BALB/c and SCID mice, intrarectal administration of *C. tyrobutyricum* prevented the reduction of MUC-2 protein observed in DSS-induced colitis.**
- ***C. tyrobutyricum* protected against impairment of the TJs and preserves the expression of ZO-1 in the colon of both BALB/c and SCID mice treated by DSS.**
- **BALB/c mice produced significantly elevated IL-18 in colon of DSS-induced colitis, whereas the severity of colitis in SCID mice was associated with limited production of biologically active form of IL-18.**
- ***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 of SCID mice which lacked inflammation-associated expression of IL-18.**
- **In BALB/c mice the expression of TNF-α in inflamed colon was lower in comparison to SCID mice. 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.**

This study demonstrated that in the DSS model, the severity of inflammatory symptoms depends on host immune response. *C. tyrobutyricum* protection against destruction of mucosal

barrier is equally effective in immunodeficient SCID mice and immunocompetent BALB/c mice. Production of cytokines IL-18 and TNF-α in acute DSS-colitis depends largely on immune cell repertoire of the host. The effect of *C. tyrobutyricum* in suppressing high levels of both Th1 cytokines appears promising in prophylaxis of acute colitis.

Article IV 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. *Vaccine*, **2013**, 31: p. 5405– 5412.

In this study we investigated the prophylactic effect of neonatal mother-to-offspring mono-colonization with *Bifidobacterium longum* ssp. *longum* CCM 7952 on subsequent allergic sensitization to allergen Bet v 1 in a model of Type I allergy.

- **Neonatal colonization of mice with *B. longum* reduced the allergic sensitization both at the cellular and humoral level.**
- **The mechanism of *B. longum* effect on allergic sensitization involved induction of regulatory rather than the Th1 immune response.**
- ***In vitro*, *B. longum* induced low maturation status and increased production of regulatory cytokines IL-10 and TGF-β from BM-DC.**
- **This induction was dependent on signalling via TLR2 and MyD88.**

Our results demonstrated that neonatal mono-colonization with *B. longum* reduces allergic sensitization, likely by activation of regulatory responses via TLR2, MyD88, and MAPK signalling pathways. Thus, *B. longum* strain might be a promising candidate for perinatal intervention strategies against the occurrence of allergic diseases in humans.

Article V Colonization of germ-free mice with the mixture of three *Lactobacillus* strains enhances the integrity of gut mucosa and ameliorates allergic sensitization.

Kozáková H, Schwarzer M, Tučková L, **Šrůtková D**, Czarnowska E, Rosiak I, Hudcovic T, Schabussová I, Hermanová P, Zákostelská Z, Aleksandrzak-Piekarczyk T, Koryszewska-

Baginska A, Tlaskalová-Hogenová H, Cukrowska B. *Cellular & Molecular Immunology*, 2015, p. 1–12.

Here, we analysed the immunomodulatory properties of three *Lactobacillus* strains *L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908 and *L. casei* LOCK0919 and the impact of their mixture on allergic sensitization to Bet v 1 using a gnotobiotic mouse model.

- **Colonization with Lmix ameliorated Bet v 1-specific allergic responses at both the humoral and cellular levels.**
- **Colonization with Lmix induced systemic and local IgA production and up-regulated the production of regulatory cytokine TGF-β.**
- **Colonization with Lmix improved the intestinal barrier by strengthening the apical junctional complexes of enterocytes and restoring the structures of microfilaments extending into the terminal web.**
- ***L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908 and *L. casei* LOCK0919 are recognized via TLR2 and NOD2 receptors and stimulated BM-DCs to produce cytokines in species- and strain-dependent manners.**
- **Strain *L. casei* LOCK0919 became the dominant strain in faeces of Lmix-colonized GF mice.**

The mixture of *L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908 and *L. casei* LOCK0919 is able to reduce sensitization to Bet v 1, and shows potential for use in the prevention of increased gut permeability and the onset of allergies in humans.

5. DISCUSSION

Mounting evidence suggests that commensal microflora plays a crucial role in health and disease in humans. It contributes to nearly every aspect of the host's physiological and immunological development; therefore many of the human diseases such as IBD, allergy, obesity, hypercholesterolemia, cancer and others are associated with an imbalance of the intestinal microflora [1]. One of the possibilities to favorably alter the intestinal microbiota composition and to contribute to the maintenance of homeostasis is the administration of probiotics [18].

5.1 Identification of *Bifidobacterium* strains by PCR-based methods

Although commensals in the gut are often source from which probiotics are isolated, until these bacterial strains are identified, characterized and their health effects are verified, they cannot be called as probiotics [26]. Therefore, there has been growing interest in the exact characterization and identification of newly isolated strains with probiotic properties. Molecular-genetic techniques based on PCR have become methods of choice for prompt and reliable bacterial identification and discrimination at both subspecies and strain levels [43]. In our work we investigated four PCR-based methods (species- and subspecies-specific PCRs, RAPD, rep-PCR and ARDRA) and biochemical analysis for the subspecies classification of twenty-eight *B. longum* isolates from the faeces of healthy breast-fed infants and adults and for identification of nine selected potentially probiotic bifidobacterial strains. According to several studies (reviewed in [43]), the 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. 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 [29, 30, 44]. Nevertheless, in our study 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 subspecies *longum* and *infantis*. (**Articles I, II)**

5.2 Immunomodulation by potentially probiotic bacteria *in vitro*

Probiotic bacteria exert their beneficial effects in different ways, among which the immunomodulation of local and systemic immune responses belongs to the most important mechanisms [26]. In this respect, we investigated nine *Bifidobacterium* strains with probiotic properties (resistance to low pH and bile toxicity) for their 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 the other. These strain-specific effects are in accordance with previous observations on human immunocompetent cells [45-47]. Nonetheless, comparative studies on the immunomodulatory properties of *Bifidobacterium* strains of the same species or subspecies are limited [47, 48]. In our study we compared the effects of two *B. longum* ssp. *longum* strains CCM 7952 and CCDM 372 on the maturation

pattern of BM-DC, as well as their ability to induce cytokine secretion. We found that the activation potential of these two strains varied significantly, suggesting their different functional roles. These results are well in line with previous study showing that stimulation of peripheral blood mononuclear cells with different strains of *B. longum* led to strain-specific production of pro-inflammatory or regulatory cytokines [48]. **(Article II)**

The strain-dependent effect of *L. rhamnosus* LOCK0900 and LOCK0908 and *L. casei* LOCK0919 and their mixture on cytokine production in BM-DC cultures was investigated in our further study. Interestingly in this 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 [49]. Furthermore, the strains, which were found to produce high amount of exopolysaccharides (*L. rhamnosus* LOCK0900 and LOCK0908, *B. longum* ssp. *longum* CCM 7952) were poor inducers of cytokines *in vitro*. This is in agreement with recent findings of Fanning *et al*. [50] 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 [51]. **(Article V)**

5.3 Recognition of probiotic bacteria by pattern recognition receptors

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 maintain intestinal homeostasis [52, 53]. 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 HEK 293 cells stably transfected with TLR2, TLR4 or NOD2. Our data 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 concentration 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 peptidoglycan of all bacteria, and even species possessing the same pentapeptide bridge can induce different NOD2-dependent immune response [54]. Nevertheless, the role of NOD2 in recognition of these strains *in vivo* remains to be evaluated. **(Articles II, IV, V)**

5.4 Prophylactic effect of probiotic bacteria in model of acute 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 [55-57]. 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 by probiotic, prebiotic and synbiotic supplementation has been demonstrated by several studies [5, 21, 58].

In our work we investigated two *B. longum* ssp. *longum* strains for their ability to prevent against 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. Our results are in agreement with the study by Mileti *et al*. [59] showing that the probiotic strain which is poor inducer of both pro- and anti-inflammatory cytokines from human monocyte-derived dendritic cells, but not strain with strong immunostimulatory properties, is protective against experimental DSS-induced colitis in mice. Recent study in human volunteers showed that administration of *L. plantarum* is able to increase the localization of occludin and ZO-1 in epithelial TJs of tissue biopsies from IBD patients [60]. Likewise, *L. casei* DN-114001 [61] and probiotic mixture VSL#3 [62] are able to sustain the intestinal barrier function. It has been described that the epithelial TLR2 activation protects against barrier disruption by enhancing ZO-1 expression in intestinal epithelial cells in a protein kinase C-dependent manner [63]. Moreover, Cario *et al*. [64] showed that administration of TLR2 ligands protect from induction of experimental colitis and thus enhance the homeostasis in the intestinal epithelium. **(Article II)**

Further, 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 [65]. Several studies have described that butyrate in low concentration is capable to decrease the intestinal permeability associated with increased expression of TJ proteins. Recently has been shown that SCFA can induce regulatory T cells in the colon and thus maintain homeostasis in the intestine [66, 67]. In this study we have demonstrated that the severity of colitis in SCID mice has been associated with limited production of biologically active form of IL-18 which was 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*. [68]. 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 [69]. **(Article III)**

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

Several clinical studies demonstrated 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 [42, 70, 71]. In this study we used previously established [41] 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. Moreover, we have demonstrated that this *B. longum* strain stimulates BM-DC to production of regulatory cytokines IL-10 and TGF-β. Our results suggest that the inhibition of sensitization was achieved by general reduction of immune responses using regulatory T cells response. This is in agreement with recent studies showing that bifidobacteria are able to induce specific subsets of FoxP3+Treg

cells [72] or IL-10-producing type 1 regulatory cells [73] which interfere with the Th2 effector cells. **(Article IV)**

The gnotobiotic mouse model was utilized in the study investigating the ability of the *Lactobacillus* mixture (Lmix) to promote the maturation of intestinal barrier and brush border structures and to modulate allergic sensitization to Bet v 1. We have shown that colonization of GF mice with Lmix ameliorates Bet v 1-specific allergic responses both on humoral and cellular levels and induces significantly higher level of regulatory cytokine TGF-β in serum, implicating the involvement of regulatory mechanisms. 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 [42, 74-76]. Nevertheless, several clinical studies demonstrated that the beneficial effects of probiotics are dependent on the use of specific strains and on the timing of treatment, which indicates the higher effectiveness of probiotics in the prevention than treatment of allergy [77-79]. **(Article V)**

6. CONCLUSION

Taken together, this thesis elucidates the effect of selected 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*. This thesis point 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 suggest their use as novel strategies in prevention of IBD and allergic diseases in humans.

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8. 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: "Bacterial strain *Bifidobacterium longum* CCM 7952 and its use in human nutrition"

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

9. PUBLICATIONS

9.1 Publications related to the present thesis

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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