



THE STUDY OF THE PROBIOTIC POTENTIAL OF THE BENEFICIAL BACTERIA ISOLATED FROM KEFIR GRAINS

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ABSTRACT

The aim of this study was to identify beneficial bacteria with probiotic potential from kefir grains. The lactobacilli isolated from kefir grains were characterised as: *Lactobacillus plantarum*, *Lactobacillus paraplantarum*, *Lactobacillus paracasei*, and *Lactobacillus kefir*. The strains *Lb. plantarum* 1Ž, *Lb. paraplantarum* S10, and *Lb. paracasei* 2Ž tolerated better the test gastric juice at pH 2 and 2.6 during 120 min of incubation in comparison with the strains *Lb. kefir*. On the other hand, the strains *Lb. kefir* were resistant to 0.3% bile acid salts. The *Lb. paracasei* 2Ž showed the significantly highest survival ($P < 0.001$) at pH 2 in comparison with all other strains tested and was also able to tolerate 0.3% concentration of the bile salts. All strains produced medium to strong biofilms on abiotic surfaces and inhibited the growth of selected potential pathogens with varying intensity. All kefir isolates were susceptible to the antibiotics tested and exhibited positive β -galactosidase activity with the exception of *Lb. paracasei* 2Ž which did not show any activity of undesirable enzymes, such as β -glucosidase and

β -glucuronidase. Additional testing and validation of the biological properties and safety of the strain *Lb. paracasei* 2Ž under *in vivo* conditions are needed to confirm the prospective use of this strain in practice.

Key words: biofilm; inhibitory activity; lactobacilli; probiotics; safety; tolerance of GIT conditions

INTRODUCTION

Kefir is a popular drink originating in the Caucasian mountains in Central Asia where it has been consumed since the middle Ages. At the present it is an important consumer commodity in many areas of the world including Africa and the Middle East. Kefir is traditionally made using kefir grains as a starter culture. The grain matrix is composed of a complex of proteins and polysaccharides and consists of densely populated lactic fermentation bacteria (*Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*), acetic fermentation bacteria and yeasts, the proportion of which is affected by geographic regions [29].

Although some of the health benefits of kefir have not yet been validated by exact scientific and clinical investigations, several *in vitro* studies conducted on animals have confirmed the positive effects of kefir on: intolerance of lactose [20], immunomodulation [21], antimicrobial activity against pathogenic micro-organisms [9] and harmonisation of intestinal microflora [44]. The functional properties of kefir have been traditionally ascribed to its biologically active proteins and bacterial exopolysaccharide — kefiran [40]. However, the potential beneficial effects could also be mediated by the microbial composition of this fermented milk or by secondary metabolites [37].

Although a considerable number of commercial well-characterised probiotic strains are available today, there is still interest in screening new productive strains [5, 36]. Strains exhibiting unique and specific properties important for health can be selected during characterisation of natural fermented dairy products such as kefir [46]. This traditional product may be an interesting source of potential probiotic bacteria with specific functional properties. Despite the fact that many authors have advocated the importance of the human origin of a strain and a selective criterion for its use by humans, the professional group at FAO/WHO [17] stressed more the probiotic activity of the strain than the source of the relevant micro-organism.

The functionality of probiotics can be determined by two groups of tests [34]. The first group of tests focuses on the physiological properties and safety, for example: the tolerance of gastric juice and bile acids; ability to adhere to intestinal mucosa and colonise the intestine; production of inhibitory substances and inhibitory activity against pathogenic bacteria; susceptibility of the strain to antibiotics and enzymatic activity; and immunomodulatory properties. The second group of tests focuses on the technological properties of probiotic micro-organisms, such as the viability during the production process and stability of the strain during storage.

The aim of this study was to identify lactobacilli isolated from kefir grains and characterise their selected properties according to instructions recommended by FAO/WHO with respect to their biological effects and safety under *in vitro* conditions.

MATERIALS AND METHODS

Isolation of lactobacilli from kefir grains and their identification

Homogenised samples of kefir grains were applied to de Man-Rogosa-Sharpe agar (MRS; Carl Roth GmbH, Germany) with addition of 200 ppm antimycotic cycloheximide (Glentham Life Sciences Ltd., UK) to suppress the growth of yeasts. The plates were cultivated in an anaerobic environment (Gas Pak Plus, BBL, Microbiology Systems, Cockeysville, USA) at 30°C, 37°C and 40°C for 48–72 hours. Individual morphologically different colonies were stained according to Gram. According to microbiological features (shape, colour, size, arrangement) selected isolates were deposited in the cryosystem Microbank™ (Pro-Lab Diagnostics, Canada) for the purpose of their preservation for additional analyses.

The isolates were identified by means of MALDI-TOF mass spectrometry (Microflex LT instrument Bruker Daltonik GmbH, Leipzig, Germany) using the method of Bessedé et al. [3]. On the basis of the obtained spectra (BioTyper software, version 2.0 Bruker Daltonik), the probability of identification was evaluated as follows: $\text{score} \geq 2.30$ — high probability of identification at the level of species; $2.30 \geq \text{score} \geq 2.00$ — high probability of identification at the level of the genus; and $2.00 \geq \text{score} \geq 1.70$ — probable identification at the level of the genus.

Testing of viability in gastric juice

Simulated gastric juice (pH 2 and 2.6) was prepared according to the method by Kos et al. [26]. A fresh suspension of a respective strain (approximately 1×10^8 CFU.ml⁻¹) obtained by multiplication in de Man Rogosa and Sharpe (MRS) broth (Carl Roth GmbH) and subsequent washing in saline solution was added to the sterile gastric juice. The incubation took place in a water bath at 37°C with shaking. The viability of the strains was determined after 0, 60, 90 and 120 min of incubation by means of a flow cytometer BD FACSCanto™ (Becton Dickinson Biosciences, USA) using BD FACS Diva™ software. For the measurement, we used a mixture of 50 µl of the strain sample in gastric juice, 5 µl of carboxyfluorescein diacetate stain and 445 µl of phosphate buffer saline (PBS) containing 1 mM dithiothreitol (Sigma-Aldrich, USA). This mixture was incubated for 30 min at 37°C. Blue laser of wavelength 488 nm was used for excitation and the subsequent emission was measured

at wavelength 530/30 nm (FL1). The percentage proportion of live and dead bacteria was evaluated by means of a histogram with FL1 fluorescence setting against the Count (cell numbers). The results were presented as the arithmetic mean of three measured values (three cultivations) \pm standard deviation (SD).

Testing of toxicity of bile salts

The testing of toxicity of the bile salts to the lactobacilli was carried out on MRS agar containing 0.3% bile acid salts (sodium taurocholate and glycocholate, Sigma-Aldrich, USA). Discs impregnated with suspensions of lactobacilli strains were placed onto agar and the plates were incubated under anaerobic conditions (Gas Pak Plus, BBL) at 30°C and 37°C for 72 hours. The ability of the lactobacilli to grow in the presence of the above salts was evaluated qualitatively.

Monitoring of the biofilm production

The production of the biofilm was observed in 96-well microtitration plates using the method of O'Toole et al. (38). The capacity of biofilm production was assessed using crystal violet, a standard stain used for the determination of biofilm production. Crystal violet bound to adhered cells (biofilm) was extracted with 200 μ l of 95% ethanol and the optic density of the solution was measured spectrophotometrically (Synergy Reader 4, BioTek, Merck, SRN) at a wavelength of 570 nm. The medium without bacterial culture was used as a control. The produced biofilm was evaluated as thick ($OD_{570} \geq 1$), medium ($0.1 \leq OD_{570} < 1$) or negative ($OD_{570} < 0.1$) (6). The strains were tested by three independent experiments, each repeated 8 times. The results are presented as the arithmetic means \pm SD. The biofilm produced and fixed to slides according to Kubota et al. [27] was detected by a scanning electron microscopy (SEM). The SEM images were obtained by a scanning electron microscope JEOL JSM-7000F (magnification: $\times 200$, $\times 2500$, $\times 15000$; high vacuum; voltage 15.0 kV; working distance 11.3 mm).

Testing of the inhibitory activity

The disc-diffusion method was used to determine the inhibitory activity of selected lactobacilli against potentially pathogenic micro-organisms. The following indicator strains were used: *Escherichia coli* 0149 F4 (Research Institute of Veterinary Medicine in Brno, CR); *Salmonella*

Typhimurium CCM 7205 (Czech Collection of Micro-organisms in Brno, CR); *Staphylococcus aureus* and *Bacillus cereus* (isolates obtained at Laboratory of gnotobiology, UVMP in Košice, SR). Sterile disks of diameter 6 mm (BBL, Cockeysville, USA) were placed on the surface of 20 ml of peptone-yeast extract-glucose (PYG) agar in Petri dishes. The composition of PYG agar was as follows: peptone for bacteriology 5 g; enzymatic casein hydrolysate 5 g; yeast extract 10 g; glucose 10 g; and agar 18 g. 1000 ml^{-1} distilled water (pH 6.9). The discs were inoculated with 5 μ l of the night cultures of lactobacilli ($1 \times 10^8 \text{ CFU. ml}^{-1}$) and the plates were then cultivated anaerobically (Gas Pak Plus, BBL) at 37°C or 30°C for 48 hours. After incubation, the plates were overlaid with 3 ml of 0.7% PYG agar, inoculated with 0.3 ml of the night culture of a respective indicator strain and incubated aerobically at 37°C for 24 hours. The results are presented as the arithmetic means of 3 measurements \pm SD.

Evaluation of safety

The minimum inhibitory concentration (MIC) of an antibiotic against the lactobacilli strains were tested by MIC Test Strip test (Liofilchem, Italy). The test is based on the use of antibiotic strips impregnated with the following concentration gradients of antibiotics: 0.016–256 mg.l^{-1} for ampicillin, vancomycin, kanamycin, erythromycin, clindamycin, tetracycline, chloramphenicol and 0.064–1024 mg.l^{-1} for gentamycin and streptomycin. The strips were placed onto solid nutrient medium Mueller-Hinton agar (Oxoid Unipath, Ltd., Basingstoke, UK) with 10% addition of MRS agar (Carl Roth) which was inoculated with 100 μ l of the lactobacilli suspension (McFarland Standard No.1). The plates were incubated anaerobically (Gas Pak Plus, BBL) at 30°C, or 37°C for 48 hours and the value of MIC was obtained after the incubation. The results obtained were compared with the critical values of MIC (mg.l^{-1}) recommended by EFSA [14].

The enzymatic activity of the lactobacilli was determined by means of a commercial semi-quantitative API ZYM test (BioMérieux, France) according to the producer's instructions. The suspension of the lactobacilli (65 μ l) of turbidity, equal to McFarland Standard No. 5, was inoculated into each well of the API ZYM strips. The enzymatic activity was evaluated after a 4-hour incubation under anaerobic conditions (Gas Pak Plus, BBL) at 37°C or 30°C

for 4 hours. The intensity of colouring ranging from 0 (no activity) up to 5 (≥ 40 nmol of hydrolysed substrate during 4-hour incubation) was recorded according to the API-ZYM colour reaction diagram.

The haemolytic activity was tested on Trypticase soy agar (TSA; Carl Roth GmbH, Germany) with 5% ram blood. The presence of α - or β -haemolysis was evaluated on the basis of the production of bright or greenish zones around the colonies.

Statistical evaluation

The results of the individual analyses were evaluated by one way variance analysis (ANOVA) supplemented with Tukey test using the software GraphPad Prism version 3.00.

RESULTS AND DISCUSSION

Lactobacilli are an important part of the microflora of kefir grains. A number of authors have described the isolation of *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus kefirianofaciens* and *Lactobacillus kefir* from kefir grains originating from various regions [2, 7, 19, 46]. With a high probability at the level of the genus and probable identification at the level of the species, we identified isolate R7 as *Lactobacillus plantarum* (score 2.091), isolate 1Ž as *Lactobacillus plantarum* (score 2.105), isolate 2Ž as *Lactobacillus paracasei* (score 2.078), isolates 4/30 and 13/30 as *Lactobacillus kefir* (scores 2.058 and 2.117, resp.). With probable identification at the level of the genus, we identified isolate S10 as *Lactobacillus paraplantarum* (score 1.725). We were not able to identify the isolate marked as 6/30; however its growth properties and morphology were almost identical with those of strains 4/30 and 13/30. In order to confirm the results obtained by MALDI-TOF mass spectrometry, it is necessary in future studies to subject the isolates also to genotype identification, such as the sequencing of 16S RNA amplicate obtained by PRC methods.

During the testing of the individual properties of isolates obtained from kefir grains, we used for comparison animal strains *Lactobacillus reuteri* 2/6 and *Lactobacillus reuteri* L26 isolated from the digestive tract of the animals in our laboratory.

The viability and survival of probiotic bacteria under unfavourable conditions in the digestive tract are the most important parameters for achieving their therapeutic effects

[18]. In order to survive in the digestive tract these bacteria must be able to resist the extreme conditions due to the presence of hydrochloric acid or bile acids. It was confirmed that the viability of bacteria exposed to such conditions are species and strain specific [28]. The first barrier these bacteria must overcome is the very low pH in the stomach (values in the range of 1–3) to which they are exposed for 90 min on average. Our observations of the incubation in simulated gastric juice at pH 2.6 showed 98–88% survival of strains *Lb. plantarum* 1Ž, *Lb. paraplantarum* S10, *Lb. paracasei* 2Ž and strains *Lb. reuteri*. These strains exhibited significantly higher tolerance ($P < 0.001$) of the gastric juice at pH 2.6 (Fig. 1a) in comparison to the strains *Lb. kefir*. A similar trend was also observed at pH 2 (Fig. 1b). During 120 min incubation we recorded higher than 60% survival of strains *Lb. plantarum* 1Ž and *Lb. paraplantarum* S10 and higher than 80% survival of strain *Lb. paracasei* 2Ž, which was significantly higher ($P < 0.001$) in comparison with the survival of strains *Lb. kefir* and animal strains *Lb. reuteri*. The *Lb. kefir* strains showed only 10% survival at 90 and 120 min incubation. A significantly highest percentage of survival ($P < 0.001$) at pH 2 compared to all tested strains was observed for *Lb. paracasei* 2Ž.

Probiotic bacteria should be able to grow in the presence of 0.15–0.3% bile acids [11]. *Lb. kefir* strains were resistant to 0.3% sodium taurocholate and glycocholate, while the growth of *Lb. plantarum* R7, *Lb. paraplantarum* S10 and *Lb. plantarum* 1Ž was inhibited by the presence of these bile salts. The exception was the strain *Lb. paracasei* 2Ž which was able to tolerate 0.3% concentration of both bile salts and, as reported earlier, exhibited a high tolerance also to simulated gastric juice with pH 2. It is well known that exposure of bacterial cells to one type of stress can induce a response protecting the cells against multiple stresses [13]. Some previously published data indicated that intestinal isolates of lactobacilli were usually more resistant to bile salts than isolates from other sources [25]. However, our results did not confirm this observation, as intestinal isolates of animal strains of *Lb. reuteri* were susceptible to the salts of bile acids.

One of the properties most frequently observed during the selection of probiotic candidates is their ability to adhere to the mucus and epithelial cells, or their congregational ability. Despite the important role of biofilm in commensal bacteria which affects immunomodulation, the exclusion of pathogens and increased contact with the in-

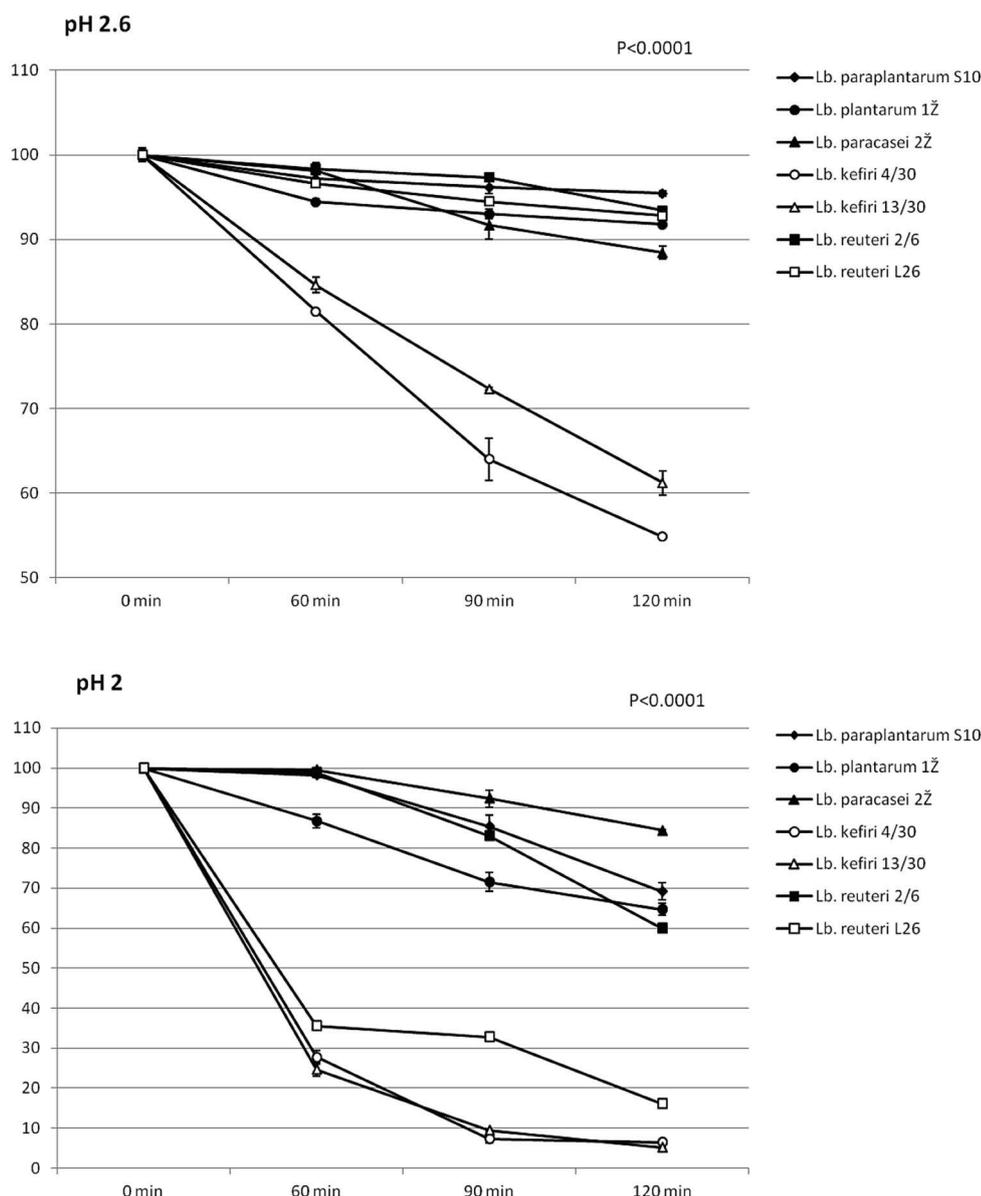


Fig. 1. Time dependence of survival (in per cent) of lactobacilli in simulated gastric juice at different pH. The values presented are means of 3 measurements \pm SD

testinal mucosa [30], the potential probiotic strains are not routinely tested for their ability to produce complex biofilm structures on various surfaces. Biofilms can protect probiotic bacteria against unfavourable conditions in the gastrointestinal tract (GIT), allow them to communicate directly with the host and support survival of these bacteria in GIT at higher beneficial metabolic effectiveness [24]. Moreover, production of biofilms would increase their stability in the process of production and storage [8]. The strains in our study produced biofilms on the abiotic surfaces in MRS medium free of Tween. These biofilms were either medium

thick (*Lb. plantarum* R7, *Lb. paraplantarum* S10, *Lb. paracasei* 2Ž, *Lb. kefir* 13/30) or thick (*Lb. plantarum* 1Ž, *Lb. kefir* 4/30, *Lactobacillus* sp. 6/30, *Lb. reuteri* 2/6, *Lb. reuteri* L26) (Fig. 2). The highest production of biofilm ($OD_{570} = 4.5$) was recorded and also confirmed by SEM with the strain *Lb. plantarum* 1Ž (Fig. 3). A similar production of biofilms by lactobacilli from various sources was observed by Terraf et al. [43] and Slířová et al. [42]. However, these authors pointed out that there are significant differences in the production of biofilms related to the composition of the medium and cultivation conditions. Although observation

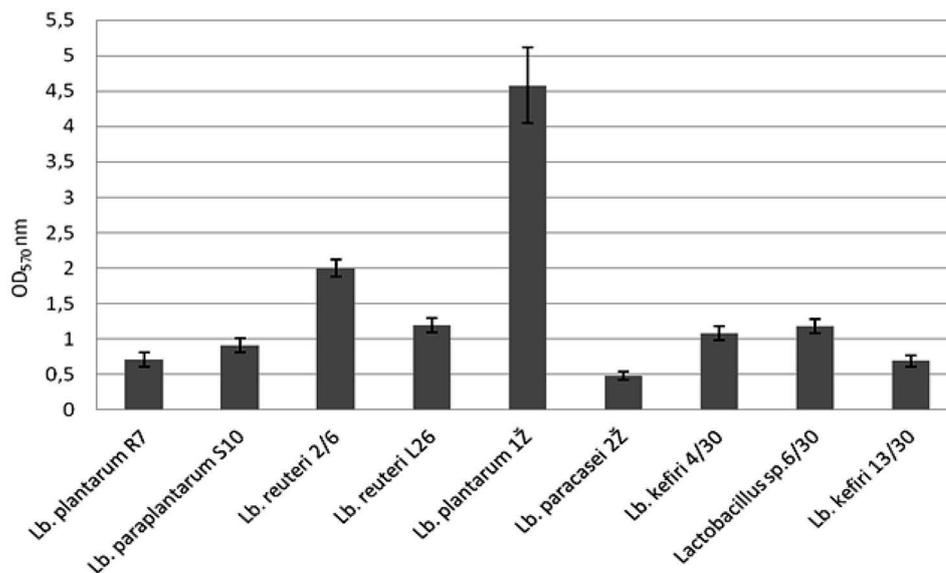


Fig. 2. Production of biofilms by lactobacilli (MRS medium free of Tween)
Results are presented as arithmetic means \pm SD

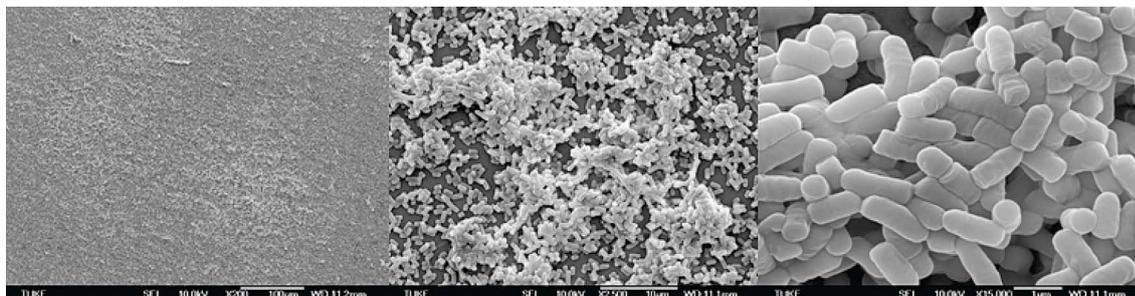


Fig. 3. SEM of biofilm produced by strain *Lb. plantarum* 1Ž.
Magn. $\times 200$; $\times 2500$; $\times 15000$.

of the production of biofilms on abiotic surfaces cannot replace the testing of the ability to adhere to biotic surfaces, several authors who tested the potential of probiotic bacteria to produce biofilms reported significant positive correlation between adherence to biotic surfaces and their ability to produce biofilms on abiotic surfaces [32, 39].

Lactobacilli are known for their ability to produce antimicrobial substances against pathogenic bacteria. The antimicrobial activity of lactobacilli is based mostly on organic acids, hydrogen peroxide and bacteriocins [23]. Our examination showed that the strains of lactobacilli inhibited the growth of indicator strains *E. coli* 0149 F4, *S. Typhimurium* CCM 7205, *S. aureus* and *B. cereus*, with the size of the inhibition zones dependent on the individual strains (Table 1). The highest inhibitory properties were exhibited by the strains *Lb. plantarum* 1Ž and *Lb. paracasei* 2Ž. The suscep-

tibility of *E. coli* 0149 F4 and *S. Typhimurium* CCM 7205 to the lactobacilli was approximately the same; however the Gram positive bacteria *B. cereus* were more susceptible than *S. aureus*. Similarly, Bilková et al. [4] reported that *S. aureus* was the strain with the highest resistance to the strains *Lb. murinus*, *Lb. mucosae* and *Lb. reuteri*, isolated from the GIT of lambs. We assume that the inhibitory effect observed in our study could be mediated by the production of organic acids. In our previous studies we observed the production of lactic acid and acetic acid by the strains tested. The antimicrobial effect of these acids may be related to the inhibition of the various metabolic functions of bacterial cells with the highest inhibitory effect ascribed to the non-dissociated form of organic acids [41].

The system of assessment of safety of probiotic micro-organisms strictly specifies criteria such as resistance

Table 1. Antimicrobial activity of the lactobacilli strains

Strains	Diameter of inhibition zones (mm)			
	<i>S. Typhimurium</i> CCM 7205	<i>E. coli</i> O149 F4	<i>S. aureus</i>	<i>B. cereus</i>
<i>Lb. plantarum</i> R7	24.67 ± 0.47	29.00 ± 0.82	14.33 ± 1.25	34.00 ± 4.32
<i>Lb. paraplantarum</i> S10	28.00 ± 1.63	29.33 ± 0.94	18.67 ± 0.94	39.00 ± 0.82
<i>Lb. plantarum</i> 1Ž	30.33 ± 0.47	34.67 ± 0.47	26.67 ± 1.25	44.67 ± 2.49
<i>Lb. paracasei</i> 2Ž	29.33 ± 0.94	30.00 ± 0.82	20.00 ± 1.63	39.33 ± 0.94
<i>Lb. kefir</i> 4/30	16.50 ± 0.50	21.00 ± 1.00	12.00 ± 2.00	25.00 ± 1.00
<i>Lactobacillus</i> spp. 6/30	23.00 ± 1.00	22.00 ± 0.00	11.00 ± 1.00	23.00 ± 1.00
<i>Lb. kefir</i> 13/30	25.00 ± 0.82	24.33 ± 0.47	11.33 ± 0.94	21.67 ± 0.47
<i>Lb. reuteri</i> 2/6	23.00 ± 2.16	28.33 ± 0.47	11.33 ± 0.94	33.00 ± 2.16
<i>Lb. reuteri</i> L26	34.67 ± 3.68	28.00 ± 1.93	20.33 ± 0.47	40.33 ± 0.47

Results are presented as arithmetic means of 3 measurements ± SD

Table 2. Minimum inhibitory concentration (MIC) of antibiotics against the lactobacilli strains

Strains	MIC mg.l ⁻¹							
	AMP	GEN	CAN	STR	ERY	CLI	TTC	CMP
<i>Lb. kefir</i> 4/30	1	0.5	16	16	0.38	0.38	6	1.5
<i>Lactobacillus</i> spp. 6/30	1.5	0.75	12	12	0.25	0.38	6	1.5
<i>Lb. kefir</i> 13/30	1.5	1	16	12	0.19	0.19	4	3
<i>Lb. paracasei</i> 2Ž	3	8	48	64	0.75	0.75	0.19	3
<i>Lb. plantarum</i> R7	0.19	2	32	24	0.50	0.016	16	8
<i>Lb. plantarum</i> 1Ž	0.19	4	96*	16	0.50	0.38	8	3
<i>Lb. paraplantarum</i> S10	0.5	2	32	24	0.75	0.032	24	8
<i>Lb. reuteri</i> 2/6	0.38	3	64	32	1	1	24*	8*
<i>Lb. reuteri</i> L26	0.25	0.75	32	24	2*	0.047	64*	4

AMP — ampicillin; GEN — gentamycin; CAN — canamycin; STR — streptomycin; ERY — erythromycin; CLI — clindamycin; TTC — tetracycline; CMP — chloramphenicol; * — values MIC exceeding critical values defined for the respective species of lactobacilli by EFSA commission [14]

to antibiotics and transfer of resistance genes. Probiotic micro-organisms should not increase the existing risk associated with normal microflora in the intestine or foods. In relation to the evaluation of resistance to antibiotics of probiotic strains, one should distinguish the type of resistance involved. Natural resistance is not transferred horizontally and raises no risk of transfer to other, particularly potentially pathogenic bacteria. On the other hand, the acquired resistance present in some strains within one species

normally susceptible to the assessed antibiotics can spread horizontally among bacteria. With regard to the therapeutic use of antibiotics, this resistance presents a serious problem [35]. The MIC values of antibiotics against strains of lactobacilli isolated from kefir grains correlated with the critical values of MIC recommended for the respective species or groups of lactobacilli by EFSA commission (Table 2) with the exception of strain *Lb. plantarum* 1Ž in the case of kanamycin. Danielsen and Wind [10] re-

Table 3. Enzymatic activity of lactobacilli by API ZYM system

Enzymes ^a	Strains								
	<i>Lb. plantarum</i> R7	<i>Lb. paraplantarum</i> S10	<i>Lb. plantarum</i> 1Ž	<i>Lb. paracasei</i> 2Ž	<i>Lb. kefir</i> 4/30	<i>Lb. sp.</i> 6/30	<i>Lb. kefir</i> 13/30	<i>Lb. reuteri</i> 2/6	<i>Lb. reuteri</i> L26
Naphthol-AS-BI-phospho- hydrolase	20	10	10	20	5	5	5	20	5
Acid phosphatase	5	5	5	10	20	20	20	30	20
Esterase (C4)	5	5	5	5	0	0	0	10	10
Esterase lipase (C8)	5	5	5	0	5	5	5	10	5
Leucine arylamidase	20	20	20	30	20	10	10	10	10
Valine arylamidase	20	20	20	30	10	5	10	0	0
Cystine arylamidase	0	0	5	0	0	0	5	0	0
α-galactosidase	0	0	5	0	20	20	20	20	20
α-fucosidase	0	0	0	0	0	0	0	0	0
β-galactosidase	5	5	5	10	30	30	30	30	30
β-glucuronidase ^b	0	0	5	0	10	20	20	0	0
α-glucosidase	5	10	10	20	10	20	10	20	20
β-glucosidase ^b	10	10	20	0	0	0	0	0	0

^a — enzyme activity measured as approximate nmol of hydrolysed substrate during 4-hour incubation; ^b — unwanted enzymatic activity; strain *Lb. paracasei* 2Ž — isolate from kefir grains; strains *Lb. reuteri* 2/6 and *Lb. reuteri* L26 — isolates from animal intestines without unwanted enzymatic activity

ported that some lactobacilli strains are naturally resistant to aminoglycosides which include also kanamycin. All kefir isolates were susceptible to ampicillin with MIC values ranging from 0.19 to 3 mg.l⁻¹, to gentamycin 0.5—8 mg.l⁻¹, to kanamycin 12—64 mg.l⁻¹, to streptomycin 12—64 mg.l⁻¹, to erythromycin 0.19—0.75 mg.l⁻¹, to clindamycin 0.016—0.75 mg.l⁻¹, to tetracycline 0.19—24 mg.l⁻¹ and to chloramphenicol 1.5—8 mg.l⁻¹. On the other hand, strains of *Lb. reuteri* of animal origin exhibited higher values of MIC, namely with *Lb. reuteri* 2/6 for tetracycline and chloramphenicol and with strain *Lb. reuteri* L26 for erythromycin and tetracycline. The phenotype results obtained for these strains should be supplemented by observation of the presence of transferrable resistance genes at the molecular level. Lactobacilli are generally susceptible to these antibiotics [16]. Plasmids encoding resistance to tetracycline, erythromycin and chloramphenicol were detected in *Lb. reuteri*,

Lb. fermentum, *Lb. acidophilus* and *Lb. plantarum* isolated from raw meat, silage and excrements. Many of these resistance genes may have been acquired by horizontal transfer [33].

The safe use of probiotic strains is related to the activity of their enzymes. The strains showed different enzymatic profiles (Table 3). From that point of safety, the absence of activity of β-glucosidase and β-glucuronidase is desirable. The activity of β-glucosidase is associated with undesirable effects in the large intestines; β-glucuronidase may release aglycons and deconjugate carcinogens conjugated with glucuronic acid [12]. The *Lb. plantarum* (R7 and 1Ž) and *Lb. paraplantarum* S10 strains exhibited medium activity (10—20 nmol of hydrolysed substrate) of β-glucosidase; *Lb. plantarum* 1Ž showed weak activity (5 nmol of hydrolysed substrate); and *Lb. kefir* (4/30, 13/30) and *Lactobacillus* spp. 6/30 medium activity of β-glucuronidase (10—

20 nmol of hydrolysed substrate). On the other hand, the activity of β -galactosidase affects favourably the human metabolism. This enzyme hydrolyses lactose to glucose and galactose and in this way alleviates the unpleasant manifestations of lactose digestion disorders. Moreover, oligomerisation of products and substrate by β -galactosidase stimulates the cytotoxic and humoral immunity through the activation of macrophages and T-cells [22]. All kefir isolates exhibited activity of β -galactosidase with the highest activity (30 nmol of hydrolysed substrate) observed with *Lb. kefir* (4/30, 13/30) and *Lactobacillus* spp. 6/30.

Haemolysis is a common factor of virulence of pathogenic bacteria which makes iron available to bacteria as a co-factor important for the action of some bacterial enzymes, and causes anaemia and oedemas in hosts [45]. Lactobacilli are able to grow in the absence of iron which is considered an ecological advantage in the environment where they have to compete with pathogenic bacteria. However, some observations indicated the presence of haemolytic activity from lactobacilli. According to Elli et al. [15] this haemolytic activity may be associated with the requirements of lactobacilli on iron in the metabolism of pyrimidine and purines in the environment with limited sources of specific nucleotides. The lactobacilli strains in our study showed no haemolysis of blood agar which is in agreement with the results of a number of authors who observed no haemolytic activity in lactobacilli isolated from clinical samples, faecal samples from children and adults and samples of dairy products [1, 31].

CONCLUSIONS

The results obtained in this study have allowed us to conclude that lactobacilli strains isolated from kefir grains could fulfil the parameters set for the required properties of probiotics. The strain *Lb. paracasei* 2Ž provided the best preliminary results as it: showed a high resistance to simulated gastric juice and bile salts; produced biofilm; exhibited strong inhibitory activity against potential pathogens; was susceptible to the test antibiotics; and showed no harmful enzymatic or haemolytic activity. In this study, we did not perform all the tests that must be used for testing of potential probiotic strains which opens additional space for further investigations focused particularly on the validation of biological properties and safety under *in vivo* conditions.

After complex characterisation and relevant *in vivo* studies, the selected strain appears prospective for the use as probiotics in functional food or clinical practice.

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