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Impact of probiotic bacteria on allergic sensitization in type I allergy model

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ABREVIATIONS

Alum	Aluminium hydroxide
BBMV	Brush border membrane vesicles
Bet v 1	Major birch pollen allergen
BM-DC	Bone marrow-derived dendritic cells
CD	Cluster of differentiation
CFU	Colony forming units
DC	Dendritic cells
FAO/WHO	Food and Agriculture Organization/ World Health Organization
FoxP3	Forkhead box P3
GF	Germ-free
HPLC	High-performance liquid chromatography
h-OVA	OVA heated to 70°C
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LAB	Lactic acid bacteria
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MLN	Mesenteric lymph nodes
MRS	de Man, Rogosa and Sharpe
MyD88	Myeloid differentiation primary response gene 88
NF-κB	Transcription factor Nuclear factor kappa B
NOD	Nucleotide oligomerization domain
OVA	Ovalbumin
PRR	Pattern recognition receptor
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	T regulatory lymphocytes

ABSTRACT

The main goal in reversing the allergy epidemic is the development of effective prophylactic strategies. Early life events, such as exposures to microbes, have a major influence on the development of balanced immune responses. Due to their ability to interact with host immune system and to modulate host immune responses probiotics, mainly bifidobacteria and lactobacilli have been used with some success in prevention of allergic disease.

In order to be referred to as probiotic, bacterial strain has to undergo rigorous testing. We have selected three new *Lactobacillus* (*L.*) strains out of 24 human isolates according to their antagonistic activity against pathogenic bacteria, resistance to low pH and milieu of bile salts. Safety of these strains was proven upon intragastric administration to mice; moreover, we have shown their ability to shift cytokine Th1 - Th2 balance towards non-allergic Th1 response in isolated splenic cells.

Allergen specific prophylaxis using probiotics as vehicles for mucosal delivery of recombinant allergen is an attractive concept for development of well-tolerated and effective allergy vaccines. We have shown that neonatal mono-colonization of germ-free mice with the *L. plantarum* NCIMB8826 strain producing the major birch pollen allergen Bet v 1 attenuates the development of birch pollen allergy later in life. The mechanisms involve a shift towards a non-allergic Th1 phenotype accompanied by increased regulatory responses, which were antigen-specific as colonization by a wild type strain exerted no such effects.

Intrinsic immunomodulatory properties of the probiotic strain play a key role in its ability to interact with the immune system of the host. We have further shown that neonatal mother-to-offspring mono-colonization with *Bifidobacterium longum* CCDM367, a human isolate with Treg rather than Th1 immunomodulatory properties, was able to reduce allergic sensitization by activating regulatory responses via TLR2 and MyD88 signaling pathways.

Understanding what makes ‘allergen an allergen’ is a key in allergy prophylaxis or treatment. We have shown in a mouse model of food allergy that even minor irreversible changes in OVA secondary structure caused by thermal processing alter both its digestion and antigenic epitopes formation. This leads to activation of different T cell subpopulations, induces shift towards Th1 response and ultimately reduces its allergenicity.

Taken together, understanding the immunomodulatory potential of bacteria in the early host development can pave a new way for probiotic use in early nonspecific prevention of type I allergy. Combining the probiotics with relevant allergen can make this approach even allergen-specific.

ABSTRAKT

Hlavním cílem v boji proti alergické epidemii je vývoj účinných preventivních strategií. Události v raném postnatálním období, jako je kolonizace bakteriemi, mají významný vliv na rozvoj vyvážených imunitních reakcí. Probiotika, zejména bifidobakterie a laktobacily, jsou vzhledem ke svým schopnostem modulovat imunitní odpověď hostitele nadějnými kandidáty pro prevenci alergických onemocnění.

Aby mohl být bakteriální kmen označen jako probiotický, musí projít přísným testováním. Z 24 lidských bakteriálních izolátů jsme vybrali tři nové kmeny rodu *Lactobacillus* (*L.*) podle jejich antagonistické aktivity vůči patogenním bakteriím, odolnosti proti nízkému pH a prostředí žlučových solí. V myším modelu jsme prokázali jejich bezpečnost a ukázali jsme jejich schopnost ovlivnit cytokinovou produkci směrem k protialergické Th1 odpovědi v izolovaných slezinných buňkách.

Využití probiotik jako vektorů pro slizniční podání rekombinantního alergenu je atraktivním přístupem ve vývoji dobře tolerovaných a účinných vakcín proti alergickým onemocněním. Ukázali jsme, že neonatální monokolonizace bezmikrobních myší rekombinantním kmenem *L. plantarum* NCIMB8826 produkujícím Bet v 1 snižuje senzibilizaci k březovému pylu v pozdějším životě. Mechanismus zahrnoval posun směrem k protialergickému Th1 fenotypu, doprovázenému zvýšenou regulační odpovědí. Tento účinek byl antigen specifický, protože kolonizace nerekombinantním kmenem žádné podobné účinky nevykazovala.

Přirozené imunomodulační vlastnosti probiotického kmene hrají klíčovou roli v jeho schopnosti interagovat s imunitním systémem hostitele. Když jsme pro neonatální monokolonizaci bezmikrobních myší použili lidský izolát s Treg spíše než Th1 imunomodulačními vlastnostmi *Bifidobacterium longum* CCDM367, pozorovali jsme celkové snížení alergické senzibilizace s aktivací regulačních odpovědí, pravděpodobně za využití TLR2 a MyD88 signálních drah.

Pochopení toho, co dělá alergen alergenem, je klíčem jak k profylaxi, tak i léčbě alergií. V myším modelu potravinové alergie jsme ukázali, že i nevelké nevratné změny v sekundární struktuře ovalbuminu, způsobené tepelným opracováním, mění způsob jeho štěpení a tvorbu antigenních epitopů, což vede k aktivaci odlišných T-buněčných subpopulací, indukuje posun směrem k Th1 odpovědi a redukuje jeho alergenicitu.

Lze tedy shrnout, že pochopení imunomodulačního potenciálu bakterií v časném stádiu vývoje hostitele nám umožní lepší využití probiotik v nespecifické prevenci alergie I. typu. Jako vektory k alergen-specifické profylaxi pak mohou sloužit probiotika produkující příslušný alergen.

1. INTRODUCTION

The prevalence of allergic diseases has increased dramatically over the past few decades, with population prevalence rates reaching 30 % in the industrialized nations (1). It has been suggested that the lack of microbial stimulation (as a consequence of increased hygiene) leads to decreased mucosal immunological defense with more prevalent sensitization to allergens (2, 3). So called 'hygiene hypothesis' (recently reviewed (4)) of allergy development claims that changes in the gut microbial ecosystem with a limited range of microbes regulating immune responses via Th1/ Treg cytokine profile might cause the Th2-biased immunological hyperresponsiveness resulting in allergies (5).

The growing interest in the role of the bacterial gut flora on health has stimulated different strategies to modify the intestinal flora. For that reason, non-pathogenic microbes were chosen to be used for administration, with the idea to confer a health benefit to the host. These microbes are indicated as probiotics (microorganisms such as lactic acid bacteria - bifidobacteria and lactobacilli with physiological benefits) and they are evaluated as an alternative way of manipulating disbalanced microflora (6).

According to the FAO/WHO probiotics are currently defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (7). In order to be used in human medicine for treatment and/ or prevention of allergies, new probiotic bacterial strains should be selected according to several criteria: they must be safe for the host organism, resistant to gastric acids and bile salts in order to survive the transit into the gut, and modulate Th1-Th2 balance in favor of anti-allergic Th1or Treg immune responses (7, 8). Probiotic therapy is based on the concept of normal healthy microflora. Specific bacterial strains have been demonstrated to exert powerful anti-allergic and anti-inflammatory properties (9, 10).

Probiotics trigger innate and adaptive immune responses and suppress colonization of the intestine by pathogenic bacteria (11) by competing with them for a limited number of receptors present on the surface epithelium (12). They also influence enhancement of mucosal barrier function and increase production of secretory IgA (10). Animal and human studies have provided unequivocal evidence that specific strains of probiotics are able to stimulate as well as regulate several aspects of natural and acquired immune responses.

The most allergenic foods are cow's milk, hen's egg, peanut, tree nuts, fish, shellfish, wheat, and soy, and these are the cause of the large majority of food allergy cases (13). There is a great deal of interest in understanding precisely how traditional food processing methods influence food allergenicity (14). Although baking, cooking, roasting, grilling, drying, pasteurization and sterilization were reported to reduce allergenicity, in some cases, these methods may increase

allergenic potential or may reveal neo-epitopes which were masked in the native protein (14). Thus insights into the relationship of allergen structure and its allergenicity are a key in allergy prophylaxis or treatment.

2. HYPOTHESIS AND AIMS

Allergy has become a significant health burden in the industrialized world, its prevalence reaching the magnitude of an epidemic. The observed rise in allergic disease cannot be explained by genomic changes; therefore it is commonly associated with reduced exposure to microbes and altered microbial communities (microbial dysbiosis) early in life. In line with the fact that the immune programming is initiated in prenatal and early postnatal life, this period represents a 'window of opportunity' for the prevention of allergic sensitization. In this respect, early interventions using probiotics appear as a valuable approach for prophylaxis of allergies. In this thesis we aimed at bringing new insides into the intimate relationship between bacteria and host immune system. We concentrated on description of immunomodulatory properties of probiotic bacteria and their possible use in primary prevention of allergic sensitization in mouse model of type I allergy.

Specific aims of the thesis:

- Selection of new probiotic bacterial strains and testing their safety and immunomodulatory properties.

- Testing the feasibility of a probiotic as mucosal antigen delivery vector in allergy prevention in gnotobiotic neonatal mother-to-offspring colonization mouse model.

- Prevention of allergic sensitization by probiotic strains in neonatal mother-to-offspring colonization mouse model and characterization of their immunomodulatory properties.

- Testing the impact of changes in allergen secondary structure on its allergenicity in mouse food allergy model.

3. MATERIAL AND METHODS

3.1 Bacterial strains, testing and preparation

Lactobacillus strains isolated from feces of healthy infants were obtained from Pure Culture Collection of Technical University, Lodz (LOCK), *Bifidobacterium longum ssp. longum* CCDM367 (*B. longum*) was provided by Culture Collection of Dairy Microorganisms (Milcom, Czech Republic) and *Lactobacillus* (*L.*) *plantarum* NCIMB8826 from National Collection of Industrial, Food and Marine Bacteria (Aberdeen, Scotland). *Lactobacillus* strains were grown in MRS medium (Oxoid, UK) for 24 hours at 37°C, *B. longum* was grown anaerobically in MRS medium supplemented with L-cysteine-hydrochloride (0.5 g/l) for 48 hours at 37°C. *Lactobacillus* strains were incubated in 0.85 % NaCl at pH 1.5, 2.5 and 3.5 for 2 h to study the impact of pH. Effect of the bile was estimated in 2 and 4 % bile salt solutions (Ox gall powder; Sigma, Germany) after a 2-d treatment. Antagonistic activity against pathogens was tested by agar slabs of 10 mm in diameter. They were aseptically cut off the MRS agar, overgrown with respective *Lactobacillus* strain and placed on plates with the agar media (Nutrient agar; Merck, Germany) inoculated with the indicator strain (10^5–10^6 CFU/ml). After 18-h incubation, the diameter of growth-inhibition zones around the agar slabs was measured. For *in vitro* experiments, bacteria were either heat (30 min, 80 °C) or 1% formaldehyde-PBS (4 h, RT) inactivated.

3.2 Construction of recombinant *Lactobacillus plantarum* strain

L. plantarum NCIMB8826 was used as the final host to carry a plasmid for constitutive intracellular Bet v 1 production and was grown at 37°C in MRS medium (Difco, USA). *Escherichia* (*E.*) *coli* MC1061 was used as intermediate host for cloning and was cultured at 37°C in Luria broth. Antibiotics were used at the following concentrations: for *E. coli*, ampicillin (100 µg/ml) and for *L. plantarum*, erythromycin (5 µg/ml). Molecular biology techniques, electrotransformation and cloning techniques were previously described (15). The resulting plasmid construct (pMEC181) carried the Bet v 1-encoding sequence under the control of pldhL. Finally, *L. plantarum* was electrotransformed with pMEC181 giving rise to an erythromycin-resistant (recLp) *L. plantarum* constitutively producing intracellular Bet v 1. To generate a control strain (conLp), *L. plantarum* NCIMB8826 was electroporated with the empty plasmid pGIT032.

3.3 Analysis of Bet v 1 production

Analysis of Bet v 1 production was performed as previously described (15).

3.4 Ovalbumin preparation, CD spectroscopy and enzymatic digestion

OVA (Worthington, USA) and heated OVA (h-OVA; prepared by exposure of OVA to 70°C for 10 minutes) were dissolved in phosphate-buffer saline (PBS) to a final concentration of 300 mg/ml containing 5 mg/ml of alum adjuvant (Sigma, Germany). For *in vitro* studies boiled OVA (b-OVA) was prepared by exposure of OVA to 95°C for 10 minutes. CD spectra were recorded in 5 mM sodium phosphate buffer (pH 7.4) with a JASCO J-815 spectropolarimeter fitted with a PTC-423S Peltier single position cell holder (Jasco, Japan). Thermal denaturation of proteins was monitored from 20°C to 70°C or from 20°C to 95°C at the fixed

wavelength of 222 nm with a temperature slope of 1°C/min. Peptides of OVA, h-OVA or b-OVA were prepared using pepsin-agarose gel similarly as described previously (16). Digested or undigested proteins were separated using SP 250/10 NUCLEOSIL 300-7 C18 column (Macherey-Nagel, Germany) on the HPLC system Gold 125NM Solvent Module (Beckman Coulter, USA). Samples were applied on columns and separated as described previously (16).

3.5 Preparation and activation of bone marrow-derived dendritic cells

BM-DC from BALB/c, wild-type and TLR2$^{-/-}$, TLR4$^{-/-}$ and MyD88$^{-/-}$ mice on a C57BL/6 background were prepared as previously described (17). BM-DC (10^6 cells/well) were stimulated with 10^6 or 10^7 CFU of formalin-inactivated *B. longum* for 18 h. As controls, BM-DC were incubated with Pam3CSK4 (1 µg/ml, InvivoGen, USA) or ultrapure LPS (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 specific inhibitors (MEK: PD98059; p38: SB203580; JNK: SP600125) or NF-κB (BAY 11-7082) at 10 µM dissolved in DMSO for 1 h at 37°C.

3.6 Stimulation of HEK293 cells stably transfected with TLRs

HEK293 cells stably transfected with plasmid carrying human (h) TLR2/CD14 and hTLR4/MD2/CD14 were stimulated with formalin-inactivated *B. longum* (10^6, 10^7, or 10^8 CFU/ml). TLR2 ligand Pam3C (1 µg/ml) and 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.

3.7 Animals

Germ-free BALB/c mice were kept under sterile conditions with a 12- h light–dark cycle at 22°C. Sterile pellet diet (ST1, Bergman, Czech Republic) and water were fed *ad libitum*. Fecal samples were weekly controlled for microbial contamination (18). Conventional BALB/c, wild-type and TLR2$^{-/-}$, TLR4$^{-/-}$ and MyD88$^{-/-}$ mice on a C57BL/6 background were kept under standard conditions.

3.8 *In vivo* stability and testing of bacterial strains

Germ-free BALB/c mice were colonized with 2 x 10^8 CFU recLp, conLp or *B. longum* by intragastric tubing. Drinking water was supplemented with erythromycin (50 µg/ml) to ensure long-term stability of the recombinant strains *in vivo*. Colonization and presence of the plasmid pMEC181 were analyzed weekly by plating on MRS agar ±5 µg/ml erythromycin; anaerobic cultivation on MRS agar supplemented with cysteine was used in case of *B. longum*. Conventional BALB/c mice received 5 x 10^8 CFU of the mixture of *Lactobacillus* strains intragastrically by soft rubber tubing twice daily for 7 consecutive days. Immune responses induced by colonization with recLp were tested in 8-week-old female BALB/c monocolonized with recLp via their mothers.

3.9 Allergic sensitization study design

Mice neonatally mono-colonized with recLp, conLp, *B. longum* or noncolonized GF mice were intraperitoneally or subcutaneously sensitized at 56 days of age three times in a 10-day or 14-day interval using 1 µg Bet v 1 (Biomay AG, Vienna) adsorbed to 2 mg aluminium hydroxide (Serva, Germany) in 200 µl saline. Mice were killed 7 days after the last immunization. Age-matched untreated GF mice served as controls when appropriate. Conventional BALB/c mice were sensitized i.p. in a 14-day interval with 60 µg of either OVA or hOVA together with 1 mg of alum in a final saline volume of 200 µl. Control mice received only 200 µl saline containing 1 mg of alum. Two weeks later, the mice were challenged 10 times at 2–3 days intervals by i.g. gavages of 15 mg of OVA in a final volume of 150 µl saline.

3.10 Cell culture and cytokine evaluation

Mesenteric lymph nodes (MLN) and spleens were removed at sacrifice. Single-cell suspensions were prepared in RPMI-1640 containing 10% fetal bovine serum (BioClot GmbH, Germany) and 1% Antibiotic-Antimycotic solution (Sigma-Aldrich). Cells ($6x10^5$/well) were cultured in a flat-bottom 96-well plate (TPP, Trasadingen, Switzerland) without any stimuli or in the presence of either OVA or h-OVA (100 µg/well) for 72 hours (37°C, 5% CO_2) or Bet v 1 (20 µg/ml) for 60 hours. Alternatively, 5 x 10^6 cells/500 µl were cultured with Bet v 1 in 48-well plates (Corning, NY, USA) for 48 h or spleen cells (5 x 10^5) were cultivated in 96-well plates with a mixture of heat inactivated *Lactobacillus* strains (10^5 CFU) for 72 hours. Supernatants were collected and stored at –40°C until analyses. Interleukin (IL)-4, IL-6, IL-13, IL-17, IL-5, IL-10, TNF-α and interferon (IFN)-γ were measured in the supernatants using ELISA kits (RnD, USA) and read on Infinite M200 (Tecan Group Ltd., Austria) or, alternatively, MILLIPLEX MAP Mouse Cytokine/Chemokine (Magnetic) Panel (Millipore, USA) according to manufacturer's instructions and analyzed with the Bio-Plex System (Bio-Rad Laboratories, USA). In order to measure the capacities of OVA, h-OVA and b-OVA and their peptic digests (100 mg/well) to induce Tregs, we cultivated them with naive mouse splenocytes for 48 hours. Proliferative responses of spleen 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.

3.11 Humoral immune responses

Allergen-specific serum IgE, IgG1, IgG2a and IgA levels were determined by ELISA as described in (19). Briefly, 96-well microtiter plates (Nunc, Denmark) coated with respective allergens and sera at appropriate dilutions were applied. Rat anti-mouse IgG1, IgG2a and IgA antibodies (Pharmingen, USA) and peroxidase-conjugated mouse anti-rat IgG antibodies (Jackson, Immuno Labs., USA) were used for the detection. Alternatively, allergen specific IgE levels in sera were quantified by degranulation of rat basophil leukemia (RBL-2H3) cells. RBL-2H3 cells were plated in 96-well tissue culture plates ($4x10^4$ cells/per well) and passively sensitized by incubation with mouse sera in a final dilution of 1/90 for 2 hours. After washing, OVA, h-OVA or b-OVA (0.6 µg/ml) or Bet v 1 (0.6 µg/ml) were added for 30 min at 37°C to induce degranulation. Supernatants were incubated with 4-methylumbelliferyl-N-acetyl-b-D-glucosaminide (Sigma-

Aldrich, USA) for analysis of β-hexosaminidase using a fluorescence microplate reader (λ_{ex}: 360 nm/ λ_{em}: 465 nm) Infinite M200. Total IgE and IgA in sera and gut lavage (prepared as previously described (15)) were measured using an ELISA kit (Bethyl, USA), levels of IL-10 and TGF-β were measured by ELISA Ready-Set-Go! kits (eBioscience, USA) according to manufacturer's instruction.

3.12 Real-time PCR

RNA was isolated from spleens of recLp colonized or GF mice sensitized to Bet v 1 using a RNeasy Minikit (Quiagen, USA). After DNase treatment, RNA integrity and purity was determined by gel electrophoresis and photometry (260/280 nm). Reverse transcription into cDNA was performed using oligo(dT)15 primers (ImProm-IITM Reverse TrancriptionSystem, Promega, USA). Universal Probe Library (UPL) (Roche, Germany) probes were used for the quantification of TGF-β1 (UPL#15), IL-10 (UPL#13), FoxP3 (UPL#13) and β-actin (UPL#101). Gene expression was determined using FastStart TaqMan Probe Master Mix (Roche) at 95°C for 10 min, followed by 45 cycles of 15 s at 95°C and 30 s at 60°C. Relative quantification was performed using GenEx software (MultiD Analyses AB, Sweden).

3.13 Flow-cytometry analysis

Single cell suspensions of spleens or MLN were stained for regulatory T cells by FoxP3 Staining Buffer Set (eBioscience, USA) using fluorochrome-conjugated monoclonal antibodies for CD4 (fluorescein-5-isothiocyanate), CD25 (allophycocyanin) and FoxP3 (phycoerythrin) (eBioscience, USA). Dendritic cells were labeled with monoclonal antibodies for CD11c (fluorescein-5-isothiocyanate), MHCII (allophycocyanin), CD40, CD80 or CD86 (phycoerythrin) (eBioscience, USA). Cells were analyzed using FACSCalibur flow cytometer (Becton-Dickinson, USA) and analyzed with FlowJo 7.6.2 software (TreeStar, USA).

3.14 Determination of enterocyte brush-border enzyme activities

Jejunum was removed, washed with cold saline and brush border membrane vesicles (BBMV) were prepared from jejunal scrapings as described by Kessler et al. (20). Protein concentration in BBMV was determined by the method of Lowry et al. (21) using bovine serum albumin, fraction V (Serva, Germany) as standard. The activity of alkaline phosphatase (EC 3.1.3.1), γ-glutamyltranspeptidase (EC 2.3.2.2), dipeptidyl peptidase IV (EC 3.4.14.5), lactase (EC 3.2.1.23/62/108) and sucrase (EC 3.2.1.48/ 10) were determined as described previously (22).

3.15 Statistical analysis

Differences between multiple experimental groups were evaluated by one-way analysis of variance (ANOVA) with Tukey's multiple comparison test, and differences between two groups were evaluated using unpaired two-tailed Student's t-test or non-parametric Mann-Whitney test. GraphPad Prism statistical software (version 5.03 GraphPad Software, La Jolla, CA, USA) was used for analyses.

4. RESULTS

Probiotic *Lactobacillus* strains: *in vitro* and *in vivo* studies.

Cukrowska B, Motyl I, Kozakova H, Schwarzer M, Gorecki RK, Klewicka E, Slizewska K, Libudzisz Z. *Folia Microbiol* (Praha) 2009;54:533-537.

In this chapter we aimed at selecting of new probiotic strains of human origin and characterizing their safety and immunomodulatory profile in animal model.

- **Out of 24 *Lactobacillus* strains, isolated from healthy breast fed infants, we selected tree strains LOCK 0900, LOCK 0908, LOCK 0919 based on their antagonistic activity against pathogenic bacteria, resistance to low pH and milieu of bile salts.**
- **We showed their safety in mouse model. They did not translocate through the intestinal barrier into blood, liver and spleen.**
- **Spleen cells, isolated from lactobacilli-treated mice and re-stimulated *in vitro* with the mixture of heat-inactivated tested strains, showed altered cytokine Th1–Th2 balance towards non-allergic Th1 response.**

Our study shows that *Lactobacillus* strains differ greatly in their ability to withstand the harsh environment of the digestive tract. The need for rigorous testing of new beneficial strains is highlighted.

Neonatal colonization of mice with *Lactobacillus plantarum* producing the aeroallergen Bet v 1 biases towards Th1 and T-regulatory responses upon systemic sensitization.

Schwarzer M, Repa A, Daniel C, Schabussova I, Hrncir T, Pot B, Stepankova R, Hudcovic T, Pollak A, Tlaskalova-Hogenova H, Wiedermann U, Kozakova H. *Allergy* 2011;66:368-375.

In gnotobiotic neonatal mother-to-offspring colonization mouse model we aimed at testing the feasibility of using the recombinant *L. plantarum* constitutively producing the major birch pollen allergen Bet v 1 as mucosal antigen delivery vector in allergy prevention.

- **We constructed a *L. plantarum* constitutively producing the major birch pollen allergen Bet v 1.**
- **We showed the stability of the strain *in vivo* and the ability to shift cell-mediated immune recall responses towards Th1 profile in neonatally colonized mice.**

- **Upon systemic sensitization, mice colonized with recombinant strain showed attenuation of allergic responses.**
- **The mechanisms involve a shift towards a non-allergic Th1 phenotype accompanied by increased regulatory responses.**

Mono-colonization of germ-free mice with recombinant *L. plantarum* specifically induced immunomodulation of allergic immune responses, thus promoting the use of recombinant lactic acid bacteria for early allergen-directed prevention of type I allergy.

Neonatal colonization of germ-free mice with *Bifidobacterium longum* prevents allergic sensitization to Bet v 1.
Schwarzer M, Srutkova D, Schabussova I, Hudcovic T, Akgün J, Wiederman U, Kozakova H. Manuscript in preparation.

Probiotic strains with the capacity to induce regulatory responses, rather than Th-1-promoting strains might be more successful in suppression of allergic responses. Here, we tested a human isolate *B. longum* in our gnotobiotic neonatal mother-to-offspring colonization mouse model of type I allergy.

- **We showed that colonization with *B. longum* dampens the induction of allergic sensitization both on cellular and humoral levels.**
- **This was accompanied by increased regulatory milieu.**
- ***In vitro, B. longum* induced increased production of regulatory cytokines IL-10 and TGF-β from bone marrow-derived dendritic cells.**
- **This induction was TLR2 and MyD88 dependent.**

Our data demonstrate that neonatal mono-colonization with *B. longum* reduces allergic sensitization by activating regulatory responses, likely via TLR2 and MyD88 signaling pathways. Thus, *B. longum* might be a promising candidate for perinatal intervention strategies against the onset of allergic diseases in humans.

Heat-induced structural changes affect OVA-antigen processing and reduce allergic response in mouse model of food allergy.

Golias J, Schwarzer M, Wallner M, Kverka M, Kozakova H, Srutkova D, Klimesova K, Sotkovsky P, Palova-Jelinkova L, Ferreira F, Tuckova L. *PLoS ONE* 2012;7(5): e37156. doi:10.1371/journal.pone.0037156.

Thermal processing influences the allergenicity of antigens. We tested the ability of thermally processed ovalbumin to induce allergic symptoms and immune responses in mouse model of food allergy.

- **Heating of OVA to 70°C caused mild irreversible changes in secondary structure compared to boiling.**
- **However, both treatments led to markedly different digestion kinetics and Tregs induction ability *in vitro*, compared to native OVA.**
- **Heating of OVA to 70°C significantly decreased clinical symptoms, immune allergic response and changed the activity of intestinal hydrolases.**
- **On the other hand OVA heated to 70°C stimulated higher IgG2a in sera and IFN-γ secretion by splenocytes.**

Minor irreversible changes in OVA secondary structure caused by thermal processing change both its digestion and formation of antigenic epitopes. This leads to activation of different T cell subpopulations, induces shift towards Th1 response and ultimately reduces its allergenicity.

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5. DISCUSSION

5.1 Selection of probiotic bacterial strains

There is a great interest in using live bacteria to improve the bacterial dysbiosis in early life, which is probably the key trigger in the increase of prevalence of allergic diseases. According to WHO (7), bacteria should be alive, although there are reports showing that even dead bacteria or lysates can modulate the host immune system (23). In order to survive the passage into the intestine, potential probiotics must be resistant to gastric acids and bile salts. Further, to be used in human medicine, they must be safe for host organisms (7). We have shown that out of 24 *Lactobacillus* strains isolated from healthy breast fed infants, only five strains presented *in vitro* both acid and

bile salt resistance. In addition to surviving the harsh environment in the gastrointestinal tract, bacterial adherence to intestinal epithelial cells is considered to be a desirable feature of a probiotic strain, as it can promote the gut residence time, pathogen exclusion, and interaction with host epithelial and immune cells (24). Therefore only three strains that showed the capacity to adhere to the epithelial Caco-2 cells were selected. We have shown that all three selected strains possess antagonistic activity against several pathogenic bacterial strains, suggesting they might play an important positive role in the modulation of intestinal microbiota.

Regarding their safety, a mixture of the bacteria was given to mice by intragastric gavage for seven consecutive days. At the end of the experiment, we observed no translocation through the intestinal barrier to blood and internal organs: bacteria translocated only to the MLN. The translocation of bacteria to MLN was well described in mice before (25); due to the fact that MLNs are now viewed as a crossroad between the peripheral and mucosal circulation pathways the translocation of bacteria might be necessary for exerting their immunomodulatory functions on systemic immunity (26). Indeed, when re-stimulated with the bacteria, spleen lymphocytes of lactobacilli-treated mice were activated to a higher production of anti-allergic Th1 cytokines and lower production of pro-allergic IL-5 than cells obtained from control mice.

Taken together we have shown that selected *Lactobacilli* strains are able to survive in the gut and to interact with the immune system of the host. *In vivo* mouse experiments showed their safety and the strains were able to shift the cytokine balance in favor of anti-allergic immune response. These findings make them promising candidates for prevention/therapy of allergic diseases.

5.2 Perinatal probiotic intervention in prevention of allergic sensitization

The use of probiotic bacteria with intrinsic Th1-promoting or immunomodulatory properties as vehicles for mucosal delivery of recombinant allergen is an attractive concept for development of well-tolerated and effective allergy vaccines (27). These interventions are of increasing interest early in life, when a so-called ‘window of opportunity’ is localized and the immune programming is initiated (28). In this study we cloned the major birch pollen allergen *bet v 1* gene under the control of a strong promoter leading to constitutive expression of the allergen in the well characterized strain with strong Th1 immunomodulatory properties *L. plantarum* NCIMB8826. In order to intervene at an early developmental stage and by using germ-free mice, we established a model of mother-to-offspring neonatal monocolonization. After confirming that the colonization and Bet v 1 production is stable, we found that the recombinant strain was able to induce a non-allergic Th1 response with significant IFN-γ but absent IL-4 and IL-5 production in spleen cells upon allergen challenge *in vitro*. This pointed out the importance of strain selection as well as

timing of colonization, because previous reports with *Lactobacillus casei* secreting beta-lactoglobulin led to both Th1 and Th2 cytokine production after colonization of adults GF mice (29). Moreover, when using Gram-negative bacteria as ovalbumin-producing vector, Dahlman *et* al. previously demonstrated an induction of allergen-specific IgE thus showing aggravation and potential participation in the induction of allergic symptoms (30).

When neonatally colonized mice were subsequently sensitized with the birch pollen allergen Bet v 1, we found a shift towards a non-allergic Th1 phenotype on the cellular level accompanied by increased regulatory responses as seen from decreased levels of both Bet v 1 specific IgG1 and IgG2a in sera and up-regulated mRNA of the regulatory marker FoxP3 in spleen. However the observed effects were antigen specific as colonization by a wild type strain exerted no such effects.

There are several recent reports suggesting that probiotic strains with the capacity to induce regulatory responses, rather than Th1-promoting strains might be more successful in suppression of allergic responses (17, 31). Due to the fact that *L. plantarum* is a strain with strong Th1 immunomodulatory properties (32) and that the wild-type *L. plantarum* itself did not exert any suppressive effects on the allergic immune response in our previous work (33), we aimed at testing a different probiotic strain with rather Treg immunomodulatory capacities. We selected *Bifidobacterium longum* spp. *longum* CCDM367 (*B. longum*), a healthy breast fed infant isolate, which has been shown to induce regulatory responses *in vitro* and to suppress the inflammatory responses in mouse models of experimental colitis (Srutkova, unpublished results). Upon neonatal mother-to-offspring mono-colonization of GF mice and subsequent sensitization, *B. longum* significantly reduced the development of allergen-specific immune responses, which was associated with induction of regulatory milieu, both on the humoral and cellular levels. This was associated with increased levels of regulatory cytokines IL-10 and TGF-β in serum. Our data are supported by the recent finding that certain bacterial strains can instruct DC to induction of FoxP3 positive regulatory T cells and enhanced IL-10 production (34). Induction of IL-10 producing Treg cells has been shown upon bifidobacteria administration to mice (35). *In vitro*, we showed that *B. longum* induces BMDC to produce IL-10 and TGF-β, with only low levels of IL-12. It is well documented, that probiotic bacteria are recognized by PRRs on different cell types (8, 32). By using the BMDC from KO mouse we showed, that *B. longum* induced IL-10 production is dependent on TLR2 and MyD88 and independent of TLR4 and that downstream signaling involves MEK, JNK, p38 and NFκB.

5.3 Altering antigen allergenicity by thermal processing

There is a great deal of interest in understanding precisely how traditional food processing methods influence food allergenicity (14). Although baking, cooking, roasting, grilling, drying, pasteurization and sterilization were reported to reduce allergenicity, in some cases, these methods may increase allergenic potential or may reveal neo-epitopes which were masked in the native protein (14).

Forming approximately 60% of the total egg white proteins, OVA is by far the most abundant of them. Allergies to eggs belong among the most frequent food allergies and their prevalence, severity and persistence have been steadily increasing during the last decades (36). We showed that heating of hen egg allergen OVA to 70°C (h-OVA) has only minor effect on its secondary structure compared to OVA heated to 95°C. However, these minor changes had a high impact on the immunological behavior of the allergen. Protein hOVA was more resistant to proteolytic digestion and after 20 minutes digests similar to OVA heated to 95°C were observed; moreover, they had a similar *in vitro* T reg inducing capacity. Similar to our results, suppressive effects of some OVA T cell epitope peptides on allergic immune responses via $FoxP3^+$ T cell generation were recently described (37). In line with the *in vitro* data we showed that the ability to induce the allergic immune responses is diminished in hOVA. Heating of OVA significantly decreased clinical symptoms (allergic diarrhea) and immune allergic response on the level of IgE, IL-4, IL-5, and IL-13. Furthermore, h-OVA induced lower activities of serum mast cell protease-1 and enterocyte brush border membrane alkaline phosphatase as compared to native OVA. On the other hand, h-OVA stimulated higher IgG2a in sera and IFN-γ secretion by splenocytes. The importance of structural epitopes in specific antibody formation was revealed when we changed the coupling allergen (h-OVA was used for OVA sensitized sera and vice versa) for specific Abs determination. Moreover, the binding was significantly higher when h-OVA antigen was used for specific IgG1 antibody determination. We assume that this is caused by heating uncovered linear epitopes (supplementing the loss of the conformational ones). On the other hand, when the OVA heated to 95°C was used we observed a strong drop in the signal in all OVA-specific antibodies.

In conclusion, we showed that even a mild change in the secondary structure of OVA after thermal processing has far-reaching consequences concerning its antigenic properties. After digestion of h-OVA, fragments with different immunogenic properties are formed leading to the shift from Th2 to Th1-type response as compared to native OVA. Nevertheless, the h-OVA fragments still have the ability to induce allergic symptoms, but these are less pronounced and need longer time to develop.

6. CONCLUSIONS

(A) We show here that the *Lactobacillus* strains LOCK 0900, LOCK 0908 and LOCK 0919 are able to withstand gut antimicrobial environment and to interact with the immune system of the host. *In vivo* mouse experiments showed cytokine shift in favor of anti-allergic immune response. Thus the mixture of *Lactobacillus* strains represents a probiotic bacterial preparation with possible use in prophylaxis and/ or therapy of allergic diseases.

(B) We have demonstrated that neonatal mono-colonization of germ-free mice with the *Lactobacillus plantarum* NCIMB8826 strain producing the major birch pollen allergen Bet v 1 attenuates the development of birch pollen allergy later in life. The mechanisms involved a shift towards a non-allergic Th1 phenotype accompanied by increased regulatory responses. Thus intervention at birth with a live recombinant *L. plantarum* producing a clinically relevant allergen reduces experimental allergy and might therefore become an effective strategy for early intervention against the onset of allergic diseases.

(C) We showed in neonatally colonized gnotobiotic mice that *Bifidobacterium longum* was a strain with the ability to induce regulatory cytokines/ T cells via TLR2 and MyD88 signaling pathways and thus prevent allergic sensitization. Therefore, *B. longum* might be considered as a promising candidate for perinatal intervention strategies against the onset of allergic diseases in humans. Moreover, our results stress the general importance of intrinsic immunomodulatory properties of bacterial strain used for intervention and shed light on the function of bifidobacteria in shaping the immune system in early human ontogeny.

(D) Aiming at elucidating the importance of secondary structure for antigen allergenicity in mouse food allergy model, we have established that heating of hen egg allergen OVA to 70°C has only minor effect on its secondary structure. Nevertheless, these minor irreversible changes in secondary structure changed both its digestion and formation of antigenic epitopes, which led to activation of different T cell subpopulations, induced shift towards Th1 response and ultimately reduced its allergenicity.

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8. PUBLICATIONS

8.1 Publications *in extenso* related to the present thesis

Cukrowska B, Motyl I, Kozakova H, **Schwarzer M**, Gorecki RK, Klewicka E, Slizewska K, Libudzisz Z: Probiotic *Lactobacillus* strains: *in vitro* and *in vivo* studies. *Folia Microbiol* (Praha) 2009;54:533-537.
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