Antibacterial potential of lactobacilli isolated from a lamb

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ABSTRACT: The antimicrobial properties of three potential probiotic strains of lactobacilli isolated from a lamb (*Lactobacillus murinus* C, *Lactobacillus mucosae* D and *Lactobacillus reuteri* E) were studied using the streak line method and the agar well diffusion assay. The probiotic lactobacilli strains *Lactobacillus rhamnosus* ATCC 53103, *Lactobacillus reuteri* ATCC 55730, *Lactobacillus reuteri* ATCC 55845 and *Lactobacillus plantarum* DSM 9843 were used for comparison. Using the streak line method the inhibitory activity of lactobacilli products towards ten Gram-positive and Gram-negative potential pathogenic bacteria under different cultivation conditions (anaerobic or microaerobic preincubation of lactobacilli for 24 h or 48 h) was tested. The strongest inhibitory activity was demonstrated by the *Lactobacillus reuteri* E strain. The most sensitive strains to the antimicrobial activity of lactobacilli were *Yersinia enterocolitica* clinical isolate (19.9 ± 6.8 mm) and *Listeria monocytogenes* ATCC 51774 (17.7 ± 6.0 mm) after microaerobic and anaerobic preincubation, respectively. Generally, microaerobic conditions and longer preincubation of lactobacilli resulted in stronger inhibition of target bacteria. The inhibitory activity of lactobacilli towards selected lactobacilli strains was also tested. Only low inhibition of growth was observed. In the agar well diffusion assay the inhibitory effect of natural and modified lactobacilli culture cell-free supernatants, obtained from MRS broth cultures, on *Staphylococcus aureus* ATCC 6538 growth was determined. Supernatants were modified by heat (10 min/60 °C; 60 min/100 °C) and protease treatment and neutralization of pH. Neutralization elicited the most significant impact on the activity of supernatants and resulted in total loss of activity. After all other modifications supernatants retained some residual activity. The highest inhibitory activity was observed for the cell-free supernatant produced by *Lactobacillus mucosae* D.

Keywords: *Lactobacillus* spp.; antibacterial activity; probiotics; cell-free supernatants

Farm animals bred in large numbers are highly susceptible to enteric disorders that lead to defects in digestion and nutrient absorption. To improve their growth different antibiotics have been used as dietary supplements. However, this resulted in another problem – animals could constitute reservoirs of resistant bacteria, potentially harmful for humans. Therefore, probiotics, with their broad-acting antimicrobial activity, hold much promise as dietary supplements (Nousiainen et al., 2004).

Probiotics for animals are defined as live microorganisms that are able to decrease the number of intestinal infections, increase production and improve food hygiene by contributing to a better gastrointestinal environment (Nousiainen et al., 2004). The FAO/WHO have stipulated several criteria for probiotic evaluation. One of the most important parameters by which potentially new probiotic strains must be characterized is the production of antimicrobial substances under *in vitro* conditions (FAO/WHO, 2002).

Lactobacilli are facultatively anaerobic, catalase-negative, non-spore forming, rod-shaped lactic acid bacteria. Several strains of the genus *Lactobacillus* are used as probiotics. The common attribute of this group is the ability to ferment saccharides to lactic acid. Lactic acid together with some other products of lactobacilli is responsible for the antimicrobial activity of these bacteria (Walencka et al., 2008). Additional antimicrobial compounds produced by

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lactobacilli include different organic acids (acetic, propionic, butyric or formic acid), hydrogen peroxide, carbon dioxide, diacetyl, acetaldehyde, reuterin, reutericyclin and different bacteriocins (Annuk et al., 2003; Ouwehand and Vesterlund, 2004). These substances have different mechanisms of action (Ouwehand and Vesterlund, 2004).

Besides the ability of probiotic microorganisms to modulate the immune system of a macroorganism, their impact on the host microflora in favour of beneficial species is also of great importance.

Three strains of lactobacilli (designated C, D and E) were isolated from the stomach mucus of a lamb in our previous study. These three strains were identified by 16S rDNA sequencing as *Lactobacillus murinus*, *L. mucosae* and *L. reuteri*, respectively (Bilkova et al., 2008). The aim of the present work was to evaluate the antimicrobial effects of these new lactobacilli isolates towards selected opportunistic pathogenic bacteria and other strains of lactobacilli.

MATERIAL AND METHODS

Bacterial cultures and growth conditions

The lactobacilli strains *L. murinus* C (Lmur C), *L. mucosae* D (Lmuc D), *L. reuteri* E (Lreu E) were isolated from the stomach mucus of a three-week old breast-fed lamb (Ocova, Slovak Republic) and characterized previously in our work Bilkova et al. (2008). The collection strains were the following: *L. rhamnosus* ATCC 53103, *L. reuteri* ATCC 55730, *L. reuteri* ATCC 55845 (American Type Culture collection, Manasas, Virginia, USA) and *L. plantarum* DSM 9843 (Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany). All lactobacilli were grown in MRS broth (according to De Man, Rogosa and Sharpe, Oxoid, Basingstoke, Hants, UK) under anaerobic conditions for 24 h at 37 °C.

Indicator (target) bacteria (*Salmonella enterica* ser. Enteritidis ATCC 1376, *Pseudomonas aeruginosa* ATCC 27853, *Listeria monocytogenes* ATCC 13932, *L. monocytogenes* ATCC 51774, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Shigella sonnei* ATCC 25931, *Enterobacter cloacae* ATCC 13047 (American Type Culture Collection, Manasas, Virginia, USA), *S. enterica* ser. Typhimurium clinical isolate, *Yersinia enterocolitica* clinical isolate (both from Institute of Microbiology, University of Tartu, Estonia) were cultivated aerobically on blood agar (Oxoid, UK) for 24 h at 37 °C. *S. aureus* ATCC 6538 was grown overnight in nutrient broth (Imuna, Sarisske Michalany, Slovak Republic) at 37 °C.

Streak line method

This assay was used to test the antibacterial activity of the substances released by lactobacilli into the solid culture medium. The technique was performed according to Hutt et al. (2006) with some modifications. Cultures of the Lmur C, Lmuc D, Lreu E, *L. rhamnosus* ATCC 53103, *L. plantarum* DSM 9843 and *L. reuteri* ATCC 55845 strains were diluted according to McFarland standard No. 3 in physiological saline and seeded in a total volume of 20 µl in a line in the middle of plates (Æ 9.6 cm) containing modified MRS agar (triammonium citrate and sodium acetate were excluded). Lactobacilli were incubated under microaerobic (10% CO_2) or anaerobic conditions for 24 h or 48 h. After incubation lactobacilli were killed by exposure to chloroform gas for 2 h.

Overnight cultures of the target strains grown on blood agar plates were diluted according to McFarland standard No. 3 in physiological saline and inoculated onto plates with killed lactobacilli in two horizontal lines – one from the line of lactobacilli to the margin of the plate and the second from the margin of the plate to the lactobacilli line in an amount of 10 µl (Figure 1). Indicator strains were incubated for 24 h at 37 °C aerobically.

The Lmur C, Lmuc D and Lreu E strains were tested for their ability to inhibit the growth of each other after anaerobic preincubation (24 h and 48 h).

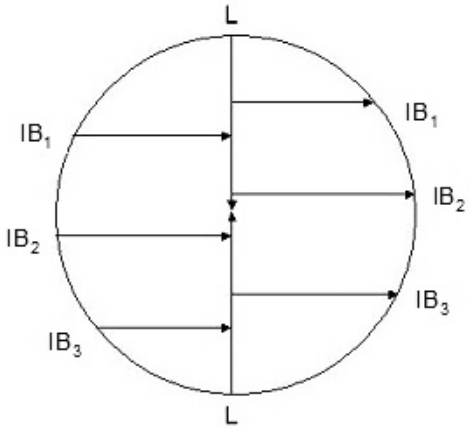

Figure 1. The streak line method. L = lines of *Lactobacillus* spp. (producer of antibacterial substances), IB1, IB2 and IB3 = lines of indicator bacteria. Each arrow indicates 10 µl of inoculated culture

The ability to inhibit the growth of a target bacterium was determined as the zone of growth inhibition, measured (mm) between the culture line of *Lactobacillus* spp. and the target bacterium. Experiments were done in three parallels and three replicates. The rate of inhibition capability was evaluated in the following manner: low > 11 mm; intermediate 18–24 mm; high > 25 mm (Hutt et al., 2006).

Agar well diffusion assay

Cell-free culture supernatants (CFSs) of Lmur C, Lmuc D, Lreu E and *L. reuteri* ATCC 55730 were examined for their antibacterial activity by the agar well diffusion assay as described by Kim and Rajagopal (2001). Culture supernatants were prepared by cultivation of lactobacilli in MRS broth anaerobically overnight at 37 °C and removal of the cells by centrifugation at 2000 × g for 10 min. The pH of culture supernatants was examined and equal portions were modified by adjusting to neutral pH 7.0 (1M NaOH), digestion with proteinase K (1 h/37 °C; final concentration 1 mg/ml; Qiagen, Hilden, Germany), and heat treatment (10 min/60 °C; 60 min/100 °C). After treatment supernatants were sterile-filtered (0.22 μm pores, MCE membrane, Millipore Ltd., Hertfordshire, UK). The indicator strain *S. aureus* ATCC 6538 was diluted in physiological saline (McFarland No. 1) and added to melted soft BHI agar (0.75% Brain Heart Infusion, Biomark Laboratories, Pune, India) at a final number of 3×10^5 CFU/ml. After allowing the soft agar to solidify on the base BHI agar (1.5%) wells of 7 mm diameter were made with a sterile cork borer. One hundred μl portions of each natural and modified CFS were added to wells. The plates were left undisturbed for a few hours so that the supernatant could diffuse into the agar. *S. aureus* was incubated for 18 h at 37 °C and afterwards the inhibition zones were measured. Experiments were carried out in four replicates.

RESULTS AND DISCUSSION

Lactobacilli are known for their production of various antimicrobial compounds (Ouwehand and Vesterlund, 2004; Pangallo et al., 2008). The production of these compounds by intestinal microflora is probably one of the most important mechanisms responsible for the antagonistic phenomenon (Gomes et al., 2006) and therefore it is essential to examine this property in probiotic candidates. The ability of three new isolates of *Lactobacillus* spp. (Lmur C, Lmuc D, Lreu E) and four probiotic lactobacilli strains (*L. rhamnosus* ATCC 53103, *L. plantarum* DSM 9843, *L. reuteri* ATCC 55845 and *L. reuteri* ATCC 55370) to produce substances that can inhibit the growth of several Gram-positive (G+) and Gram-negative (G–) bacteria was determined in our study.

The secretion of antibacterial substances into solid medium was examined by the streak line method (Hutt et al., 2006). Lactobacilli were preincubated for 24 h or 48 h under anaerobic or microaerobic conditions. Their ability to inhibit the growth of target bacteria varied according to strain, incubation time and cultivation conditions (Tables 1 and 2). In general, no significant differences were observed between anaerobically and microaerobically grown lactobacilli cultures, but prolonged incubation (48 h) increased inhibition activity.

The highest inhibitory activity was observed in the case of *L. reuteri* E against the *Y. enterocolitica* clinical isolate after 48 h preincubation of the lactobacillus under microaerobic conditions (29.4 ± 3.6 mm). The lowest sensitivity was shown by *L. monocytogenes* ATCC 13932 after 24 h preincubation of Lmur C in a microaerobic environment (6.8 ± 1.0 mm). Other strains mostly displayed intermediate inhibitory activity towards target bacteria (Tables 1 and 2).

The antagonistic effects of culture supernatants of Lmur C, Lmuc D, Lreu E and *L. reuteri* ATCC 55730 on the indicator organism *S. aureus* were tested by the agar well diffusion assay. The diameter of growth inhibition zones varied among the lactobacilli strains; however, differences were not significant (Table 3). CFS derived from Lmuc D was the strongest inhibitor of *S. aureus* ATCC 6538 growth.

Further, the antibacterial activity of CFS's was evaluated after neutralization of pH, heat inactivation and protease-treatment of CFS's. Neutralization of CFS's resulted in a complete loss of their antibacterial activity. These results lead us to suggest that the main extracellular antibacterial agents of lactobacilli are organic acids, produced during fermentation. Proteinase K and heat treatment had little or no impact on the inhibitory effect of CFS's. In the case of *L. reuteri* ATCC 55730 reduced inhibition was observed after heat treatment. Therefore, some heat labile antibacterial agent could also be produced by this strain.

Bactericins, extracellularly released antibacterial peptides or proteins, display a limited inhibitory

Table 1. Antibacterial activity of lactobacilli towards selected potential pathogens after microaerobic preincubation

Microaerobic preincubation	Lmur C		Lreu E		*L. rhamnosus*		*L. reuteri*		*L. plantarum*		A
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	
S. aureus	8.4 ± 0.9	13.2 ± 2.3	17.0 ± 1.1	21.8 ± 2.9	11.3 ± 1.6	22.2 ± 2.5	11.3 ± 2.4	18.0 ± 2.1	13.5 ± 1.6	21.7 ± 0.8	15.8 ± 5.0
L. monocytogenes 13932	6.8 ± 1.0	12.7 ± 1.3	18.4 ± 1.5	25.4 ± 3.6	12.2 ± 1.2	22.9 ± 1.9	13.4 ± 1.9	21.0 ± 1.1	13.7 ± 1.5	25.6 ± 3.0	17.2 ± 6.4
L. monocytogenes 51774	8.0 ± 0.7	14.3 ± 1.8	19.2 ± 1.5	24.4 ± 2.5	12.4 ± 0.8	22.8 ± 1.5	13.4 ± 2.1	22.2 ± 1.3	14.1 ± 1.6	25.8 ± 2.4	17.7 ± 6.0
G+ average	7.7 ± 0.9	13.4 ± 0.8	18.2 ± 1.1	23.9 ± 1.8	11.9 ± 0.6	22.6 ± 0.4	12.7 ± 1.2	20.4 ± 2.1	13.8 ± 0.3	24.4 ± 2.3	17.2 ± 5.7
P. aeruginosa	7.1 ± 1.0	12.6 ± 1.6	19.1 ± 1.8	27.3 ± 2.0	9.4 ± 1.0	22.0 ± 2.2	12.1 ± 1.8	25.2 ± 1.6	10.9 ± 1.0	22.2 ± 1.3	16.8 ± 7.2
E. coli	7.8 ± 0.9	12.2 ± 1.5	20.1 ± 1.3	26.4 ± 3.0	9.9 ± 0.8	22.6 ± 2.5	13.2 ± 2.4	23.8 ± 1.8	12.0 ± 0.9	23.5 ± 1.6	17.2 ± 6.8
S. typhimurium	6.4 ± 1.0	9.8 ± 2.6	19.0 ± 1.2	25.2 ± 3.1	9.7 ± 1.0	21.1 ± 2.5	12.8 ± 1.8	20.7 ± 1.5	11.9 ± 1.3	22.1 ± 1.9	15.9 ± 6.5
S. enteritidis	8.5 ± 0.5	11.8 ± 2.5	20.9 ± 1.4	24.2 ± 2.9	9.7 ± 1.1	19.3 ± 1.5	12.6 ± 1.9	22.5 ± 2.6	11.4 ± 0.9	21.5 ± 1.0	16.2 ± 6.0
Sh. sonnei	8.6 ± 1.2	12.4 ± 1.2	21.1 ± 1.6	26.4 ± 3.2	10.5 ± 0.8	22.4 ± 2.0	13.4 ± 2.7	24.7 ± 1.2	12.8 ± 0.9	24.9 ± 2.2	17.7 ± 6.8
E. cloacae	5.6 ± 0.8	9.9 ± 1.0	18.7 ± 1.8	25.1 ± 2.4	9.1 ± 0.9	22.8 ± 3.6	12.2 ± 1.8	21.2 ± 1.8	10.8 ± 1.1	23.0 ± 2.9	15.8 ± 7.0
Y. enterocolitica	10.1 ± 1.4	16.1 ± 0.9	22.7 ± 1.9	29.4 ± 3.6	12.5 ± 1.0	22.5 ± 1.8	15.9 ± 2.5	28.5 ± 2.3	15.2 ± 1.9	25.8 ± 1.8	19.9 ± 6.8
G– average	7.7 ± 1.5	12.1 ± 2.1	20.2 ± 1.4	26.3 ± 1.7	10.1 ± 5.6	21.8 ± 1.2	13.2 ± 1.3	23.8 ± 2.7	12.1 ± 1.5	23.3 ± 1.6	17.1 ± 6.6

The values are arithmetical means ± SD of inhibition zones (mm)
L. rhamnosus – *L. rhamnosus* ATCC 53103, *L. reuteri* – *L. reuteri* ATCC 55845, *L. plantarum* – *L. plantarum* DSM 9843. A = mean of inhibition zones in one indicator strain

Table 2. Antibacterial activity of lactobacilli towards selected potential pathogens after anaerobic preincubation

Anaerobic preincubation	Lmur C		Lmuc D		Lreu E		*L. rhamnosus*		*L. reuteri*		*L. plantarum*		A
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	
S. aureus	8.9 ± 1.3	13.4 ± 1.1	12.2 ± 0.9	20.4 ± 2.4	14.3 ± 1.4	21.6 ± 1.6	13.0 ± 1.5	20.5 ± 1.7	13.4 ± 1.2	20.6 ± 2.1	13.3 ± 2.0	21.9 ± 2.2	16.1 ± 4.5
L. monocytogenes 13932	8.5 ± 1.1	12.2 ± 1.1	13.4 ± 1.0	20.7 ± 2.6	14.3 ± 1.6	24.1 ± 2.0	12.7 ± 0.9	21.1 ± 1.3	12.8 ± 1.8	22.1 ± 1.2	14.1 ± 1.1	23.3 ± 1.2	16.6 ± 5.3
L. monocytogenes 51774	9.6 ± 1.2	13.3 ± 1.2	13.5 ± 1.1	21.2 ± 1.9	14.9 ± 1.2	23.5 ± 1.9	13.4 ± 1.3	21.2 ± 1.5	13.4 ± 1.8	22.3 ± 1.5	13.8 ± 1.2	23.6 ± 1.6	17.0 ± 5.0
G+ average	9.6 ± 1.8	13.0 ± 0.8	13.0 ± 0.7	20.8 ± 0.4	14.5 ± 0.4	23.1 ± 1.3	16.3 ± 0.4	20.9 ± 0.4	13.2 ± 0.3	21.7 ± 1.0	13.7 ± 0.4	22.9 ± 0.9	16.6 ± 4.8
P. aeruginosa	7.8 ± 1.2	12.3 ± 1.4	10.9 ± 1.4	19.1 ± 3.3	13.4 ± 0.9	22.8 ± 1.4	11.2 ± 1.5	19.4 ± 2.1	12.2 ± 1.3	20.6 ± 2.3	11.3 ± 1.6	20.9 ± 0.9	15.2 ± 5.0
E. coli	7.9 ± 1.4	12.1 ± 1.0	11.9 ± 0.9	19.6 ± 2.5	13.7 ± 1.1	23.5 ± 2.8	11.6 ± 1.2	20.4 ± 2.1	11.5 ± 1.9	21.8 ± 1.9	12.2 ± 1.7	21.8 ± 1.7	15.7 ± 3.9
S. typhimurium	7.1 ± 1.6	10.2 ± 0.9	10.4 ± 1.2	18.8 ± 2.5	13.2 ± 1.1	22.1 ± 1.4	10.7 ± 0.8	18.9 ± 1.7	11.2 ± 1.2	21.1 ± 1.8	11.2 ± 1.6	20.4 ± 1.0	14.6 ± 5.2
S. *enteritidis*	7.7 ± 1.4	12.6 ± 1.6	11.6 ± 1.1	19.8 ± 1.7	13.9 ± 1.3	22.7 ± 1.6	12.2 ± 1.3	19.4 ± 1.1	12.1 ± 2.0	20.9 ± 1.6	12.4 ± 1.8	20.9 ± 1.2	13.5 ± 4.9
Sh. sonnei	9.1 ± 1.1	12.6 ± 1.4	11.7 ± 1.6	20.9 ± 1.8	13.5 ± 1.0	23.2 ± 2.1	12.2 ± 1.2	20.9 ± 1.8	12.0 ± 2.2	22.8 ± 2.1	12.6 ± 1.2	22.3 ± 1.3	16.2 ± 5.3
E. cloacae	8.7 ± 1.3	10.1 ± 1.9	10.6 ± 0.8	19.1 ± 2.1	13.1 ± 0.7	22.3 ± 2.2	10.8 ± 0.9	19.2 ± 1.5	10.6 ± 1.3	20.8 ± 1.5	12.3 ± 1.1	20.7 ± 1.0	14.8 ± 5.1
Y. enterocolitica	11.6 ± 1.5	16.6 ± 2.2	15.7 ± 1.5	20.6 ± 2.3	17.2 ± 1.6	28.6 ± 2.9	13.9 ± 1.2	24.2 ± 3.0	14.8 ± 1.8	20.5 ± 3.1	14.5 ± 1.8	26.6 ± 2.3	16.4 ± 5.4
G– average	8.5 ± 1.5	12.4 ± 2.2	11.8 ± 1.8	19.7 ± 0.8	14.0 ± 1.4	23.6 ± 2.3	11.8 ± 1.1	20.3 ± 5.0	12.1 ± 1.3	21.2 ± 0.8	12.3 ± 1.1	21.9 ± 2.2	15.8 ± 5.2

The values are arithmetical means ± SD of inhibition zones (mm)
L. rhamnosus = *L. rhamnosus* ATCC 53103, *L. reuteri* = *L. reuteri* ATCC 55845, *L. plantarum* = *L. plantarum* DSM 9843; A = mean of inhibition zones in one indicator strain

Table 3. Antibacterial activity of cell-free supernatants of selected Lactobacillus strains towards *S. aureus* ATCC 6538

Cell free supernatant	Lmur C	Lmuc D	Lreu E	*L. reuteri* ATCC 55730
Untreated	12.0 ± 1.4	11.8 ± 0.5	11.0 ± 0.8	10.8 ± 1.0
pH 7.0	7.0 ± 0.0	7.0 ± 0.0	7.0 ± 0.0	7.0 ± 0.0
10 min/60 °C	11.5 ± 0.6	11.3 ± 1.0	9.8 ± 1.0	10.3 ± 0.5
60 min/100 °C	10.0 ± 0.8	10.8 ± 0.5	9.3 ± 0.0	8.8 ± 1.0
Proteinase K	11.3 ± 1.5	11.0 ± 0.8	11.0 ± 0.8	10.0 ± 0.8

The values are arithmetical means ± SD of inhibition zones (mm). Diameter of well was 7 mm

spectrum towards closely related G+ bacteria (De Vuyst and Vandamme, 1994). In our experiments G– target bacteria were more sensitive towards the antibacterial activity of lactobacilli than G+. However, there was no large difference in the inhibition of G+ and G– (mean 16.7 ± 5.2 and 16.4 ± 5.9 mm, respectively). The most sensitive target bacterium to anaerobically grown lactobacilli was *L. monocytogenes* ATCC 51774 (17.0 ± 5.0 mm). Interestingly, after lactobacilli precultivation in a microaerobic environment, the most sensitive bacterium was the G– *Y. enterocolitica* clinical isolate (19.9 ± 6.8 mm). The most resistant strains were *S. aureus* ATCC 25923 (15.8 ± 5.0 mm) and *E. cloacae* ATCC 13047 (15.8 ± 7.0 mm) after microaerobic preincubation of lactobacilli and *S. enterica* ser. Enteritidis ATCC 13076 (13.5 ± 4.9 mm) after anaerobic preincubation.

According to the cultivation conditions, a longer preincubation of lactobacilli resulted in a stronger inhibitory effect towards target bacteria. The concentration of carbon dioxide during cultivation also had a significant impact on the ability of lactobacilli to inhibit the growth of indicator bacteria. Altogether, a higher inhibitory activity was noted in a microaerobic environment, in comparison with anaerobic conditions. This allows us to venture that the presence of oxygen is necessary for or supports the production of some antibacterial substance or substances. As the antibacterial activity of lactobacilli was noted in different environments (anaerobic and microaerobic), it is possible that these compounds will be produced also in different parts of the gastrointestinal tract, as both of these environments are present there (Annuk et al., 2003). It is interesting that *L. mucosae* D was not able to grow even after 48 h incubation in a microaerobic environment. Thus, it seems that this strain is strictly anaerobic.

The ability of new isolates to inhibit the growth of other strains of lactobacilli was also determined by the streak line method (Table 4). Overall, 24 h preincubation of lactobacilli had a weaker influence on the growth inhibition of the "indicator" strain. On the other hand, two day-long preincubation resulted in only low inhibition. These results are important with regard to the combined use of probiotic preparations.

We were further interested to determine the composition and concentration of the main substances contributing to the antibacterial effects of lactobacilli CFS's. After overnight incubation of lactobacilli in MRS broth, a significant acidification of the medium was observed (from pH 6.5 to ca. pH 4.4). This low pH most likely affected the inhibitory activity of lactobacilli supernatants. However, Kim and Rajagopal (2001), who tested the CFS's of lactobacilli against several G+ and G– indicator bacteria using the agar well diffusion assay, neutralized the pH of these supernatants before their experiments and observed inhibition of indicator strain growth. As we mention above, different extracellular products could contribute to the inhibitory effects of CFS's.

Table 4. Ability of new isolates of lactobacilli to inhibit the growth of other lactobacilli after anaerobic preincubation

Producer strain →	Lmur C		Lmuc D		Lreu E	
Indicator strain ↓	24 h	48 h	24 h	48 h	24 h	48 h
Lmur C			1.6 ± 1.5	3.3 ± 1.5	2.0 ± 2.3	5.8 ± 1.9
Lmuc D	2.0 ± 1.2	9.4 ± 1.9			1.6 ± 1.7	6.0 ± 1.8
Lreu E	2.4 ± 1.5	9.1 ± 1.5	1.4 ± 1.4	4.1 ± 1.6		

The values are arithmetical means ± SD of inhibition zones (mm)

The results we obtained with treated supernatants of lactobacilli indicate that besides organic acids, these could possibly include several heat labile substances, resistant to proteinase K cleavage.

In conclusion, the presented results show that the newly isolated lactobacilli strains Lmur C, Lmuc D and Lreu E can inhibit the growth of potential pathogens as strongly as or even stronger than the probiotic strains *L. rhamnosus* ATCC 53103, known as LGG, *L. reuteri* ATCC 55730, used in preparation BioGaiaa, *L. reuteri* ATCC 55845, and *L. plantarum* DSM 9843 designated also as *L. reuteri* RC-14 and *L. plantarum* 299v, respectively. This attribute is advantageous when considering the use of these strains in food preservation (Kecerova et al., 2004) as well as feed supplements or in veterinary medicine.

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