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Probiotic *Lactobacillus* Strains: *in vitro* and *in vivo* Studies

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ABSTRACT. Three *Lactobacillus* strains (LOCK 0900, LOCK 0908, LOCK 0919) out of twenty-four isolates were selected according to their antagonistic activity against pathogenic bacteria, resistance to low pH and milieu of bile salts. Intragastric administration of a mixture of these strains to Balb/c mice affected cytokine T_H1–T_H2 balance toward nonallergic T_H1 response. Spleen cells, isolated from lactobacilli-treated mice and re-stimulated *in vitro* with the mixture of heat-inactivated tested strains, produced significantly higher amounts of anti-allergic tumor necrosis factor-α and interferon-γ than control animals whereas the level of pro-allergic interleukin-5 was significantly lower. *Lactobacillus* cells did not translocate through the intestinal barrier into blood, liver and spleen; a few *Lactobacillus* cells found in mesenteric lymph nodes could create antigenic reservoir activating the immune system. The mixture of *Lactobacillus* LOCK 0900, LOCK 0908 and LOCK 0919 strains represents a probiotic bacterial preparation with possible use in prophylaxis and/or therapy of allergic diseases.

Abbreviations

CFU	colony forming units	PBS	phosphate-buffered saline
Con A	concanavalin A	T_H	T helper (lymphocytes)
IFN	interferon	TGF	transforming growth factor
IL	interleukin	TNF	tumor necrosis factor
MLN	mesenteric lymph nodes		

The evidence of the importance of intestinal microflora on the development of immune responses leads to the theory that well-balanced microbial composition can improve the health of the host. Application of probiotics, *i.e*. viable nonpathogenic microorganisms exhibiting beneficial health effects when consumed in adequate amount, seems to be a perspective way for maintenance of balanced microflora (Schrezenmeier and de Vrese 2001). Probiotics are believed to trigger innate and adaptive immune responses and suppress colonization of the intestine by pathogenic bacteria by competing with them for a limited number of receptors present on the surface epithelium (Ostad *et al*. 2009). They also influence enhancement of mucosal barrier function, increase production of secretory IgA (Vanciková *et al*. 2003) and elicit production of short-chain fatty acids with health benefits related to colitis and cancer prevention (Fedorak and Madsen 2004; Kokesova *et al.* 2006).

Adaptive mucosal immune defense is induced by $CD4^+$ T lymphocytes, which differ in their secreted cytokine profile: T_H1 lymphocytes (IFN-γ, IL-2, TNF-α), T_H2 lymhocytes (IL-4, IL-5, IL-13, IL-9, IL-6) and regulatory $CD25^+$ $CD4^+$ T cells (TGF-β, IL-10). Disturbed balance between T_H1 and T_H2 lymphocyte subpopulations with elevated production of T_H2 cytokines leads to allergy development (Umetsu and DeKruyff 2006). On the other hand, production of T_H1 cytokines correlates with allergy prophylaxis and amelioration of allergic symptoms (Pohjavuori *et al.* 2004).

Lactic acid bacteria – mostly strains of the genus *Lactobacillus* – and bacteria of the genus *Bifidobacterium* are the most widely used probiotic bacteria in allergy therapy studies for their potency to modulate the T_H1–T_H2 balance (Ljungh and Wadstrom 2006). In clinical trials, probiotics have been shown to improve clinical syndromes of food allergy (Isolauri *et al*. 2000), strengthen T_H1 response in children with IgE mediated atopic dermatitis (Pohjavuori *et al.* 2004), protect the development of allergic diseases in infants

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(Lodinova-Zadnikova *et al*. 2003, Kocourkova *et al*. 2007) and even protect infants from developing atopic dermatitis when given to atopic mother during pregnancy (Kalliomaki *et al*. 2001*a*).

In order to be used in human medicine for treatment and/or prevention of allergies, probiotic bacteria 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 T_H1–T_H2 balance in favor of anti-allergic T_H1 immune responses. In this study we report a selection of probiotic *Lactobacillus* strains by *in vitro* tests and subsequent verification of their immunomodulating properties in *in vivo* mouse model.

MATERIAL AND METHODS

Survival of Lactobacillus *strains at low pH and in the presence of bile salt. Lactobacillus* strains isolated from feces of healthy infants were obtained from *Pure Culture Collection of Technical University*, Lodz (LOCK). Bacteria were cultivated for 1 d in MRS medium (*Oxoid*, UK), centrifuged and re-suspended to final concentration 10^7–10^8 CFU/mL in PBS. Bacteria 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. The bacteria were then plated on MRS agar medium for 2 d at 37 °C. The measurement was repeated at least three times in two independent experiments.

Lactobacillus survival (in %) was calculated as follows: $N_i/N_x \times 100$, where N_i = log CFU/mL after incubation, N_x = log CFU/ mL before incubation.

Antagonistic activity against pathogens. Antagonistic properties toward pathogens were analyzed by a slab method. Agar slabs of 10 mm in diameter 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 a 18-h incubation, the diameter of growth-inhibition zones around the agar slabs was measured. The following strains were used as indicator pathogens: *Escherichia coli* LOCK 105, *Enterococcus faecalis* LOCK 105, *Pseudomonas aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *E. coli* 018 (obtained from the *Serum and Vaccine Producer Biomed*, Cracow, Poland), *Salmonella enterica* subsp. *enterica* sv. Enteritidis (clinical isolate obtained from the *Institute of Sea and Tropical Medicine*, Gdynia, Poland), *Staphylococcus aureus* ATCC 25923, *Salmonella enterica* sv. Typhimurium, *Listeria monocytogene*s, *L. innocua* (clinical isolates obtained from *Voivodeship Sanitary-Epidemiological Station*, Bydgoszcz, Poland). The measurement was repeated four times in two independent experiments.

Sequencing of 16S rDNA. Total DNA was extracted (Tailliez *et al*. 1998) to analyze rDNA sequences for 16S ribosomal RNA. The 1063-bp fragment of the 16S rDNA gene was amplified by PCR reaction using primers: 1406R and 343F (5′-TAC GGG AGG CAG CAG-3′), the inverse complementary to primer 343aR (all described by Salama *et al.* 1991). Sequencing of the PCR products was done using BigDye Terminator Cycle Sequencing (*Applied Biosystems*, USA). Sequences were read by ABI 377 apparatus (*Applied Biosystems*) and subjected to the BLAST search program (NCBI) (Krauthammer *et al*. 2000).

Experimental design in vivo. Eight-week-old inbred BALB/c mice (n = 5 per group) were used for *in vivo* experiments. Strains selected in *in vitro* tests were mixed in equal proportions, lyophilized with hydrolyzed milk formula and resuspended in PBS in final concentration 10^9 CFU/mL. The mixture of bacteria (0.5 mL) was given intragastrically by soft rubber tubing twice daily for 7 consecutive days. Control mice received PBS-reconstituted hydrolyzed milk.

The experiment was approved by the *Animal Experimentation Ethics Committee of the Institute of Microbiology, Academy of Sciences of the Czech Republic* and conducted in accordance with the *European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes* (CETS no. 123).

Bacteria translocation. MLN, spleen and liver were removed aseptically, weighed and diluted 5× in sterile PBS. After homogenization, suspensions were plated onto MRS agar and incubated for 3 d at 37 °C. The blood was taken intracardially and plated undiluted. The number of *Lactobacillus* CFU was also established in colon and cecum contents. Results were expressed as the number of CFU/g of intestine contents or the number of CFU per organ or pooled MLN from each group.

Lymphocyte cultivation for cytokine production. Cytokine production was measured in supernatants after cultivation of spleen lymphocytes. Spleens were minced in RPMI 1640 medium (*Sigma-Aldrich*, USA), filtered by nylon mesh and washed thrice in the medium. Cell concentration was adjusted to 5×10^6 per mL in complete RPMI 1640 medium supplemented with 5 % fetal calf serum and antibiotics (*Sigma-Aldrich*). Lymphocytes (100 μL) were applied to 96-well cultivation microplates (*Nunc*, Denmark) and cultivated in RPMI 1640 medium with a mixture of heat inactivated (30 min, 80 °C) *Lactobacillus* cells (10^6 per mL in

total volume of 300 μL of RPMI 1640 medium). Polyclonal lymphocyte activator Con A (*Sigma-Aldrich*) in 5 μg/mL final concentration was used as a positive control. Cells cultivated with RPMI 1640 medium only were used as negative controls. At least 10 replicates for each activator from one mouse were done. Plates were incubated for 72 h under 5 % CO_2 atmosphere at 37 °C; the supernatants were then collected and stored at –40 °C until cytokine production analysis.

Detection of cytokine production. The levels of IL-5, TNF-α and IFN-γ were measured in culture media. Cytokine production was determined by mouse ELISA kits (*R&D System*, USA) according to manufacturer's instructions. The results were read on Infinite M200 (*Tecan Group Ltd.*, Austria) and expressed in pg/mL.

Statistical analysis was done using nonparametric Mann–Whitney test; $p < 0.05$ was considered as statistically significant.

RESULTS

Characterization of selected strains. Only five out of 24 tested strains presented *in vitro* both acid and bile salt resistance. As we have previously reported that three out of these five strains also adhere to Caco-2 epithelial cell line (Marewicz *et al*. 2000), these three strains (LOCK 900, LOCK 0908, LOCK 0919) were selected for *in vivo* experiments.

The survival of selected strains after incubation at pH 2.5 did not change markedly and remained 67.5–84.2 % (10^6–10^8 CFU/mL) (Table I), whereas in other strains it decreased to 1–10^5 CFU/mL. All 24 examined strains exhibited high survival rate at pH 3.5 (>90 %), whereas pH 1.5 was lethal (*data not shown*). The incubation with 2 % bile salt solution resulted in survival >80 % for all examined strains, whereas 4 % solution markedly decreased the amount of living bacteria (*data not shown*). However, the surviving of LOCK 0900, LOCK 0908 and LOCK 0919 after incubation in 4 % solution was ≥88.8 % (*i.e.* ≥10^6 CFU/mL) (Table II). Selected strains showed antagonistic activity against all used pathogenic bacteria (Table III) but no antagonistic properties towards each other were observed.

Table I. The survival (%[a])of selected *Lactobacillus* strains after a 2-h incubation at low pH

Strain	pH 1.5	pH 2.5	pH 3.5
LOCK 0900	32.8 ± 3.6	67.5 ± 4.6	99.3 ± 3.6
LOCK 0908	13.2 ± 4.1	80.9 ± 3.5	93.5 ± 4.1
LOCK 0919	13.1 ± 3.7	84.2 ± 5.2	98.3 ± 3.9

[a]Arithmetical means ±SD.

Table II. The survival (%[a])of selected *Lactobacillus* strains after 48 h of incubation with 2 and 4 % bile salt solution

Strain	2 %	4 %
LOCK 0900	97.2 ± 3.8	88.8 ± 5.1
LOCK 0908	94.6 ± 4.2	90.8 ± 4.9
LOCK 0919	97.0 ± 5.6	95.8 ± 4.4

[a]Arithmetical means ±SD.

Table III. Antagonistic activity of selected *Lactobacillus* strains[a]

Strain LOCK	*Ec*A	*Ec*L	*Ec*0	*Pa*	*S*E	*S*T	*Ef*	*Sa*	*Lm*	*Li*
0900	15.5 ± 1.8	11.0 ± 1.6	14.0 ± 2.0	21.5 ± 1.3	10.0 ± 1.8	12.0 ± 1.9	8.5 ± 1.7	17.5 ± 1.8	11.0 ± 0.8	11.0 ± 1.1
0908	16.0 ± 1.8	13.0 ± 1.5	12.0 ± 1.6	18.0 ± 1.6	14.0 ± 1.6	13.0 ± 1.6	9.5 ± 1.9	12.0 ± 1.6	12.0 ± 1.0	12.0 ± 1.3
0919	13.5 ± 1.2	18.0 ± 1.4	16.0 ± 1.0	19.5 ± 1.2	13.0 ± 1.9	14.0 ± 1.5	6.5 ± 2.1	14.0 ± 1.8	11.0 ± 1.1	9.0 ± 0.7

[a]Arithmetical means ±SD of inhibition zones (mm).

*Ec*0 *E. coli* 018
*Ec*L *E. coli* LOCK 105
*Ec*A *E. coli* ATCC 25922
Ef *Enterococcus faecalis*
Li *Listeria innocua*
Lm *Listeria monocytogenes*
Pa *Pseudomonas aeruginosa* ATCC 27853
Sa *Staphylococcus aureus* ATCC 25923
*S*E *Salmonella enterica* ssp. *enterica* sv. Enteritidis
*S*T *Salmonella enterica* sv. Typhimurium

Analysis of 16S rDNA sequences demonstrated that the studied strains belong to the genus *Lactobacillus* and, more precisely, to the group of closely related species including *L. casei*, *L. rhamnosus* and *L. paracasei.* The strains LOCK 0900 and LOCK 0908 exhibited 97 and 98 % homology, respectively, to the sequence in *GenBank* accession no. D16552 (*L. casei*), whereas LOCK 0919 showed 99 % similarity to the sequence D79212 (*L. paracasei*).

Bacteria translocation through the intestinal barrier. The number of *Lactobacillus* CFU in colon ($3.3 \pm 0.5 \times 10^9$) and cecum ($2.3 \pm 0.2 \times 10^9$) of lactobacilli-treated mice was similar to control animals (colon – $2.8 \pm 0.3 \times 10^9$, cecum – $3.2 \pm 0.5 \times 10^9$). No bacteria were cultivated from blood, liver and spleen of both experimental and control groups. However, in MLN of lactobacilli-treated mice, 225 CFU per pooled MLN were grown in contrast to control group where no bacteria were detected.

Cytokine production in cultures of spleen lymphocytes. Lymphocytes from both lactobacilli-treated mice and control group were stimulated by Con A to a high secretion of anti-allergic T_H1 cytokines (IFN-γ and TNF-α) and pro-allergic IL-5 whereas stimulation with inactivated lactobacilli induced mainly secretion of T_H1 cytokines (Table IV). There were no significant differences in the cytokine levels between experimental groups after stimulation of spleen lymphocytes with Con A. Nevertheless, after co-cultivation of cells with lactobacilli a significant increase in IFN-γ and TNF-α levels in lactobacilli-treated mice was found compared with control animals. In contrast, these cells produced significantly lower amounts of IL-5 than control cells.

Table IV. The cytokine production in spleen cell cultures[a]

Cytokine	Lactobacilli-treated mice		Control mice	
	Lm	Con A	Lm	Con A
IFN-γ	442 ± 98**	4054 ± 1002	151 ± 23	4001 ± 987
TNF-α	1012 ± 223*	449 ± 85	881 ± 132	444 ± 93
IL-5	29 ± 3‡	1146 ± 289	37 ± 3	1285 ± 298

[a]The level of cytokine (pg/mL) was measured by ELISA in supernatants of spleen lymphocytes cultivated with the heat inactivated mixture of tested *Lactobacillus* strains (Lm) or Con A. Spleen cells of lactobacilli-treated mice incubated with Lm produced significantly higher amounts of IFN-γ (**$p = 0.01$), TNF-α (*$p = 0.04$) and significantly lower of IL-5 (‡$p = 0.04$) than cells of control mice. There were no statistical differences between experimental groups after Con A activation. Values are expressed as aritmetical means ± SD.

DISCUSSION

In the selection of prospective probiotic *Lactobacillus* strains we used the criteria (pH and bile resistance) similar to those published by Kurman (1988), who stated that the resistance of bacteria to 4 % bile can be considered a good characteristic for probiotics.

Selected strains presented different levels of antagonistic activities against tested pathogens (11 strains of 8 species) but, finally, the strains were chosen to ensure the highest antagonistic activity in the mixture. The main antagonistic metabolites produced by the tested strains were lactic and acetic acids (*unpublished data*), both of which were shown to reduce pH and thus inhibit growth of pathogenic bacteria (Ljungh and Wadstrom 2006). Moreover, the LOCK 0900 and LOCK 0919 strains also synthesize bacteriocins resistant to proteolytic enzymes, *e.g.*, biosurfactants with described antimicrobial function (Walencka *et al.* 2008).

The disturbances in gut ecosystem that occur in early life are supposed to cause a misbalanced development of the immune system favoring pro-allergic reactions. As an increased number of genus *Clostridium* was found in the intestine of allergic patients (Kalliomaki *et al*. 2001*b*), antagonistic properties of *Lactobacillus* bacteria can play an important positive role in the modulation of the gut microbiota.

We observed that in human blood-cell cultures the selected strains (LOCK 0900, LOCK 0908, LOCK 0919) induced higher synergistic effects on cytokine production compared with individual strains (*unpublished results*); the mixture of bacteria was, therefore, used for *in vivo* experiment in mice. During studies in mouse model the selected strains were safe and affected significantly the cytokine profile toward anti-allergic response. They did not translocate through the intestinal barrier to blood and internal organs (CFU of *Lactobacillus* were found only in MLN of lactobacilli-treated mice). The translocation of bacteria to MLN was described in mice given high doses of these bacteria (Macpherson and Uhr 2004). After being caught by dendritic cells, bacteria are shuttled to MLN where they can activate the immune system. Thus, the presence of probiotic bacteria in MLN creates the antigenic reservoir with an ability to induce immune responses. We also showed that the spleen lymphocytes of lactobacilli-treated mice are activated to a higher production of anti-allergic T_H1 cytokines and lower production of pro-allergic IL-5 than cells obtained from control mice. The systemic immune responses to ingested antigens are considered to be probably generated

in MLN (Mowat 2003) and thus the observed shift in the T_H1–T_H2 balance can be attributed to translocated lactobacilli.

Our results showed that the *Lactobacillus* strains LOCK 0900, LOCK 0908 and LOCK 0919 are able to withstand the gut conditions and to interact with the immune system of the host. *In vivo* mouse experiments showed that, on the cellular level, they can shift the cytokine balance in favor of anti-allergic immune response.

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