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Screening and Evaluation of Human Intestinal Lactobacilli for the Development of Novel Gastrointestinal Probiotics

Piret Kõll · Reet Mändar · Imbi Smidt · Pirje Hütt · Kai Truusalu · Raik-Hiio Mikelsaar · Jelena Shchepetova · Kasper Krogh-Andersen · Harold Marcotte · Lennart Hammarström · Marika Mikelsaar

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Abstract The aim of this study was to screen intestinal lactobacilli strains for their advantageous properties to select those that could be used for the development of novel gastrointestinal probiotics. Ninety-three isolates were subjected to screening procedures. Fifty-nine percent of the examined lactobacilli showed the ability to auto-aggregate, 97% tolerated a high concentration of bile (2% w/v), 50% survived for 4 h at pH 3.0, and all strains were unaffected by a high concentration of pancreatin (0.5% w/v). One *Lactobacillus buchneri* strain was resistant to tetracycline. None of the tested strains caused lysis of human erythrocytes. Six potential probiotic strains were selected for safety evaluation in a mouse model. Five of 6 strains caused no translocation, and were considered safe. In conclusion, several strains belonging to different species and fermentation groups were found that have properties required for a potential probiotic strain. This study was the first phase of a multi-phase study aimed to develop a novel, safe and efficient prophylactic and therapeutic treatment system against gastrointestinal infections using genetically modified probiotic lactobacilli.

Introduction

Lactobacilli have been used as probiotics in food products and dietary supplements for decades. According to the expert panel commissioned by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [7]. Today, there is increasing interest in developing novel genetically engineered probiotics, or "designer probiotics" to combat pathogenic microorganisms [30]. With the emergency of new technology, it has become possible to produce antibody fragments from recombinant lactobacilli [15, 19, 24]. The use of these constructs in the gastrointestinal tract could provide an efficient therapy at low cost.

Several requirements have been proposed for novel probiotic strains. Isolates from healthy humans are advised and their functional properties and safety should be assessed by in vitro and in vivo tests. The effect of probiotics is greatly influenced by their functional properties, such as antimicrobial activity and the ability to persist in gut [3, 7, 22, 34]. The properties differ significantly among various *Lactobacillus* species and strains [1, 11, 14, 32], therefore, besides *Lactobacillus casei*, *Lactobacillus paracasei, Lactobacillus plantarum* and *Lactococcus lactis* that have most frequently been used for genetic engineering [18, 24, 29], more species and strains should be tested to choose the ones with good functional properties. Another important issue in selecting and developing new probiotics is the safety of probiotic strains [34]. There is growing concern about the development of antibiotic resistance in pathogenic microorganisms. The spread of antibiotic-resistance genes between bacterial species through lateral gene transfer may occur [20] and therefore, knowledge of the resistance pattern of the probiotic strains would be useful to avoid inducing strains that carry transferable resistance genes. Safety considerations require also animal trials prior to human trials [7].

P. Kõll · R. Mändar (✉) · I. Smidt · P. Hütt · K. Truusalu · R.-H. Mikelsaar · J. Shchepetova · M. Mikelsaar
Department of Microbiology, University of Tartu, Tartu, Estonia
e-mail: reet.mandar@ut.ee

K. Krogh-Andersen · H. Marcotte · L. Hammarström
Department of Laboratory Medicine, Division of Clinical Immunology, Karolinska University Hospital Huddinge, Karolinska Institutet, Stockholm, Sweden

The aim of this study was to screen human intestinal lactobacilli to select suitable strains that could eventually be used for the development of novel gastrointestinal probiotics.

Materials and Methods

Lactobacilli Strains

Ninety-three lactobacilli isolated from the faecal samples of 1–2-year-old Estonian and Swedish children, belonging to the culture collection of Department of Microbiology, University of Tartu were used in this study (Table 1). Study design, isolation, and identification of lactobacilli have been described elsewhere [23]. Briefly, provisional identification of lactobacilli was based on the ability of the isolate to grow in the MRS broth, Gram-positive rod-shaped non-sporing cell morphology and negative catalase reaction. For detecting fermentation group, some physiological properties were assessed. The fermentation of glucose without gas, growth at 37°C and no growth at 15°C identifies obligately homofermentative lactobacilli (OHOL); growth both at 15 and 37°C without gas production is characteristic of facultatively heterofermentative lactobacilli (FHEL), whereas gas production at 37°C and variable growth at 15°C are characteristic of obligately heterofermentative lactobacilli (OHEL). The species of lactobacilli were initially identified using an API 50 CHL kit (bioMérieux, Marcy l'Etoile, France); the OHEL isolates were also tested for their ability to produce reuterin. Next, the isolates were subjected to internal transcribed spacer polymerase chain reaction (ITS-PCR) followed by enzymatic restriction analysis as described by Jacobsen et al. [12]. Partial sequencing of the 16S-rDNA fragment was performed for strains with uncertain identity using Genbank database (http://www.ncbi.nlm.nih.gov/blast/). The latter include *L. acidophilus* 821-1—GenBank Accession No. GU454851; *L. acidophilus* 821-2—GU454852; *L. delbrueckii* subsp. *bulgaricus* E9D2-1—GU454854; *L. gasseri* E9B4-1—GU454855; *L. gasseri* E101G2-4-2—GU454857 and all strains that are mentioned in Animal trial section.

Testing of Auto-Aggregation Ability, Acid, Bile and Pancreatin Tolerance

Auto-aggregation assay was performed according to Pascual et al. [25] with certain modifications. Lactobacilli were grown for 48 h at 37°C on MRS agar (Oxoid) plates in microaerobic environment (10% CO_2). A loopful (10 µl) of culture was suspended on a glass microscope slide in 1 ml of 0.9% saline solution (pH 6.7) to a final concentration that corresponded to McFarland Nephelometer Standard 3. Auto-aggregation was determined as the ability to form aggregates (clearly visible sand-like particles) within 2 min at room temperature. The results were expressed as: score 0—no auto-aggregation, score 1—intermediate auto-aggregation (presence of some flakes), and score 2–strong auto-aggregation.

The effect of low pH, bile, and pancreatin on the survival of lactobacilli was examined in microwell plates (Costar® 96 Well Cell Culture Clusters, Myriad Industries, San Diego, CA). MRS broth (Oxoid) was adjusted to a pH range between pH 5.0 and pH 2.0 to test acid tolerance and contained oxgall (2% w/v) (Sigma, Steinheim, Germany) and pancreatin (0.5% w/v) (Sigma) to test bile and pancreatin tolerance. Each 180-µl volume of adjusted and non-adjusted MRS broth (as control; pH 6.0) was inoculated with 20 µl of suspension of lactobacilli (McFarland 1.0 turbidity standard) and incubated in microaerobic environment at 37°C for 4 h. The number of cells in the suspension of lactobacilli (CFU ml^{-1}) and the number of surviving cells following incubation in pH-, bile- and pancreatin-adjusted media was determined by plating 100 µl of tenfold serially diluted sample onto the MRS agar [12, 14]. Strains with viable cell counts equal to viable counts before incubation in pH-, bile- and pancreatin-adjusted media were considered as resistant to a particular pH, bile and pancreatin concentration.

Testing of Antibiotic Susceptibility

Minimum inhibitory concentrations (MICs) of 13 antibiotics were determined by E-test method. Wilkins-Chalgren (Oxoid) agar plates with 5% horse blood, E-test antibiotic strips (AB Biodisk, Solna, Sweden) and 48 h of incubation at 37°C in an anaerobic glove chamber were applied. The breakpoints were determined in accordance with the CLSI guidelines for gram-positive microorganisms [13] as follows: ciprofloxacin and rifampicin (4 µg ml^{-1}); erythromycin (8 µg ml^{-1}); ampicillin, imipenem, gentamicin and tetracycline (16 µg ml^{-1}); cefoxitin, cefuroxime, vancomycin, chloramphenicol and metronidazole (32 µg ml^{-1}); and trimethoprim-sulfamethoxazole (4/76 µg ml^{-1}).

Testing of Haemolytic Activity

A single line of lactobacilli culture (grown in MRS broth (Oxoid) for 48 h) was streaked onto blood agar plates containing either human or horse blood. Haemolysis was evaluated following 24 and 48 h of incubation in aerobic, microaerobic (10% CO_2) and anaerobic (90% N_2, 5% CO_2, 5% H_2) environment. One *Staphylococcus aureus* strain (ATCC 25923) and two *Streptococcus pyogenes* strains (ATCC 19615 and a human clinical isolate) were used as positive controls.

Table 1 Auto-aggregative properties and acid tolerance of intestinal lactobacilli, expressed as a number (*n*) of aggregating and surviving strains

Lactobacilli	No. of strains tested	No. of strains (*n*) with auto-aggregation score[a]			No. of strains tested	Survival of strains (*n*) at pH			
Fermentation type/species		0	1	2		6.0[b]	3.0	2.5	2.0
OHOL									
L. acidophilus	36	13	4	19	20	20	6	3	0
L. crispatus	1	1	0	0	0	0	0	0	0
L. delbrueckii[c]	2	0	0	2	2	2	0	0	0
L. gasseri	4	0	0	4	4	4	2	0	0
L. salivarius	1	1	0	0	1	1	1	0	0
FHEL									
L. paracasei	22	5	13	4	22	22	9	0	0
L. plantarum	6	3	3	0	6	6	5	0	0
OHEL									
L. brevis	3	3	0	0	3	3	1	0	0
L. buchneri	10	7	3	0	10	10	8	0	0
Weissella confusa	3	3	0	0	3	3	1	0	0
L. fermentum	5	2	2	1	5	5	5	0	0
Total (*n*)	93	38	25	30	76	76	38	3	0

OHOL obligately homofermentative lactobacilli, *FHEL* facultatively heterofermentative lactobacilli, *OHEL* obligately heterofermentative lactobacilli

[a] Auto-aggregation: score 0, no aggregation; score 1, intermediate aggregation; and score 2, strong aggregation

[b] Survival in non-adjusted MRS broth (as control; pH 6.0)

[c] The two *L. delbrueckii* isolates include one *L. delbrueckii* subsp. *bulgaricus* and one *L. delbrueckii* subsp. *lactis*

In Vivo Animal Trial

The animal trial was approved by the Ethic Committee on Animal Experiments of the Ministry of Agriculture of Estonia. Ten BALB/c mice (Scanbur BK AB, Sweden) were fed a mixture of six *Lactobacillus* strains (with each freshly cultured strain being present at a concentration of 10^7 CFU per daily dose) in their drinking water for 5 consecutive days. Throughout the trial, the animal's activity, behaviour and general health were observed daily. Five randomly selected mice were sacrificed on Day 5, and the other five mice on Day 15. Samples for histological and microbiological analyses were collected.

For histological analysis, tissue sections of liver, spleen, kidney and lungs of the sacrificed mice were fixed in 10% of formaldehyde and embedded in paraffin. The samples were stained with haematoxylin and eosin, and by using van Gieson method. Alterative and inflammatory changes in tissues were evaluated.

For microbiological analysis, heart blood (10 µl) and homogenized tissue of liver, spleen, kidney and lungs were plated onto blood agar and MRS agar (Oxoid). After 72 h of incubation in aerobic (blood agar plates) and microaerobic environment (MRS plates), colonies were enumerated and lactobacilli identified. *Lactobacillus* strains were typed by using arbitrarily primed polymerase chain reaction (AP-PCR) with three different primer sets: ERIC1R (5′-ATG TAA GCT CCT GGG GAT TCA C-3′) and ERIC2 (5′-AAG TAA GTG ACT GGG GTG AGC G-3′) [21], primer 5′-ACG CGC CCT-3′ [10] and primer 5′-ATG TAA CGC C-3′ [8].

Results

Auto-Aggregation Ability, Acid, Bile and Pancreatin Tolerance

Fifty-five strains (59%) showed the ability to auto-aggregate. Thirty strains were strongly aggregative (19 *Lactobacillus acidophilus*, two *Lactobacillus delbrueckii*, four *Lactobacillus gasseri*, four *L. paracasei*, and one *Lactobacillus fermentum* strain), while 25 strain showed intermediate auto-aggregation (Table 1).

For further experiments, 76 strains were selected including all strains of obligately heterofermentative lactobacilli (OHEL) and facultatively heterofermentative lactobacilli (FHEL) groups as well as the best auto-aggregating strains and some randomly selected strains of obligately homofermentative lactobacilli (OHOL) group. Since the latter was initially the most numerous group

(mainly at the expense of *L. acidophilus*—36 strains), then, for evening up the groups, not all strains of this species were included in further experiments.

The tested strains showed relatively high tolerance of acidic environment, bile salts and pancreatin. Half of the strains (38 of 76) survived at pH 3.0 and the lowest pH tolerated by lactobacilli was 2.5 (three *L. acidophilus* strains; Table 1). Nearly all strains (74 of 76) were resistant to bile at a concentration (2% w/v), and all tested strains were resistant to pancreatin at a concentration (0.5% w/v).

Lactobacilli strains were scored according to the results of auto-aggregation and acid, bile, and pancreatin tolerance. Data for 24 potential probiotic strains with the highest scores are shown in Table 2. *L. acidophilus* strain 821-3 showed the best potential probiotic characteristics.

Antibiotic Susceptibility

The 24 selected strains were subjected to antibiotic susceptibility testing. No resistance was found to ampicillin, cefuroxime, imipenem, gentamicin, erythromycin, chloramphenicol, and rifampicin (Table 3). Although most of the strains had low MICs to tetracycline, one resistant *Lactobacillus buchneri* strain appeared. All the tested lactobacilli were resistant to metronidazole and the majority of the strains, belonging to different species, were resistant to cefoxitin (14 of 24 strains), vancomycin (12 strains),

Table 2 The intestinal *Lactobacillus* strains with the highest scores according to auto-aggregative properties and acid, bile and pancreatin tolerance

Lactobacilli		Auto-aggregation[a]	Survival scores			Total score[e]
Strain	Species		pH[b]	Oxgall[c]	Pancreatin[d]	
821-3	*L. acidophilus*	2	2	2	2	8
821-2	*L. acidophilus*	1	2	2	2	7
821-1	*L. acidophilus*	1	2	2	2	7
E27G2-7	*L. acidophilus*	2	1	2	2	7
E16B7	*L. gasseri*	2	1	2	2	7
E101G2-4-2	*L. gasseri*	2	1	2	2	7
R37E4	*L. acidophilus*	2	0	2	2	6
E7C4-3	*L. acidophilus*	2	0	2	2	6
E3B2-3	*L. delbrueckii*	2	0	2	2	6
E9D2-1	*L. delbrueckii*	2	0	2	2	6
177	*L. gasseri*	2	0	2	2	6
E9B4-1	*L. gasseri*	2	0	2	2	6
317	*L. paracasei*	1	1	2	2	6
1-4-2A	*L. paracasei*	1	1	2	2	6
1-4-1A	*L. paracasei*	1	1	2	2	6
13-2-1(96)	*L. paracasei*	1	1	2	2	6
A30-2-1	*L. paracasei*	1	1	2	2	6
15-2-4A	*L. paracasei*	1	1	2	2	6
148-7-1	*L. plantarum*	1	1	2	2	6
13-2-2	*L. buchneri*	1	1	2	2	6
A7-2-1	*L. buchneri*	1	1	2	2	6
338-1-1	*L. fermentum*	1	1	2	2	6
338-1-2	*L. fermentum*	1	1	2	2	6
180-2-1	*L. salivarius*	0	1	2	2	5

[a] Auto-aggregation: score 0, no aggregation; score 1, intermediate aggregation; and score 2, strong aggregation

[b] Acid tolerance: score 0, survival only at pH >3.0; score 1, survival at pH 3.0; and score 2, survival at pH ≤2.5

[c] Bile tolerance: score 0, no survival at oxgall concentration 2% (w/v); score 2, survival at oxgall concentration 2%

[d] Pancreatin tolerance: score 0, no survival at pancreatin concentration 0.5% (w/v); score 2, survival at pancreatin concentration 0.5%

[e] Total score for a *Lactobacillus* strain based on data of auto-aggregation and acid, bile and pancreatin tolerance (max. value 8)

Table 3 Antibiotic susceptibility of the selected intestinal *Lactobacillus* strains

Lactobacilli		Antibiotic with the MIC (μg ml^{-1})												
Strain	Species	AM	FX	XM	VA	IP	GM	EM	TC	CL	CI	MZ	RI	TS
821-3	*L. acidophilus*	0.023	6	0.5	0.25	0.064	2	0.25	1	2	**>32**	**≥256**	0.38	0.75
821-2	*L. acidophilus*	0.047	8	0.5	0.25	0.094	8	0.25	1.5	3	**>32**	**≥256**	0.75	**4**
821-1	*L. acidophilus*	0.016	8	0.125	0.25	0.094	4	0.125	0.75	1.5	**>32**	**≥256**	0.25	0.125
E27G2-7	*L. acidophilus*	0.094	**≥256**	0.75	2	0.19	12	1	2	6	**>32**	**≥256**	0.094	**>32**
R37E4	*L. acidophilus*	0.094	**≥256**	0.5	1.5	1	4	0.38	4	8	**>32**	**≥256**	0.125	**>32**
E7C4-3	*L. acidophilus*	0.19	**≥256**	0.75	1	0.064	8	0.047	0.75	1.5	**>32**	**≥256**	0.023	**>32**
E3B2-3	*L. delbrueckii*	0.125	24	0.25	0.5	0.047	1.5	0.016	0.25	0.75	**>32**	**≥256**	0.016	**>32**
E9D2-1	*L. delbruekcii*	0.094	8	0.125	0.75	0.032	1.5	0.032	0.5	1.5	**>32**	**≥256**	0.38	**>32**
E16B7	*L. gasseri*	0.19	**≥256**	0.38	1	0.125	4	0.19	3	2	**>32**	**≥256**	0.047	**>32**
E101G2-4-2	*L. gasseri*	0.016	**≥256**	0.125	0.38	0.064	0.125	<0.016	0.023	0.5	**>32**	**≥256**	0.047	**>32**
177	*L. gasseri*	0.031	1.5	0.19	0.75	0.047	2	0.047	0.5	0.75	**>32**	**≥256**	0.064	**>32**
E9B4-1	*L. gasseri*	0.25	**≥256**	0.5	2	1.5	8	0.25	0.75	4	**>32**	**≥256**	0.094	**>32**
180-2-1	*L. salivarius*	0.125	3	0.125	**≥256**	0.032	4	0.25	0.25	1	1	**≥256**	0.125	0.38
317	*L. paracasei*	1	**≥256**	1.5	**≥256**	0.75	1.5	0.38	0.75	3	**>32**	**≥256**	0.125	**>32**
1-4-2A	*L. paracasei*	0.064	**≥256**	1	**≥256**	0.19	0.75	0.25	0.75	3	1	**≥256**	0.094	**>32**
1-4-1A	*L. paracasei*	0.25	**≥256**	2	**≥256**	0.38	0.75	0.25	0.75	3	1	**≥256**	0.125	**>32**
13-2-1(96)	*L. paracasei*	0.5	**≥256**	3	**≥256**	1.5	4	0.5	0.75	3	1	**≥256**	0.19	**>32**
A30-2-1	*L. paracasei*	0.5	**≥256**	12	**≥256**	0.25	0.5	0.38	0.5	3	1	**≥256**	0.25	**>32**
15-2-4A	*L. paracasei*	0.75	12	2	**≥256**	0.19	0.125	0.25	0.75	3	3	**≥256**	0.064	**>32**
148-7-1	*L. plantarum*	0.016	**≥256**	0.125	**≥256**	0.047	0.094	0.75	8	2	2	**≥256**	1.5	0.38
13-2-2	*L. buchneri*	0.5	4	0.125	**≥256**	0.047	0.125	0.094	**24**	3	0.5	**≥256**	0.38	0.38
A7-2-1	*L. buchneri*	0.064	8	0.047	**≥256**	0.064	0.064	0.125	6	2	0.5	**≥256**	0.064	0.19
338-1-1	*L. fermentum*	0.125	**≥256**	1	**≥256**	0.047	0.5	0.125	2	2	2	**≥256**	0.19	0.5
338-1-2	*L. fermentum*	0.125	**≥256**	0.75	**≥256**	0.047	0.38	0.094	1.5	1	1	**≥256**	0.094	0.38

AM ampicillin, *FX* cefoxitin, *XM* cefuroxime, *VA* vancomycin, *IP* imipenem, *GM* gentamicin, *EM* erythromycin, *TC* tetracycline, *CL* chloramphenicol, *CI* ciprofloxacin, *MZ* metronidazole, *RI* rifampicin, *TS* trimethoprim-sulfamethoxazole

Strains resistant to a respective antibiotic are represented in *bold*

ciprofloxacin (13 strains), and trimethoprim-sulfamethoxazole (16 strains).

Haemolytic Activity

None of the tested lactobacilli caused the lysis of erythrocytes of human and horse blood in neither of the environments whereas complete lysis (β-haemolysis) was caused by strains of *Streptococcus pyogenes* and *Staphylococcus aureus* that were used as positive controls.

In Vivo Animal Trial

Ten strains belonging to all three fermentation groups were randomly selected from the set with the highest scores (score 6–8) and subjected to lyophilisation. The viable cell counts of lyophilizates ranged from 8×10^7 to 2×10^{11} CFU/g. Six *Lactobacillus* strains with the count of at least 10^{10} CFU/g; all identified by sequence analysis and belonging to different fermentation groups were selected for an animal trial. The selected strains were *L. acidophilus* 821-3—GenBank Accession No. GU454853; *L. gasseri* E16B7—GU454856, *L. gasseri* 177—GU454858, *L. paracasei* 317—HM035544, *L. paracasei* 1-4-2A—HM035542 and *L. fermentum* 338-1-1—HM035544.

Oral administration of these lactobacilli caused no adverse effect on the activity and general health status of the treated mice.

Heart blood, liver, kidney and lung samples obtained at autopsy were sterile in all mice treated with lactobacilli. The spleen culture of one mouse (mouse H5, sacrificed on Day 5) was positive both on MRS and blood agar media. Two different species were identified by API—*L. paracasei* subsp. *paracasei* and *L. plantarum.* AP-PCR typing revealed that the banding pattern of *L. plantarum* showed no similarity to the fingerprint patterns of any of the *Lactobacillus* strains that were administered to mice but the banding pattern of *L. paracasei* subsp. *paracasei* was identical to *L. paracasei* strain 1-4-2A (Fig. 1).

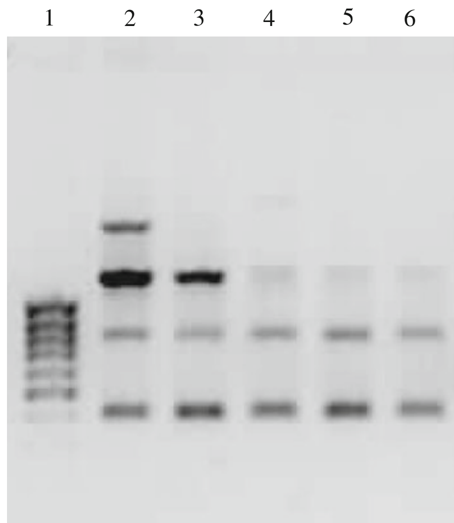

Fig. 1 PCR generated fingerprints for *L. paracasei* DSM5622 (*line* 2), *L. paracasei* DSM20020 (*line* 3), *L. paracasei* 1-4-2A (*line 4*), and *L. paracasei* strain isolated from spleen of mouse H5 (*lines 5 and 6*). *Line 1* contains 100 bp DNA ladder

No pathological changes were found in the samples of kidney and lung of the treated mice. Some non-specific changes were observed in liver and spleen samples of five mice. Small droplet steatosis was detected in the liver of one mouse sacrificed on Day 5 and in four mice sacrificed on Day 15. The latter four mice had also increased granulocytic infiltration in lymphoid follicles of spleen. The mouse H5 with positive spleen culture had no changes in any of the organs.

Discussion

This study was the first phase of a multi-phase study aimed to develop a novel, safe and efficient prophylactic and therapeutic treatment system against gastrointestinal infections using genetically modified probiotic lactobacilli. In this current study, we screened intestinal lactobacilli of healthy children and selected five potential probiotic strains for further studies.

To claim that a bacterial strain is a potential probiotic strain, several guidelines have been suggested [7, 22, 34]. Although these working groups did not address issues related to genetically modified microorganisms, the concepts and principles should be equally applicable to all potential probiotics. Testing of auto-aggregative ability as an indicator of adhesion ability [3, 26, 27] and tolerance of gastrointestinal environmental conditions [7, 22] were considered as a prerequisite for screening intestinal lactobacilli for their functional properties. As we aimed to select lactobacilli strains for subsequent genetic transforming, the antagonistic activity of non-transformed lactobacilli was not primary. However, previously we have shown that strains used in this study inhibited the growth of pathogens and potential pathogens (e.g. *Salmonella* Typhimurium, *Shigella sonnei*, *Escherichia coli*, *Enterococcus faecalis* and *Staphylococcus aureus*) [1].

It is recommended that the antibiotic resistance pattern of potential probiotic strains should be determined to avoid introducing strains containing transferable antibiotic-resistance genes [20, 28]. We screened selected strains for 13 antibiotics, including inhibitors of cell wall, protein and nucleic acid synthesis. High level of resistance to cefoxitin, vancomycin, ciprofloxacin, metronidazole and trimethoprim-sulfamethoxazole is similar to some other reports [5, 6] that could be interpreted as high natural resistance to these antibiotics. Plasmid-encoded erythromycin, tetracycline and chloramphenicol resistance has been reported in lactobacilli [2, 9, 16]. We observed no resistance to erythromycin and chloramphenicol but one *L. buchneri* strain was resistant to tetracycline and excluded from further experiments.

Although lactobacilli have a long history of safe use, in rare cases they could cause clinical conditions like bacteraemia and endocarditis [31]. Therefore, screening of new probiotics should include safety assessment. In our study none of the tested strains caused the lysis of erythrocytes of human blood. In animal trial, no translocation into blood and organs was detected in case of five strains but one of the two *L. paracasei* strains translocated into spleen in one mouse and was considered non-safe. Probiotic translocation is difficult to induce in healthy humans, and even if it does occur, detrimental effects are rare. Despite this, various reports have documented health-damaging effects of probiotic translocation in immunocompromised patients [17] and therefore each potential probiotic strain should be addressed individually in order to confirm its safety.

Although most of the examined tissues of lactobacilli-treated mice showed normal morphology, we detected some non-specific changes in liver of five mice. These changes were diagnosed as small droplet steatosis—a term that is used to describe accumulations of triglycerids within parenchymal cells, and is often seen in the liver because it is the major organ involved in fat metabolism [4]. We have frequently observed small droplet steatosis in healthy control mice in our previous animal trials [33], suggesting that it may occur during normal metabolic process.

In conclusion, our study identified several human intestinal *Lactobacillus* strains belonging to different species and fermentation groups that have properties required for a potential probiotic strain and therefore they could be used for the development of novel gastrointestinal probiotics. In the next phase of characterization, human trials are

necessary to study their survivability in human gut and confirm their safety in humans.

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 94:403–412
2. Axelsson LT, Ahrne SE, Andersson MC, Stahl SR (1988) Identification and cloning of a plasmid-encoded erythromycin resistance determinant from *Lactobacillus reuteri*. Plasmid 20:171–174
3. Collado MC, Meriluoto J, Salminen S (2007) Adhesion and aggregation properties of probiotic and pathogen strains. Eur Food Res Technol 226:1065–1073
4. Cotran RS, Kumar V, Collins T (1999) Pathologic basis of disease. WB Saunders Co, Philadelphia
5. Danielsen M, Wind A (2003) Susceptibility of *Lactobacillus* spp. to antimicrobial agents. Int J Food Microbiol 82:1–11
6. Delgado S, Flórez AB, Mayo B (2005) Antibiotic susceptibility of *Lactobacillus* and *Bifidobacterium* species from the human gastrointestinal tract. Curr Microbiol 50:202–207
7. FAO/WHO (2006) Probiotics in food. Health and nutritional properties and guidelines for evaluation. FAO Food and Nutrition Paper 85. FAO, Rome, Italy. ftp://ftp.fao.org/docrep/fao/009/a0512e/a0512e00.pdf
8. Gardiner G, Ross RP, Collins JK, Fitzgerald G, Stanton C (1998) Development of a probiotic cheddar cheese containing human-derived *Lactobacillus paracasei* strains. Appl Environ Microbiol 64:2192–2199
9. Gevers D, Huys G, Swings J (2003) In vitro conjugal transfer of tetracycline resistance from Lactobacillus isolates to other Gram-positive bacteria. FEMS Microbiol Lett 8:125–130
10. Hayford AE, Petersen A, Vogensen FK, Jakobsen M (1999) Use of conserved randomly amplified polymorphic DNA (RAPD) fragments and RAPD pattern for characterization of *Lactobacillus fermentum* in Ghanaian fermented maize dough. Appl Environ Microbiol 65:3213–3221
11. 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 100:1324–1332
12. Jacobsen CN, Rosenfeldt Nielsen V, Hayford AE, Møller PL, Michaelsen KF, Paerregard A, Sandström B, Tvede M, Jakobsen M (1999) Screening of probiotic activities of forty-seven strains of *Lactobacillus* sp. by in vitro techniques and evaluation of the colonisation ability of five selected strains in humans. Appl Environ Microbiol 65:4949–4956
13. Jorgensen JH, Turnidge JD (2003) Susceptibility test methods: general considerations. In: Murray PR, Baron EJ, Jorgensen JH, Pfaller MA, Yolken RH (eds) Manual of clinical microbiology. ASM Press, Washington, pp 1108–1125
14. Kõll P, Mändar R, Marcotte H, Leibur E, Mikelsaar M, Hammarström L (2008) Characterization of oral lactobacilli as potential probiotics for oral health. Oral Microbiol Immunol 23:139–147
15. Krüger C, Hu Y, Pan Q, Marcotte H, Hultberg A, Delwar D, van Dalen PJ, Pouwels PH, Leer RJ, Kelly CG, van Dollenweerd C, Ma JK, Hammarström L (2002) In situ delivery of passive immunity by lactobacilli producing single-chain antibodies. Nat Biotechnol 20:702–706
16. Lin CF, Fung ZF, Wu CL, Chung TC (1996) Molecular characterization of a plasmid-borne (pTC82) chloramphenicol resistance determinant (cat-TC) from *Lactobacillus reuteri* G4. Plasmid 36:116–124
17. Liong MT (2008) Safety of probiotics: translocation and infection. Nutr Rev 66:192–202
18. Maassen CBM, Laman JD, Heijne den Bak-Glashouwer MJ, Tielen FJ, van Holten-Neelen JCPA, Hoogteijling L, Antonissen C, Leer RJ, Pouwels PH, Boersma WJA, Shaw DM (1999) Instruments for oral disease-intervention strategies: recombinant *Lactobacillus casei* expressing tetanus toxin fragment C for vaccination or myelin proteins for oral tolerance induction in multiple sclerosis. Vaccine 17:2117–2128
19. Marcotte H, Kõll-Klais P, Hultberg A, Zhao Y, Gmür R, Mändar R, Mikelsaar M, Hammarström L (2006) Expression of single-chain antibody against RgpA protease of *Porphyromonas gingivalis* in Lactobacillus. J Appl Microbiol 100:256–263
20. Mathur S, Singh R (2005) Antibiotic resistance in food lactic acid bacteria—a review. Int J Food Microbiol 105:281–295
21. Matsumiya Y, Kato N, Watanabe K, Kato H (2002) Molecular epidemiological study of vertical transmission of vaginal Lactobacillus species from mothers to newborn infants in Japanese, by arbitrarily primes polymerase chain reaction. J Infect Chemother 8:43–49
22. Mercenier A, Lenoir-Wijnkoop I, Sanders ME (2008) Physiological and functional properties of probiotics. Bull Int Dairy Fed 429:1–7
23. Mikelsaar M, Annuk H, Shchepetova J, Mändar R, Sepp E, Björksten B (2002) Intestinal lactobacilli of Estonian and Swedish children. Microb Ecol Health Dis 14:75–80
24. Pant N, Hultberg A, Zhao Y, Svensson L, Pan-Hammarström Q, Johansen K, Pouwels PH, Ruggeri FM, Hermans P, Frenken L, Boren T, Marcotte H, Hammarström L (2006) Lactobacilli expressing variable domain of Llama heavy-chain antibody fragments (lactobodies) confer protection against rotavirus-induced diarrhoea. J Infec Dis 194:1580–1588
25. Pascual LM, Daniele MB, Ruiz F, Giordano W, Pajaro C, Barberis L (2008) *Lactobacillus rhamnosus* L60, a potential probiotic isolated from the human vagina. J Gen Appl Microbiol 54:141–148
26. Perez PF, Minnaard Y, Disalvo EA, De Antoni GL (1998) Surface properties of bifidobacterial strains of human origin. Appl Environ Microbiol 64:21–26
27. Rahman Md D, Kim W-S, Kumura H, Shimazaki K (2008) Autoaggregation and surface hydrophobicity of bifidobacteria. World J Microbiol Biotechnol 24:1593–1598
28. Saarela M, Mogensen G, Fonden R, Mättö J, Mattila-Sandholm T (2000) Probiotic bacteria: safety, functional and technological properties. J Biotechnol 84:197–215
29. Seegers JFML (2002) Lactobacilli as live vaccine delivery vectors: progress and prospects. Trends Biotechnol 20:508–515
30. Sleator RD, Hill C (2009) Rational design of improved pharmabiotics. J Biomed Biotechnol 2009:275287
31. Snydman DR (2008) The safety of probiotics. Clin Inf Dis 46:S104–S111
32. Strahinic I, Busarcevic M, Pavlica D, Milasin J, Golic N, Topisirovic L (2007) Molecular and biochemical characterizations of human oral lactobacilli as putative probiotic candidates. Oral Microbiol Immunol 22:111–117
33. Truusalu K, Naaber P, Kullisaar T, Tamm H, Mikelsaar R-H, Zilmer K, Rehema A, Zilmer M, Mikelsaar M (2004) The influence of antibacterial and antioxidative probiotic lactobacilli on gut mucosa in a mouse model of Salmonella infection. Microb Ecol Health Dis 16:180–187
34. Vankerckhoven V, Huys G, Vancanneyt M, Vael C, Klare I et al (2008) Biosafety assessment of probiotics used for human consumption: recommendations from the EU-PROSAFE project. Trends Food Sci Technol 19:102–114