

## IDENTIFICATION AND BIOLOGICAL ACTIVITY OF POTENTIAL PROBIOTIC BACTERIUM ISOLATED FROM THE STOMACH MUCUS OF BREAST-FED LAMB

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### ABSTRACT

The lactic acid bacterium E isolated from the stomach mucus of breast-fed lamb was identified by sequencing of 16S rDNA fragment and species-specific PCR as *Lactobacillus reuteri*. Its potential antimicrobial activity and ability to modulate immune system *in vitro* and *in vivo* was determined. The growth inhibition of potential pathogens decreased from *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella enterica* ser. *Minnesota* to *Escherichia coli*. The lowest inhibition activity was observed in the case of *Candida albicans*. The ability of *L. reuteri* E to modulate biological activities of human and mouse mononuclear cells was estimated *in vitro* and *in vivo*, respectively. The production of IL-1 $\beta$  by monocytes *in vitro* was significantly induced by *L. reuteri* E (relative activity 2.47). The ability to modulate biological activities of mononuclear cells by living *L. reuteri* E cells *in vitro* in comparison to disintegrated *L. reuteri* E cells *in vivo* differed. For example lysozyme activity *in vitro* was inhibited while *in vivo* was stimulated (relative activities 0.30 and 1.83, respectively). The peroxidase activity *in vitro* was stimulated while *in vivo* was inhibited (relative activities 1.53 and 0.17, respectively). Obtained results indicate that *L. reuteri* E is potential candidate to be used in probiotic preparations for animals and/or human.

**Key words:** *Lactobacillus reuteri* - mononuclear cells - PCR - antagonistic activity - phagocytosis

### INTRODUCTION

*Lactobacillus* spp. are the natural inhabitants of human and animal colon and vagina. Lactobacilli belong to the group of lactic acid bacteria that ferment saccharides to lactic acid. Specific strains of lactobacilli that are able to colonize body cavities seem to play an important role in maintaining a balanced microflora or preventing and lowering the incidence of recurrent urinary or digestive tract infections (18). Presence of *Lactobacillus* strains has positive effect on some digestive

diseases, e.g. lactose intolerance, inflammatory bowel disease, constipation or post-antibiotic diarrhea (15).

Nowadays many researchers are looking for different probiotic bacteria that exhibit advantageous properties on human and/or animal health. These can be used in human or veterinary medicine in food supplements and/or probiotic preparations. According to FAO/WHO (5) guidelines, probiotic microorganisms must be exactly identified and characterize on strain level in several points: acid and bile salt resistance, ability to produce antimicrobial substances, adhere to mucus

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and modulate immune system.

The aim of this study was to identify and characterize bacterial isolate E derived from the stomach mucus of breast-fed lamb (Očová, Slovakia). According to microbiological and biochemical tests bacterium E showed several characteristics of lactic acid bacteria. For exact identification of isolate E, sequencing of fragment of 16S rDNA and PCR were used. To detect inhibition activity of isolate E against potential pathogens the streak line method on agar plates was performed. The ability of strain E to affect immune system of host was tested by *in vitro* and *in vivo* experiments.

## MATERIAL AND METHODS

### Bacterial strains and growth conditions

Bacterium E was isolated from the stomach mucus of three weeks old breast-fed lamb (Očová, Slovakia). As the reference strains for lactobacilli identification *Lactobacillus reuteri* CCM 3625 and *L. acidophilus* CCM 4833 (Czech Collection of Microorganisms, Brno, Czech Republic) were used. Lactobacilli were cultivated in de Man-Sharpe-Rogosa (MRS) broth (Oxoid, UK) 18 h in anaerobic conditions at 37°C. Antagonistic activity of strain E was tested against potential pathogens: *Staphylococcus aureus* CNCTC Mau 29/58 (Czech National Collection of Type Cultures, Prague, Czech Republic), *Pseudomonas aeruginosa* CNCTC Ps 79/70, *Escherichia coli* ATCC 11229 (American Type Culture Collection, Manassas, Virginia, USA), *Salmonella enterica* ser. *Minnesota* SK 99/39 (Faculty of Pharmacy, Comenius University, Bratislava, Slovak Republic) and *Candida albicans* CCM 8180. Strains of potential pathogens were cultivated in peptone water (peptone 10.0 g/L; sodium chloride 5.0 g/L; disodium hydrogen phosphate dodecahydrate 9.0 g/L; potassium dihydrogenphosphate 1.5 g/L) for 18 h at 37°C. For testing immunomodulatory activity *E. coli* ATCC 11229, *Micrococcus luteus* ATCC 4698, *Enterococcus faecalis* CCM 2122 and *Candida albicans*

CCM 8180 were used.

### Identification of lactobacilli

Chromosomal DNA was isolated from 3 mL of an overnight culture of bacteria using DNA Easy<sup>®</sup> Tissue Kit (Qiagen, Germany), according to the manufacturer's protocol. For identification of new isolate E on the species level amplification of the part of 16S rDNA by PCR and sequencing of PCR product were used. The PCR was performed according to Heilig *et al.* (10). Sequence analysis was performed at the Faculty of Natural Sciences (Comenius University, Bratislava, Slovakia). The nucleotide sequence was processed in computer software Vector NTI and compared with known sequences in GenBank ([www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST)). Species-specific PCR for *L. reuteri* was performed according to Kwon *et al.* (14).

### Electrophoresis of PCR products

The PCR products (8 µL) were separated in 1.5% agarose gel containing 1xTAE (18) in electric field of 80 V. DNA was stained by "GoldView" (SBS Genetech Co. Ltd., China) and visualised under UV light ( $\lambda = 254$  nm).

### Antagonistic activity

The turbidity of the broth cultures of strain E and potential pathogens was adjusted to equal that of 4 and 3 McFarland standard, respectively. 10 µL of *Lactobacillus* culture were seeded in line to the middle of Petri dish containing MRS agar. Cultures were cultivated for 24 h in anaerobic conditions at 37°C. After incubation, lactobacilli were killed by exposure to chloroform gases for 2 h. Then the diluted cultures of potential pathogens were inoculated in volume 1 µL, in duplicate, perpendicular to the streak line of dead lactobacilli. Pathogens were incubated 24 h in aerobic conditions at 37°C. After final incubation, zones of growth inhibition between lines of lactobacilli and pathogens were measured. Experiments were performed in twelve parallels. The degree of antagonistic

activity was evaluated as following: 0 – 12 mm as low inhibition, 13 – 25 mm as intermediate inhibition and more than 25 mm as high inhibition according to Hütt *et al.* (12).

#### **Isolation of human mononuclear cells and their pre-incubation with strain E**

The human mononuclear cells (MOs) from 6 healthy independent volunteers (OHT-Derer's Hospital, Bratislava, Slovakia) were isolated and purified by HistoPaque-1077 (Sigma, USA) according to Böyum *et al.* (2). MOs used were suspended in RPMI medium (diluted in saline 1:4) supplemented with 10% fetal bovine serum to concentration  $2 \times 10^6$  cells/mL. In the next step, they were cultivated 18 h (at 37°C in 5% CO<sub>2</sub> atmosphere) exposed to  $3 \times 10^2$  CFU/mL and  $6 \times 10^2$  CFU/mL of strain E suspension according to Bukovský *et al.* (3). Control samples contained untreated human MOs.

#### **Administration of mouse model**

For the experiments male C<sub>57</sub> BL<sub>6</sub> inbred mice (25–27 g) (Velaz, Czech Republic) were used. Bacterial suspensions of strain E (the dose of strain E was  $3 \times 10^5$  CFU/kg of mouse) were administered intraperitoneally to mice daily during 5 days period. To animals in control group physiological saline was applied. Mice were weighted before and after experiment. All experiments were performed in accordance with the Proclamation of the Government of Slovak Republic in July 9, 2003, Law N<sup>o</sup> 289/2003 in the Collection of Laws of the Slovak Republic, and in accordance to the local Ethical Committee of Faculty of Pharmacy (Comenius University, Bratislava, Slovakia). Mononuclear cells were isolated from mouse peritoneum using 5 mL of physiological saline.

#### **Phagocytic activity and index**

Phagocytic activity and index were determined microscopically. 100 µL of MOs ( $2 \times 10^6$  cells/mL) were incubated 1 h at 37°C with 50 µL of heat inactivated *E. faecalis*

( $5 \times 10^8$  cells/mL) (3). The used ration strain/Wright's staining was performed according to the conventional methodology. Phagocytic activity was calculated as a percentage of the phagocytosing mononuclear cells. Phagocytic index was determined as the average number of heat inactivated bacteria ingested per one active MO.

#### **Microbicidal and metabolic activities**

MOs, influenced *in vivo* and *in vitro* with strain E, were ultrasonically disintegrated (18 kHz, 10 s). The microbicidal activity of homogenate was tested on *S. aureus*, *E. coli* and *C. albicans*. 24 h cultures of *S. aureus* (grown on blood agar) and *E. coli* (grown on Endo agar) and 48 h culture of *C. albicans* (cultured on Sabouraud's agar) were washed twice in physiological saline and adjusted to OD<sub>540</sub> = 0.35 (all culture media were purchased from Imuna, Slovakia). Bacterial suspensions were resuspended to  $1 \times 10^5$  cells/mL. The turbidity of *C. albicans* was adjusted to  $1 \times 10^4$  cells/mL. Microbial cultures in different amounts (250 µL of *E. coli*, 100 µL of *S. aureus*, 100 µL of *C. albicans*) were incubated with 100 µL of crude homogenate of MOs. Incubation at 37°C lasted 1 h. The number of surviving microbes was estimated after cultivation on appropriate media (bacteria – 24 h at 37°C on blood agar or Endo agar, yeast 48 h at 25°C on Sabouraud's agar). In control sample untreated MOs were used. Microbicidal activity was expressed as difference in number of CFU/mL between control and test samples.

The ultrasonically disintegrated monocytes (18 kHz, 10 s) were centrifuged (2500 g, 10 min, 4°C). For lysozyme activity 150 µL of supernatant were mixed with 50 µL of the suspension of *M. luteus* (OD<sub>410</sub> = 0.8) in phosphate buffer (pH 6.2, 0.07 mol/L KH<sub>2</sub>PO<sub>4</sub> and 0.07 mol/L Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O). After 20 min absorbance at 410 nm was measured (MR 5000, Dynatech, USA). For the determination of peroxidase activity 50 µL of the peroxidase substrate (benzene-1,2-diamine – 0.5 mg/mL, freshly diluted H<sub>2</sub>O<sub>2</sub> – 10 µL/mL and 0.1 M sodium citrate tribasic dihydrate in distilled H<sub>2</sub>O, pH 5.0) was

added to 150  $\mu$ L monocyte supernatant. After incubation at 22°C for 20 min reaction was stopped with H<sub>2</sub>SO<sub>4</sub> (50  $\mu$ L, 4 mol/L) and absorbance was measured at 490 nm (MR 5000, Dynatech, USA).

*In vitro* experiments were performed on human monocytes and repeated at least 4 times. *In vivo* experiments were carried out in two groups of 6 mice.

### Influence on IL-1 $\beta$ production

Ability of bacterium E to induce production of IL-1 $\beta$  by phagocytes was determined spectrophotometrically (A<sub>450</sub>; MR 5000, Dynatech, USA). 2x10<sup>6</sup> phagocytes per mL were cultivated with strain E (6x10<sup>2</sup> CFU/mL) in total volume 50  $\mu$ L in RPMI medium supplemented with 10% fetal bovine serum 18 h at 37°C. Untreated monocytes were used as a control. The amount of IL-1 $\beta$  was determined by enzyme immunoassay method according to Immunotech's recommendations (Immunotech, France).

### Statistical analysis

For calculations and statistical analysis OriginPro 7.5 was used. Data are expressed as mean values  $\pm$  standard deviations or as relative activities (the control sample was considered as

1.00). The statistical comparison between control and samples was performed by Student's t-test. The value  $p \leq 0.05$  was regarded as statistically significant.

## RESULTS

### Identification of strain E

Several bacteria were isolated from the stomach mucus of breast-fed lamb. Isolate E showed attributes typical for genus *Lactobacillus* by microbiological and biochemical tests (Bukovský *et al.* - data unpublished). For unambiguous identification the partial nucleotide sequence of the 16S rDNA was used. Amplification of this gene was performed using primer set Bact-0011f and Lab-0677r (10). Nucleotide sequence of cca 700 bp PCR product of strain E was used for the analysis of sequence similarity through BLAST ([www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST)). It showed a significant homology to the 16S rDNA of known *Lactobacillus* spp. The highest homology (99%) was achieved with strain *L. reuteri* LU3 (AY 735 406) (Figure 1). Identification of isolate E on species level was evaluated by species-specific PCR for *L. reuteri* according to Kwon *et al.* (14). Specific PCR product of 1100 bp length was observed. Finally the new isolate was identified as *L. reuteri* and named *L. reuteri* E.

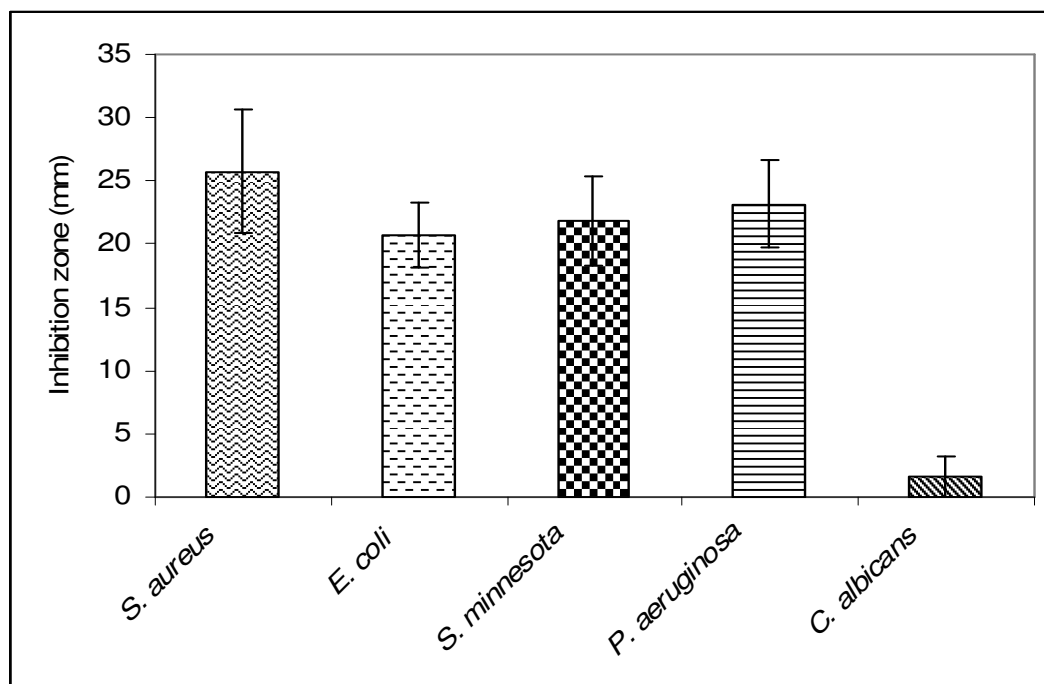
|                             |  |              |
|-----------------------------|--|--------------|
| E (1)                       | 6  | 55           |
| <i>L. reuteri</i> LU3 (6)   | ACGCCGGCGGTGTGCCTAATACATGCAAGTCGTACGCACTGGCCCAACT  | <u>R</u>     |
|                             | 56   | 105          |
| E (51)                      | ATTGATGGTGCTTGCACCTGATTGACGATGGATCACCAGTGAGTGGCGGA |              |
| <i>L. reuteri</i> LU3 (56)  | ATTGATGGTGCTTGCACCTGATTGACGATGGATCACCAGTGAGTGGCGGA |              |
|                             | 106  | 155          |
| E (101)                     | CGGGTGAGTAACACGTAGGTAACCTGCCCGGAGCGGGGGATAACATTTG  |              |
| <i>L. reuteri</i> LU3 (106) | CGGGTGAGTAACACGTAGGTAACCTGCCCGGAGCGGGGGATAACATTTG  |              |
|                             | 156  | 205          |
| E (151)                     | GAAACAGATGCTAATACCGCATAACAACAAAAGCC                | <u>R</u>     |
| <i>L. reuteri</i> LU3 (156) | GAAACAGATGCTAATACCGCATAACAACAAAAGCC                | <u>G</u>     |
|                             |  | CATGGCTTTTGT |

**Figure 1.** Comparison of nucleotide sequences of 16S rDNA fragment of *L. reuteri* E and *L. reuteri* LU3 (AY 735406, BLAST). Differences were found only in positions 55 and 191. R – any of purines, A/G.

**Antagonistic activity of *L. reuteri* E**

Antagonistic activity of *L. reuteri* E was tested against several potential pathogens. The streak line method on agar plates with few modifications was used (12). The highest inhibition activity was detected against *S. aureus*; the inhibition

zone was  $25.75 \pm 4.90$  mm from the line of lactobacilli. Other pathogens were inhibited weaker: *P. aeruginosa* ( $23.17 \pm 3.42$  mm), *S. enterica* ser. *Minnesota* ( $21.83 \pm 3.47$  mm), *E. coli* ( $20.75 \pm 2.58$  mm) and the lowest inhibition zone was observed in the case of *C. albicans*,  $1.67 \pm 1.61$  mm (Figure 2).



**Figure 2.** Antagonistic activity of *L. reuteri* E against selected potential pathogens.

Values are averages  $\pm$ SD from six parallels.

**The effect of *L. reuteri* E on human mononuclear cells *in vitro***

MOs were incubated with *L. reuteri* E overnight. After incubation the number of MOs was not significantly changed, in comparison to control sample (Table 1). The ability of *L. reuteri* E to affect the phagocytic activity and index of phagocytosis was estimated. Significant increase of both phagocytic activity and index was observed. The relative phagocytic activity was 1.09 and relative phagocytic index was 1.36. Microbicidal activity was presented as a number of killed

CFU by MOs, that were pre-treated by *L. reuteri* E. Microbicidal activities of MO cells treated with *L. reuteri* E against *E. coli* and *C. albicans* were decreased, relative activities were 0.72 and 0.42, respectively. The lysozyme activity of induced MOs was significantly decreased (relative activity 0.30). Another protective metabolic activity of MOs against infection, peroxidase activity, was significantly stimulated by *L. reuteri* E (relative activity 1.53).

The phagocytes IL-1 $\beta$  production was significantly induced *in vitro*, relative activity was 2.47 (Table 1).

**Table 1.** Effect of *L. reuteri* E on biological activities of MOs *in vitro*.

| <i>In vitro</i> experiment  | Control sample, mean $\pm$ sd         | Treated sample, mean $\pm$ sd                       | Relative activity |
|---|---------------------------------------|---|-------------------|
| Number of surviving MOs after incubation with <i>L. reuteri</i> E <sup>a)</sup> | 1.63 $\pm$ 0.36 x 10 <sup>6</sup> /mL | 1.70 $\pm$ 0.45 x 10 <sup>6</sup> /mL <sup>NS</sup> | 1.04              |
| Phagocytic activity <sup>a)</sup>   | 80 $\pm$ 0.00 %                       | 87 $\pm$ 2.00 % <sup>**</sup>                       | 1.09              |
| Phagocytic index <sup>a)</sup>  | 5.9 $\pm$ 0.6                         | 8.0 $\pm$ 0.1 <sup>**</sup>                         | 1.36              |
| The number of killed <i>S. aureus</i> <sup>a)</sup>                             | 44 $\pm$ 2 x 10 <sup>6</sup> /mL      | 40 $\pm$ 16 x 10 <sup>6</sup> /mL <sup>NS</sup>     | 0.91              |
| The number of killed <i>E. coli</i> <sup>a)</sup>                               | 146 $\pm$ 16.4 x 10 <sup>6</sup> /mL  | 105 $\pm$ 6.8 x 10 <sup>6</sup> /mL <sup>*</sup>    | 0.72              |
| The number of killed <i>C. albicans</i> <sup>a)</sup>                           | 25 $\pm$ 1.1 x 10 <sup>5</sup> /mL    | 10 $\pm$ 0.5 x 10 <sup>5</sup> /mL <sup>***</sup>   | 0.42              |
| Lysozyme activity <sup>a)</sup>   | 0.01 $\pm$ 0.004                      | 0.003 $\pm$ 0.000 <sup>**</sup>                     | 0.30              |
| Peroxidase activity <sup>a)</sup>   | 0.163 $\pm$ 0.004                     | 0.250 $\pm$ 0.036 <sup>***</sup>                    | 1.53              |
| IL-1 $\beta$ <sup>b)</sup>  | 282.6 $\pm$ 49.1 pg/mL                | 698.2 $\pm$ 213.5 pg/mL <sup>**</sup>               | 2.47              |

<sup>a)</sup> dose 0.5 mg of dry weight of *L. reuteri* E per mL; <sup>b)</sup> dose 1.0 mg of dry weight of *L. reuteri* E per mL. The number of phagocytes was 2x10<sup>6</sup>/mL for each sample; experiments were done in 6 parallels. NS – non-significant sample, p – means statistically significant sample compared with control. p < 0.001 (\*\*\*); p < 0.01 (\*\*); p < 0.05 (\*)

#### The effect of *L. reuteri* E on mouse macrophages *in vivo*

*L. reuteri* E exhibited no lethal activity after 5 days of intraperitoneal application to mice. The average weight of mice in control group was not significantly changed after 5 days (from 24.8  $\pm$  0.9 g to 25.6  $\pm$  1.8 g). The average weight of mice pre-treated with *L. reuteri* E was significantly increased in comparison to control group (from 23.6  $\pm$  1.5 g to 27.4  $\pm$  0.1 g). Isolated peritoneal mouse macrophages were tested for several biological

activities (Table 2). Pre-treatment with *L. reuteri* E increased phagocytic activity and phagocytic index of mouse phagocytes. Relative phagocytic activity was 1.13 and relative phagocytic index was 1.51. Microbicidal activities of macrophages isolated from pre-treated mice were not influenced. The lysozyme activity of effected macrophages isolated from pre-treated mice was significantly increased (relative activity 1.83). Peroxidase activity was significantly inhibited (relative activity 0.17) (Table 2).

**Table 2.** The effect of *L. reuteri* E on biological activities of macrophages isolated from *in vivo* pretreated mice.

| <i>In vivo</i> experiment   | Control sample, mean $\pm$ sd         | Treated sample, mean $\pm$ sd                       | Relative activity |
|---|---------------------------------------|---|-------------------|
| Number of surviving macrophages after incubation with <i>L. reuteri</i> E | 1.62 $\pm$ 0.45 x 10 <sup>6</sup> /mL | 1.86 $\pm$ 0.33 x 10 <sup>6</sup> /mL <sup>NS</sup> | 1.16              |
| Phagocytic activity   | 84 $\pm$ 4 %                          | 94 $\pm$ 2 % <sup>**</sup>                          | 1.13              |
| Phagocytic index  | 7.33 $\pm$ 2.45                       | 9.69 $\pm$ 1.1 <sup>**</sup>                        | 1.51              |
| The number of killed <i>S. aureus</i>                                     | 16 $\pm$ 2 x 10 <sup>6</sup> /mL      | 16 $\pm$ 6 x 10 <sup>6</sup> /mL <sup>NS</sup>      | 1.00              |
| The number of killed <i>E. coli</i>                                       | 59 $\pm$ 11 x 10 <sup>6</sup> /mL     | 66 $\pm$ 13 x 10 <sup>6</sup> /mL <sup>NS</sup>     | 1.17              |
| The number of killed <i>C. albicans</i>                                   | 60 $\pm$ 18 x 10 <sup>5</sup> /mL     | 50 $\pm$ 12 x 10 <sup>5</sup> /mL <sup>NS</sup>     | 0.86              |
| Lysozyme activity   | 0.03 $\pm$ 0.004                      | 0.055 $\pm$ 0.000 <sup>***</sup>                    | 1.83              |
| Peroxidase activity   | 0.404 $\pm$ 0.018                     | 0.07 $\pm$ 0.045 <sup>***</sup>                     | 0.17              |

NS – non-significant sample, p – mans statistically significant sample in comparison with control. p < 0.001 (\*\*\*); p < 0.01 (\*\*). The number of macrophages was 2x10<sup>6</sup>/mL for each sample, the number of mice in group was 6 for each sample. Dose of *L. reuteri* E was 25 mg/kg.

## DISCUSSION

For identification of new isolate E derived from the stomach mucus of lamb the nucleotide sequencing was performed. 665 bp nucleotide sequence of PCR product of isolate E showed highest homology (99%) with the 16S rDNA of *L. reuteri* LU3 (AY 735 406). Two nucleotides in obtained sequence were identified ambiguously (any of purines, A or G). In the reference sequence from GenBank there is G on both of these positions (Figure 1). Identification was confirmed by species-specific PCR (14), 1100 bp product was observed. This verifies the previous results, which recognized new isolate E as *Lactobacillus reuteri*.

The inhibition activity of *L. reuteri* E against potential pathogens decreased as follows: *S. aureus* > *P. aeruginosa* > *S. enterica* ser. *Minnesota* > *E. coli* > *C. albicans* (Figure 2). According to Ocaña *et al.* (15) *S. aureus* growth is inhibited by *Lactobacillus* strains, which produce hydrogen peroxide. This might be one of the reasons of high growth inhibition of *S. aureus* by *L. reuteri* E. Production of bacteriocins should be another explanation of this effect. Bacteriocins, the antimicrobial proteinaceous substances, are generally defined as substances that kill related bacteria (4). Bacteriocins produced by Gram-positive (G+) bacteria are active mostly against other G+ bacteria. Weaker inhibition activity of *L. reuteri* E was observed in the case of Gram-negatives (G-) than in *S. aureus* (G+). *C. albicans* that has diametrically different composition of cellular coating was inhibited the least.

Besides the exclusion of pathogens from the gut mucus lactobacilli can induce also host resistance to microbial pathogens by stimulation of non-specific immunity (6).

For evaluation of immunomodulatory potential of *L. reuteri* E *in vitro* and *in vivo* tests on mammalian cells were performed. The ability of induced MOs by *L. reuteri* E to phagocyte *E. faecalis* was comparable in both experiments (relative activity *in vivo* 1.13 and *in vitro* 1.09). Phagocytes

release toxic agents, e.g., reactive oxygen intermediates and lytic enzymes, in various inflammatory reactions. Phagocytic activity results in the further recruitment of immunocompetent cells and the generation of inflammatory response. On the other hand microbicidal effect of MOs is limited to the production of cationic peptide molecules of different size and structure. During their interaction with bacteria they tend to find specific target in the bacterial cell membrane (9, 16). Modification of lipopolysaccharid (in G- bacterial membrane) or teichoic acid (in G+ bacterial membrane) often makes bacteria resistant to microbicidal activity of mononuclear cells (8, 16). The difference in bactericidal activity of modulated phagocytosing cells against *E. coli* and *S. aureus* can be explained by the different mechanism of action of MOs on G- and G+ bacteria. Microbicidal activity of pre-treated human MO cells against *E. coli* and *C. albicans in vitro* was inhibited (Table 1). The obtained results in the case of *C. albicans* are in good agreement with data obtained by streak line method.

Results of metabolic activities *in vitro* (human MO cells) and *in vivo* (mouse macrophages) showed the opposite course. Lysozyme activity *in vitro* was reduced, while *in vivo* increased. Vice versa, the peroxidase activity of MOs was increased *in vitro* while *in vivo* was decreased. Explanation of differences in peroxidase and lysozyme activities *in vivo* and *in vitro* could be in different administration of lactobacilli to mononuclear cells. In *in vitro* experiment live lactobacilli were used while in *in vivo* test lactobacilli were disintegrated before administration. Alive lactic acid bacteria can produce several extracellular substances e.g. organic acids, H<sub>2</sub>O<sub>2</sub>, bacteriocins etc. (1) which can affect metabolic activities of immunocompetent cells. Moreover organic acids produced by lactobacilli can reduce pH of environment (in our experiment phosphate buffer, pH 6.2) and simultaneously influence activity of lysozyme. Optimal pH for lysozyme activity is 6.2.

Anyway, there are also other agents and biochemical systems, except H<sub>2</sub>O<sub>2</sub> production, which can play a role in the modulation of the immune system of mouse.

Pro-inflammatory cytokines, including interleukin 1, play a pivotal, yet ambiguous, role in inflammation. *L. reuteri* induced several proinflammatory and/or Th1 cytokines, such as IL-1, IL-2, and TNF, but not antiinflammatory or Th2 cytokines, such as IL-10. The authors are open to dispute, that outcome of probiotic treatment may depend on the immunologic state of the host and that changes in cytokine profile induced by probiotic strains may be site specific and dependent on the experimental system used (7).

Immunomodulating effects of probiotic bacteria may depend on the immunologic state of the host. It was further suggested that differences between specific strains of probiotic bacteria may exist. It was also observed that whole bacterial cells enhance immune cells proliferation and induce pro-inflammatory cytokines production, concretely tumor necrosis factor and interleukin 6. In contrast probiotic bacteria mediate suppression of lymphocyte proliferation and T cells cytokine production (13).

Isolauri *et al.* (13) attempted to compare the anti-proliferative effect of several probiotic bacterial strains in their nonviable forms. The suppressive effect of 10  $\mu\text{mol/L}$  dexamethasone was compared with that of successful probiotics. Results indicated that some probiotic bacteria possess significant anti-inflammatory properties comparable to the therapeutic pharmaceutical agent. These findings further implicate the potential use of probiotic bacteria as immunomodulatory agents.

To estimate anti- or pro-inflammatory properties of *L. reuteri* E, bacterial cells were tested for ability to affect IL-1 $\beta$  production of phagocytes *in vitro* (Table 1). There are several studies investigating the usage of probiotic cells components for cytokine production. Lactobacilli, bifidobacteria and *E. coli* Nissle 1917 can affect pro- and anti-inflammatory cytokines production in peripheral blood MOs (11).

Our results indicate that *L. reuteri* E isolated from the stomach mucus of lamb has auspicious probiotic properties, for

example ability to inhibit the growth of several potential pathogens and modulate mammal's MOs activities *in vitro* as well as *in vivo*. Taken together, obtained data indicate that *L. reuteri* E is potential candidate for use in probiotic preparations for animals and/or human. However, additional experiments and testing are necessary.

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## REFERENCES

1. Annuk, H.; Shchepetova, J.; Kullisaar, T.; Songisepp, E.; Zilmer, M.; Mikelsaar, M. (2003): Characterization of intestinal lactobacilli as putative probiotic candidates. *J. Appl. Microbiol.* 94, p. 403-412.
2. Böyum, A. (1968): Isolation of leucocytes from human blood. Further observations. Methylcellulose dextran and ficoll as erythrocyte aggregating agents. *Scand. J. Clin. Lab. Invest. Suppl.* 97, p. 31-50.
3. Bukovský, M.; Mlynářčík, D.; Ondráčková, V. (1996). Immunomodulatory activity of amphiphilic antimicrobials on mouse macrophages. *Int. J. Immunopharmacol.* 18, p. 423-426.
4. Cleveland, J.; Montville, T.J.; Nes, I.F.; Chikindas, M.L. (2001). Bacteriocins: safe, natural antimicrobials for food preservation. *Int. J. Food Microbiol.* 71, p. 1-20.
5. FAO/WHO. 2002. Guidelines for the Evaluation of Probiotics in Food. Available at: <ftp://ftp.fao.org/es/esn/food/wgreport2.pdf>. Accessed 8 July 2010.
6. Foligne, B.; Nutten, S.; Grangette, C.; Dennin, V.; Goudercourt, D.; Poiret, S.; Dewulf, J.; Brassart, D.; Mercenier, A.; Pot, B. (2007). Correlation between *in vivo* and *in vitro* immunomodulation properties of lactic acid bacteria. *World J. Gastroenterol.* 13, p. 236-243.
7. Forsythe, P.; Inman, M. D.; Bienenstock, J. (2007). Oral treatment with live *Lactobacillus reuteri* inhibits the allergic airway response in mice. *Am. J. Respir. Crit. Care Med.* 175, p 561-569.
8. Gunn J.S. (2001). Bacterial modification of LPS and resistance to antimicrobial peptides. *J. Endotox. Res.* 7, p. 57-62.
9. Hancock, R.E.W.; Rozek, A. (2002). Role of membranes in the activities of antimicrobial cationic peptides. *FEMS Microbiol. Lett.* 206, p. 143-149.
10. Heilig, H.G.H.J.; Zoetendal, E.G.; Vaughan, E.E.; Marteau, P.;



- Akkermans, A.D.L.; de Vos, W.M. (2002). Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. *Appl. Environ. Microbiol.* 68, p. 114-123.
11. Helwig U.; Lammers K.M.; Rizzello F.; Brigidi P.; Rohleder V.; Caramelli E.; Gionchetti P.; Schrezenmeir J.; Foelsch U.R.; Schreiber S.; Campieri M. (2006). Lactobacilli, bifidobacteria and *E. coli* Nissle induce pro- and anti-inflammatory cytokines in peripheral blood mononuclear cells. *World J. Gastroenterol.* 12, p. 5978-5986.
  12. Hütt, P.; Shchepetova, J.; Lõivukene, K.; Kullisaar, T.; Mikelsaar, M. (2006). Antagonistic activity of probiotic lactobacilli and bifidobacteria against entero- and uropathogens. *J. Appl. Microbiol.* 100, p. 1324-1332.
  13. Isolauri E.; Sütas Y.; Kankaanpää P.; Arvilommi H.; Salminen S. (2001) Probiotics: effects on immunity, *Am. J. Clin. Nutr.* 73, p. 2001, 444-450.
  14. Kwon, H.S.; Yang, E.H.; Yeon, S.W.; Kang, B.H.; Kim, T.Y. (2004). Rapid identification of probiotic *Lactobacillus* species by multiplex PCR using species – specific primers based on the region extending from 16S rRNA through 23S rRNA. *FEMS Microbiol. Lett.* 239, p. 267-275.
  15. Ocaña, V.S.; Pesce de Ruiz Holgado, A.A.; Nader-Macías, M.E. (1999). Growth inhibition of *Staphylococcus aureus* by H<sub>2</sub>O<sub>2</sub>-producing *Lactobacillus paracasei* subsp. *paracasei* isolated from human vagina. *FEMS Immunol. Med. Microbiol.* 23, p. 87-92.
  16. Pesch A. (2002). How do bacteria resist human antimicrobial peptides? *Trends Microbiol.* 10, p. 179-185.
  17. Tannock, G.W.; Tilsala-Timisjarvi, A.; Rodtong, S.; Ng, J.; Munro, K.; Alatossava, T. (1999). Identification of *Lactobacillus* isolates from the gastrointestinal tract, silage, and yoghurt by 16S – 23S rRNA intergenic spacer region sequence comparisons. *Appl. Environ. Microbiol.* 65, p. 4264-4267.
  18. Versalovic, J.; Koeuth, T.; Lupski, J.R. (1991). Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucl. Acid Res.* 19, p. 6823-6831.
  19. Wilks, M.; Wiggins, R.; Whiley, A.; Hennessy, E.; Warwick, S.; Porter, H.; Corfield, A.; Millar, M. (2004). Identification and H<sub>2</sub>O<sub>2</sub> production of vaginal lactobacilli from pregnant women at high risk of preterm birth and relation with outcome. *J. Clin. Microbiol.* 42, p. 713-717.



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