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ORAL MICROBIOLOGY
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Characterization of oral lactobacilli as potential probiotics for oral health

P. Kõll[1], R. Mändar[1], H. Marcotte[2], E. Leibur[3], M. Mikelsaar[1], L. Hammarström[2]

[1]Department of Microbiology, Faculty of Medicine, University of Tartu, Tartu, Estonia, [2]Department of Laboratory Medicine, Division of Clinical Immunology, Karolinska University Hospital Huddinge, Karolinska Institutet, Stockholm, Sweden, [3]Department of Stomatology, Faculty of Medicine, University of Tartu, Tartu, Estonia

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Introduction: Intestinal lactobacilli have been successfully used as probiotics to treat gastrointestinal disorders, but only limited data are available for the probiotic properties of oral lactobacilli to combat oral diseases. We aimed to characterize oral lactobacilli for their potential probiotic properties according to the international guidelines for the evaluation of probiotics, and to select potential probiotic strains for oral health.
Methods: The study included 67 salivary and subgingival lactobacilli of 10 species, isolated from healthy humans. All strains were identified using amplified ribosomal DNA restriction analysis, tested for antimicrobial activity against oral pathogens, tolerance of low pH and bile content. Thereafter, the lysozyme tolerance and antibiotic susceptibility of 22 potential probiotic strains were assessed.
Results: The majority of strains suppressed the growth of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Streptococcus mutans*, but none inhibited *Candida albicans*. The lowest pH tolerated by lactobacilli following 4 h of incubation was pH 2.5, but none of the strains grew at this pH. All strains tolerated a high concentration of lysozyme (10 mg/ml) and half of the strains tolerated a high concentration of human bile [5% volume/volume (V/V)]. Four *Lactobacillus plantarum* and two *Lactobacillus oris* strains expressed resistance to tetracycline and/or doxycycline.
Conclusions: Strains of *L. plantarum*, *Lactobacillus paracasei*, *Lactobacillus salivarius*, and *Lactobacillus rhamnosus* expressed both high antimicrobial activity and high tolerance of environmental stress. The absence of transferable antibiotic-resistance genes in *L. plantarum* strains remains to be confirmed. These results suggest a potential for oral lactobacilli to be used as probiotics for oral health.

Key words: acid tolerance; antibiotic susceptibility; antimicrobial activity; bile tolerance; lysozyme tolerance; oral lactobacilli; probiotic

Piret Kõll, Department of Microbiology, University of Tartu, Ravila 19, 50411 Tartu, Estonia
Tel.: +372 7374173;
fax: +372 7374172;
e-mail: piret.koll-klais@ut.ee
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Lactobacilli, the acidophilic and aciduric gram-positive bacteria of the genus *Lactobacillus*, belong to the indigenous microflora of humans and colonize various parts of the body (5). Lactobacilli are known to play an important role in the maintenance of human health by stimulating the natural immunity and contributing to the balance of microflora, mainly through competitive exclusion and antimicrobial activity against pathogenic bacteria (31, 33, 38). Several species of obligately homofermentative, facultatively heterofermentative, and obligately heterofermentative lactobacilli have been found in the oral cavity, with *Lactobacillus gasseri*, *Lactobacillus salivarius*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, and *Lactobacillus fermentum* being the most prevalent (2, 10, 20, 26).

Lactobacilli are widely used for the manufacture of fermented foodstuffs and, as such, have been consumed for centuries. During recent decades lactobacilli have gained importance as probiotics, 'live microorganisms which when administered confer a health benefit on the host' (15). Few studies are available on the role and effects of probiotics in the mouth (32). Consumption of products containing probiotic lactobacilli has been shown to reduce caries risk and oral carriage of mutans streptococci (1, 8, 36). In the field of oral immunology, administration of transformed lactobacilli, expressing single-chain antibody fragments against

Streptococcus mutans, has been shown to protect rats against the development of dental caries (27). Furthermore, we have expressed functional single-chain antibody fragments against *Porphyromonas gingivalis* in *Lactobacillus paracasei* to combat periodontal diseases (29). Thus, these studies highlight the possibility of using lactobacilli as probiotics not only against gastrointestinal disorders but also against oral diseases. However, little is known about the probiotic properties of oral lactobacilli (43).

Several requirements have been proposed for novel probiotic strains (15). Isolates from healthy humans are advised and their functional properties and safety should be assessed by *in vitro* tests (15, 39). It has been shown that good antimicrobial properties of probiotic strains are necessary to eradicate or inhibit pathogenic bacteria. At the same time, significant fermentation type-, species-, and strain-specific variability in functional probiotic properties of lactobacilli, such as antimicrobial activity as well as acid and bile tolerance of lactobacilli, has been observed (3, 42, 43). Therefore, several strains from various fermentation types and species should be tested to choose the best, those with high antimicrobial activity and high tolerance of environmental stress. Furthermore, one of the important issues is the safety of probiotic strains. 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 (13) and therefore, knowledge of the resistance pattern of the probiotic strains would be useful to avoid inducing strains that carry transferable resistance genes.

The aim of the present study was to characterize oral lactobacilli for their potential probiotic properties according to the international guidelines for the evaluation of probiotics, and to select strains that could eventually be used as probiotics for oral health.

Material and methods

Sampling, isolation, and identification of lactobacilli

Sixty-seven oral lactobacilli strains used in this study (Table 1) were isolated from saliva and subgingival samples of 11 healthy humans (six female and five male; mean age 36.2 ± 10.5 years) participating in a prospective study of oral microflora in periodontitis and periodontal health. The study design, selection of patients, and isolation and identification of lactobacilli have been thoroughly described elsewhere (26). Briefly, salivary lactobacilli (60 strains) were obtained by using the Dentocult®LB dip-slide (Orion Diagnostica, Espoo, Finland) method (6) and subgingival lactobacilli (seven strains) were obtained by plating the gingival crevice lavage samples (7) on de Man–Rogosa–Sharpe (MRS) agar. Provisional identification of *Lactobacillus* species and strains was based on colony and cell morphology, physiological and biochemical properties, such as the ability of isolates to grow in MRS broth for 24 h in a 10% CO_2 environment at 15°C and 37°C, the ability to produce gas in MRS agar containing 1% glucose, negative catalase reaction, sugar fermentation pattern (sorbitol, tagatose, melezitose, ribose), and arginine hydrolysis (23). The species were identified using rapid amplified ribosomal DNA restriction analysis (ARDRA) (45). Partial sequencing of the 16S-rDNA fragment was performed for strains with uncertain identity.

Table 1. Species and origin of oral lactobacilli characterized for probiotic use

Lactobacilli	Strains* (*n*) isolated from	
Fermentation type/species	Saliva	Subgingival sites
OHOL	19	3
L. acidophilus	2	0
L. crispatus	2	0
L. delbrueckii	1	0
L. gasseri	9	3
L. salivarius	5	0
FHEL	17	1
L. paracasei	7	1
L. plantarum	7	0
L. rhamnosus	3	0
OHEL	24	3
L. fermentum	16	0
L. oris	8	3

OHOL, obligately homofermentative lactobacilli; FHEL, facultatively heterofermentative lactobacilli; OHEL, obligately heterofermentative lactobacilli.

*Lactobacilli strains were isolated from saliva and subgingival samples of 11 healthy adults (six female and five male; mean age 36.2 ± 10.5 years).

Testing of antimicrobial activity

Target bacterial strains used for antimicrobial activity testing were *S. mutans* NG8 (wild-type), *Aggregatibacter actinomycetemcomitans* 31-2-1A (wild-type), *P. gingivalis* ATCC 49 417, *Prevotella intermedia* ATCC 25 611, and *Candida albicans* 048 (wild-type).

Antimicrobial activity of lactobacilli against the microaerophilic target bacteria *S. mutans* and *A. actinomycetemcomitans* (67 lactobacilli strains were tested) was assessed using a deferred antagonism method (35), and against the anaerobic target bacteria *P. gingivalis* and *P. intermedia* (42 lactobacilli strains were tested) using a streak line method (3). Both methods have been thoroughly described in our previous paper (26).

Antimicrobial activity of lactobacilli against *C. albicans* was assessed by the deferred antagonism method (35). The medium used as underlying base agar (1.4%) was MRS agar without tri-ammonium citrate and sodium acetate (pH 7.1) (3). The medium used as top agar (0.7%) was Sabouraud–2% dextrose (Merck, Darmstadt, Germany). Lactobacilli were stab-inoculated on the surface of the bottom agar and incubated anaerobically (BBL®GasPakPlus™; BBL Microbiology Systems, Cockeysville, MD) for 24 h at 37°C to develop visible macrocolonies. A maximum of four *Lactobacillus* strains were grown on one agar plate. The target yeasts were precultivated in Sabouraud–2% dextrose (Merck) broth and suspensions of cells were adjusted to a predetermined optical density (OD 0.10 at 600 nm) to yield confluent growth in the top agar. Thereafter, the melted (and cooled to 42°C) top agar was seeded with the precultivated target yeast suspension and poured over the macrocolonies of lactobacilli. The plates were incubated under microaerobic conditions (BBL® CampyPakPlus™; BBL Microbiology Systems) at 37°C for 24 h to yield inhibitory zones. The tests were performed in duplicate, and the results were reported as the mean width of two inhibition zones measured from the edge of the colony of the *Lactobacillus* strain to the margin of the inhibition zone.

Testing of acid tolerance

Survival testing

The effect of low pH on the survival of lactobacilli was examined in flat-bottom microwell plates (Costar® 96-Well Cell Culture Clusters; Myriad Industries, San Diego, CA) with MRS broth (Merck) adjusted to a pH range between pH 3.5 and 1.5 with 6 mol/l HCl, and a non-adjusted MRS broth (pH 5.6) as control. Each 180-µl volume of pH-adjusted or non-adjusted MRS broth was inoculated with 20 µl of overnight culture of lactobacilli and incubated aerobically at 37°C for 4 h. The number of cells in the overnight culture of lactobacilli, measured as colony-forming units/ml and the

number of surviving cells following incubation in pH-adjusted media was determined by plating 100 μl of 10-fold serially diluted sample onto the MRS agar (21). Strains with viable cell counts equal to or higher than viable counts before incubation in pH-adjusted media were considered resistant to a particular pH. In total, 67 strains were tested at pH 3.5 and pH 3.0, and 31 strains were additionally tested at pH 2.5, pH 2.0, and pH 1.5.

Growth testing

In parallel, growth at pH 3.5 to pH 1.5 was measured as changes in OD in a 180-μl volume of pH-adjusted and non-adjusted (as control) MRS broth, inoculated with 20 μl of overnight culture of lactobacilli with pre-determined density. For each strain, the OD at 630 nm (OD_{630}) was measured at different time-points: at baseline (0 h) and following 3, 6, 9, 12, and 24 h of incubation at 37°C in aerobic conditions (21). OD_{630} values at different incubation intervals were compared to baseline value and strains with an increase in OD_{630} were considered as growing at a particular pH. All experiments were performed in duplicate. In total, 67 strains were tested at pH 3.5 and 3.0, and 23 strains were additionally tested at pH 2.5, 2.0, and 1.5.

Testing of bile tolerance

The effect of bile salts on the growth of lactobacilli (67 strains) was examined by adding human bile to MRS broth to a final concentration of 0.08, 0.16, 0.3, 0.6, 1.25, 2.5, and 5.0% (V/V). A 180-μl volume of bile-adjusted and non-adjusted (as control) MRS broth was inoculated with the 20 μl of overnight culture of lactobacilli and incubated aerobically at 37°C for 24 h. For each strain, the OD_{630} was measured at baseline (0 h) and following 3, 6, 9, 12, and 24 h of incubation (21). Strains with an increase in OD_{630} at incubation intervals compared to the baseline value were considered as growing at a particular bile concentration. All experiments were performed in duplicate.

Selection of lactobacilli strains with best potential probiotic characteristics

Each particular *Lactobacillus* strain (in total 42 strains) was scored according to the results of antimicrobial activity, and acid and bile tolerance with maximum value of 19. Antimicrobial activity of a strain determined by the deferred antagonism method (*S. mutans*, *A. actinomycetemcomitans* and *C. albicans*) was scored as follows: 0, inhibition <1 mm; 1, ≥1 but <2 mm of inhibition; 2, between 2 and 5 mm of inhibition; 3, ≥5 mm of inhibition; and by the streak line procedure (*P. gingivalis* and *P. intermedia*) as follows: 0, inhibition <1 mm; 1, ≥1 but <7 mm of inhibition; 2, between 7 and 20 mm of inhibition; 3, ≥20 mm of inhibition. Acid tolerance was scored as follows: 0, survival or growth only at pH >3.5; 1, survival or growth at pH 3.5; 2, survival or growth at pH ≤3.0. Bile tolerance was scored as follows: 0, growth only at bile concentrations <0.6%; 1, growth at bile concentrations 0.6 and/or 1.25%; 2, growth at bile concentration ≥2.5%.

Testing of lysozyme tolerance

The effect of lysozyme on the growth of lactobacilli (22 potential probiotic strains) was examined by a well diffusion assay (41). Eighteen milliliters of melted (and cooled to 50°C) MRS agar (2%) was seeded with 2 ml lactobacilli suspension (McFarland 1.0) and poured onto Petri dishes. Wells, 4-mm diameter, were cut into these agar plates and 25 μl lysozyme solution was placed into each well. Lysozyme was tested at concentrations of 0.2 mg/ml, 0.4 mg/ml, 0.6 mg/ml, 0.8 mg/ml, 1 mg/ml and 10 mg/ml. The plates were incubated under microaerobic conditions at 37°C for 24 h to yield inhibitory zones. The tests were performed in duplicate, and the results were reported as the mean width of two inhibition zones measured from the edge of the well to the margin of the inhibition zone. MRS agar plates inoculated with *Micrococcus luteus* were used as a positive control.

Testing of antibiotic susceptibility

Twenty-two strains were tested. Minimum inhibitory concentrations (MICs) of inhibitors of cell wall synthesis (amoxicillin, cefoxitin, cefprozil, cefotaxime, vancomycin), protein synthesis (gentamicin, erythromycin, doxycycline, tetracycline, clindamycin, chloramphenicol), and nucleic acid synthesis (ciprofloxacin, metronidazole) were determined by E-test method (28). Saline solution for suspending bacteria (McFarland 0.5 turbidity standard), Wilkins–Chalgren (Oxoid) agar plates with 5% horse blood and E-test antibiotic strips (AB Biodisk, Solna, Sweden) were used. After 48 h of incubation at 37°C in an anaerobic glove chamber (Sheldon Manufacturing, Inc. Shel LAB, Cornelius, OR), the elliptical zones of growth inhibition were examined and the MICs were interpreted as the value on the E-test strip scale where the inhibition zone intersected the edge of the strip. The breakpoints (susceptible/resistant) were determined in accordance with the Clinical and Laboratory Standards Institute (formerly NCCLS) guidelines for gram-positive microorganisms (22) as follows: clindamycin and ciprofloxacin (4 μg/ml); amoxicillin and erythromycin (8 μg/ml); gentamicin, doxycycline, and tetracycline (16 μg/ml); cefoxitin, cefprozil, vancomycin, chloramphenicol, and metronidazole (32 μg/ml); and cefotaxime (64 μg/ml). Strains with MICs equal to or higher than the breakpoints were considered resistant.

Statistical methods

The antimicrobial activity for different fermentation groups of lactobacilli was compared using either Student's *t*-test or Mann–Whitney rank sum test. The choice of tests was made automatically using the SIGMASTAT (Jandel Scientific, San Rafael, CA) program according to the distribution of the data. The differences were considered significant when $P < 0.05$.

Results

Identification of *Lactobacillus* species

The distribution of different fermentation types of lactobacilli was as follows: 22 obligately homofermentative, 18 facultatively heterofermentative, and 27 obligately heterofermentative lactobacilli (Table 1). All 67 strains were subjected to ARDRA analysis, and of those, 66 isolates were identified by ARDRA as *Lactobacillus acidophilus*, *Lactobacillus crispatus*, *L. gasseri*, *L. salivarius*, *Lactobacillus casei*, *L. plantarum*, *L. rhamnosus*, and *L. fermentum*. Nineteen strains were later reassigned based on results from the *16S-rRNA* gene sequencing (first 500 bases of the *16S-rRNA* gene). The latter included eight strains of *L. casei* to *L. paracasei* subsp. *paracasei* and 11 strains of *L. fermentum* to *Lactobacillus oris*. One strain, which showed an unknown restriction pattern by ARDRA, was identified following sequencing of the *16S-rRNA* gene as *Lactobacillus delbrueckii*.

Antimicrobial activity

The majority of *Lactobacillus* strains suppressed the growth of periodontal pathogens *A. actinomycetemcomitans* (60

strains of 67 tested strains), *P. gingivalis* (35 of 42 strains), *P. intermedia* (26 of 42 strains), and the cariogenic *S. mutans* (37 of 67 strains), but none inhibited *C. albicans*. The antimicrobial activity of lactobacilli was mainly species-specific; however, some strain-specific differences were observed, particularly among strains of *L. fermentum*, *L. oris*, and *L. gasseri*. The highest antimicrobial activity against all tested periodontal pathogens and *S. mutans* was associated with facultatively heterofermentative lactobacilli and homofermentative *L. salivarius* (Table 2). In addition, homofermentative *L. crispatus* and *L. gasseri* had quite high activity against *P. gingivalis* and *P. intermedia*, whereas *L. fermentum* (obligately heterofermentative) inhibited neither of these anaerobic bacteria. Lactobacilli from all fermentation types showed higher antimicrobial activity against *P. gingivalis* than against *P. intermedia* (inhibition zone 18.2 ± 5.5 vs. 6.5 ± 3.8 mm, $P < 0.001$, in the obligately homofermentative group; 22.4 ± 3.4 vs. 10.6 ± 1.4 mm, $P < 0.001$, in the facultatively heterofermentative group; 7.1 ± 7.5 vs. 0 mm, $P < 0.05$, in the obligately heterofermentative group).

The antimicrobial activity of subgingival strains (*L. gasseri*, *L. paracasei*, and *L. oris*) was comparable to the same species isolated from saliva (Table 2).

Acid tolerance

Acid tolerance of lactobacilli was found to be strain-, species-, and fermentation type-specific (Table 3). Nearly all strains (65 of 67) survived for 4 h at pH 3.0 but only 28 were able to grow at this pH. Survival following 4 h of incubation at pH 2.5 was observed for 24 (nine facultatively heterofermentative and 15 obligately heterofermentative) of 31 strains but none of the strains tested grew at this pH. Heterofermentative *L. plantarum* and *L. fermentum* were the most tolerant species. The acid tolerance of subgingival strains was comparable to the same species isolated from saliva.

Bile tolerance

The tested strains showed relatively high tolerance of bile salts: half of the strains (11 obligately homofermentative, 14 facultatively heterofermentative and eight obligately heterofermentative strains) were able to grow at a bile concentration of 5% (V/V) following 24 h of incubation (Table 4). The most tolerant species were heterofermentative *L. paracasei* and *L. rhamnosus*, and homofermentative *L. acidophilus*. The bile tolerance of subgingival strains was comparable to the same species isolated from saliva.

Selection of lactobacilli strains with best potential probiotic characteristics

Each particular *Lactobacillus* strain was scored according to the results of antimicrobial activity, and acid and bile tolerance. Lactobacilli originating from the same person and belonging to the same species group were considered as different strains based on their different phenotypic characteristics. Data for 18 salivary and four subgingival strains are shown in Table 5. Strains of species of *L. plantarum*, *L. paracasei*, *L. salivarius*, and *L. rhamnosus* showed both high antimicrobial activity and good tolerance of low pH and high concentration of bile.

Lysozyme tolerance

All the tested strains showed high tolerance of lysozyme. Lysozyme at concentrations of 0.2–10 mg/ml had no inhibitory effect on the growth of lactobacilli whereas inhibition of *M. luteus* was observed. The mean widths of inhibition zones for *M. luteus* were 8.0 ± 0 mm at 0.2 mg/ml, 8.5 ± 0 mm at 0.4 mg/ml, 8.8 ± 0.3 mm at 0.6 mg/ml, 9.0 ± 0 mm at both 0.8 and 1 mg/ml, and 10.8 ± 0.3 mm at 10 mg/ml of lysozyme.

Antibiotic susceptibility

Data regarding the susceptibility of 22 *Lactobacillus* strains to 13 antibiotics are presented in Table 6. No resistance was found to amoxicillin, cefprozil, cefotaxime, erythromycin, and chloramphenicol. Although most of the strains had low MICs to gentamicin, doxycycline, tetracycline, and clindamycin, some resistant strains appeared. One strain of *L. gasseri* was resistant to gentamicin, all four *L. plantarum* and two *L. oris* strains were resistant to doxycycline and/or tetracycline

Table 2. Antimicrobial activity of oral lactobacilli originating from saliva and subgingival sites, expressed as inhibition zone values (mm)

Lactobacilli		Inhibition of target bacteria: zone values (mm) mean† ± SD				
Origin	Fermentation type/species	*S. mutans*	*A. actinomycetemcomitans*	*P. gingivalis*	*P. intermedia*	*C. albicans*
Salivary	OHOL, *n** =	*19*	*19*	*15*	*15*	*19*
	L. acidophilus	0	2.5 ± 0	12.0 ± 0	0	0
	L. crispatus	0	1.0 ± 0.7	26.7 ± 0	9.5 ± 0	0
	L. delbrueckii	1.5 ± 0	4.3 ± 0	11.3 ± 0	0	0
	L. gasseri	0.1 ± 0.2	1.5 ± 0.9	17.1 ± 4.0	6.7 ± 2.4	0
	L. salivarius	2.7 ± 2.1	4.2 ± 0.8	24.4 ± 4.2	11.2 ± 1.9	0
	FHEL, *n* =	*17*	*17*	*9*	*9*	*17*
	L. paracasei	2.2 ± 1.5	3.7 ± 1.2	24.0 ± 2.1	12.0 ± 1.9	0
	L. plantarum	3.0 ± 0.8	6.1 ± 0.7	21.7 ± 5.4	9.6 ± 1.3	0
	L. rhamnosus	2.0 ± 0.5	4.4 ± 1.0	22.1 ± 1.2	11.2 ± 0.3	0
	OHEL, *n* =	*24*	*24*	*12*	*12*	*24*
	L. fermentum	1.3 ± 0.8	3.1 ± 2.4	0	0	0
	L. oris	0.1 ± 0.4	2.4 ± 1.5	12.1 ± 5.7	0	0
Subgingival	OHOL, *n* =	*3*	*3*	*3*	*3*	*3*
	L. gasseri	0	1.8 ± 0.4	17.4 ± 5.1	4.5 ± 3.0	0
	FHEL, *n* =	*1*	*1*	*1*	*1*	*1*
	L. paracasei	2.5 ± 0	6.0 ± 0	23.0 ± 0	10.3 ± 0	0
	OHEL, *n* =	*3*	*3*	*2*	*2*	*3*
	L. oris	0	1.1 ± 1.9	7.4 ± 7.9	0	0

OHOL, obligately homofermentative lactobacilli; FHEL, facultatively heterofermentative lactobacilli; OHEL, obligately heterofermentative lactobacilli.
**n* = number of *Lactobacillus* strains tested.
†Data for each *Lactobacillus* species were averaged within a strain and then across strains.

Table 3. Survival and growth of lactobacilli in acidic environment, expressed as a number (*n*) of surviving and growing strains

Lactobacilli			Growth* of strains (*n*) at pH			Survival‡ of strains (*n*) at pH						
Origin	Fermentation type/species	No. strains tested	5.6	3.5	3.0†	5.6	3.5	3.0	No. strains tested	2.5	2.0	1.5
Salivary	OHOL											
	L. acidophilus	2	2	0	0	2	2	2				
	L. crispatus	2	2	0	0	2	2	2				
	L. delbrueckii	1	1	0	0	1	0	0				
	L. gasseri	9	9	2	0	9	9	8	1	0	0	0
	L. salivarius	5	5	4	0	5	5	5	3	0	0	0
	FHEL											
	L. paracasei	7	7	6	2	7	7	7	1	0	0	0
	L. plantarum	7	7	7	7	7	7	7	7	7	0	0
	L. rhamnosus	3	3	3	1	3	3	3	3	2	0	0
	OHEL											
	L. fermentum	16	16	16	16	16	16	16	15	15	0	0
	L. oris	8	8	8	2	8	8	8				
Subgingival	*L. gasseri*	3	3	0	0	3	3	3				
	L. paracasei	1	1	1	0	1	1	1	1	0	0	0
	L. oris	3	3	3	0	3	3	3				

OHOL, obligately homofermentative lactobacilli; FHEL, facultatively heterofermentative lactobacilli; OHEL, obligately heterofermentative lactobacilli.
*Data presented following incubation for 24 h.
†No growth was observed at pH lower than 3.0.
‡Data presented following incubation for 4 h.

Table 4. Growth of lactobacilli at various concentrations of bile following incubation for 24 h, expressed as a number (*n*) of growing strains

Lactobacilli			Growth of strains (*n*) at bile concentration (% V/V)						
Origin	Fermentation type/species	No. strains tested	0.08	0.16	0.3	0.6	1.25	2.5	5
Salivary	OHOL								
	L. acidophilus	2	2	2	2	2	2	2	2
	L. crispatus	2	2	2	2	2	2	1	0
	L. delbrueckii	1	1	1	1	1	1	1	0
	L. gasseri	9	9	9	9	8	8	7	5
	L. salivarius	5	5	5	5	5	5	5	3
	FHEL								
	L. paracasei	7	7	7	7	7	7	7	7
	L. plantarum	7	7	7	7	7	7	7	3
	L. rhamnosus	3	3	3	3	3	3	3	3
	OHEL								
	L. fermentum	16	16	16	16	16	15	3	0
	L. oris	8	8	8	8	8	8	8	6
Subgingival	*L. gasseri*	3	3	3	3	3	2	1	1
	L. paracasei	1	1	1	1	1	1	1	1
	L. oris	3	3	3	3	3	3	3	2

OHOL, obligately homofermentative lactobacilli; FHEL, facultatively heterofermentative lactobacilli; OHEL, obligately heterofermentative lactobacilli.

and all four *L. gasseri* strains were resistant to clindamycin. All the studied lactobacilli were resistant to metronidazole and the majority of the strains, belonging to different species, were resistant to cefoxitin, vancomycin, and ciprofloxacin. All vancomycin-susceptible strains belonged to *L. gasseri*, while cefoxitin- and ciprofloxacin-susceptible strains belonged to species of *L. gasseri*, *L. salivarius*, and *L. paracasei*. When testing for ciprofloxacin (two *L. salivarius*, one *L. paracasei* and two *L. rhamnosus* strains), cefotaxime (two *L. oris*), and cefprozil (two *L. fermentum*), pinpoint colonies were observed within the inhibition zone of the E-test. Following re-testing of the strains, these colonies were considered resistant subpopulations within the same pure strain (heteroresistance) and were included in the MICs.

Discussion

In the current study we showed that by characterizing oral salivary and subgingival lactobacilli of healthy humans according to the international guidelines for the evaluation of probiotics, several strains had the properties required for a potential probiotic strain.

To claim that a bacterial strain is a potential 'probiotic' strain, several guidelines have been suggested by a joint Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) working group (15). These guidelines emphasize the necessity of correct identification of a probiotic strain and the use of various *in vitro* tests to evaluate the functionality and safety of a probiotic strain. The current state of evidence suggests that probiotic effects are generally strain-specific and therefore, precise identification of isolates is an important consideration in the selection of probiotic strains. It is recommended that a combination of phenotypic and genetic tests be used (15). In this study, we followed these recommendations and were able to identify 60 salivary and seven

Table 5. Antimicrobial activity, acid and bile tolerance of 22 selected salivary and subgingival *Lactobacillus* strains

Lactobacilli			Antimicrobial activity toward					Survival[‡] at	Growth[**] at		
Origin	Strain	Species	Sm[*]	Aa[*]	Pg[†]	Pi[†]	Ca[*]	pH	pH	bile (% V/V)	Score[††]
Saliva	25-DLB-3A-A	*L. plantarum*	+	++	++	+	–	2.5	3.0	2.5	16
	27-DLB-1	*L. plantarum*	+	++	++	+	–	2.5	3.0	2.5	16
	35-DLB-2	*L. plantarum*	+	++	++	+	–	2.5	3.0	2.5	16
	21-DLB-4-B	*L. paracasei*	+	++	++	+	–	3.0[§]	3.0	5	16
	37-DLB-2A	*L. plantarum*	+	++	+	+	–	2.5	3.0	5	15
	27-DLB-2A	*L. salivarius*	++	+	++	+	–	3.0	3.5	2.5	15
	43-DLB-3-A	*L. salivarius*	+	++	++	+	–	3.0	3.5	5	15
	21-DLB-5-B	*L. rhamnosus*	+	++	++	+	–	2.5	3.5	5	15
	35-DLB-5	*L. rhamnosus*	–	++	++	+	–	3.0	3.0	5	15
	21-DLB-6-B	*L. paracasei*	+	+	++	+	–	3.0[§]	3.5	5	14
	37-DLB-1	*L. salivarius*	+	+	++	+	–	3.0	3.5	5	14
	21-DLB-7	*L. rhamnosus*	+	+	++	+	–	2.5	3.5	5	14
	13-DLB-6A	*L. gasseri*	–	+	++	+	–	3.0[§]	3.5	1.25	11
	33-DLB-2	*L. gasseri*	–	+	+	+	–	3.0	3.5	5	11
	13-DLB-4A	*L. fermentum*	+	++	–	–	–	2.5	3.0	1.25	10
	37-DLB-2B	*L. fermentum*	–	++	–	–	–	2.5	3.0	2.5	10
	21-DLB-1B-2	*L. oris*	–	+	+	–	–	3.0[§]	3.0	5	10
	25-DLB-4A-1	*L. oris*	–	++	+	–	–	3.0[§]	3.5	5	10
Subgingival	8-2-16A-B	*L. paracasei*	+	++	++	+	–	3.0	3.5	5	15
	8-2-1A	*L. gasseri*	–	–	+	+	–	3.0[§]	5.6	5	9
	37-2-10-A	*L. gasseri*	–	+	++	–	–	3.0[§]	5.6	1.25	9
	8-2-16B	*L. oris*	–	+	+	–	–	3.0[§]	3.5	5	9

[*]Antimicrobial activity: –, no inhibition or <2 mm; +, between 2 and 5 mm of inhibition; ++, 5 mm of inhibition and above; Sm, *S. mutans*; Aa, *A. actinomycetemcomitans*; Ca, *C. albicans*.
[†]Antimicrobial activity: –, no inhibition or <7 mm; +, between 7 and 20 mm of inhibition; ++, 20 mm of inhibition and above; Pg, *P. gingivalis*; Pi, *P. intermedia*.
[‡]Data presented following incubation for 4 h: [§], not determined at pH lower than pH 3.0.
[**]Data presented following incubation for 24 h.
[††]Total score for a *Lactobacillus* strain based on data of antimicrobial activity, acid and bile tolerance (max. value 19).

Table 6. Antibiotic susceptibility of 22 selected salivary and subgingival *Lactobacillus* strains

Lactobacilli		Antibiotic[*] with the MIC (μg/ml) as follows												
Strain	Species	AC	FX	FP	CT	VA	GM	EM	DC	TC	CM	CL	CI	MZ
Salivary strains														
25-DLB-3A-A	*L. plantarum*	0.25	≥256	0.5	0.25	≥256	2	0.38	16	24	0.38	3	≥32	≥256
27-DLB-1	*L. plantarum*	0.38	≥256	0.5	0.38	≥256	1.5	0.5	24	24	0.25	4	≥32	≥256
35-DLB-2	*L. plantarum*	0.19	≥256	0.38	0.25	≥256	1.5	0.25	6	24	1.5	4	≥32	≥256
21-DLB-4-B	*L. paracasei*	1.0	≥256	6	6	≥256	4	0.19	0.75	0.75	0.09	3	2	≥256
37-DLB-2A	*L. plantarum*	0.12	≥256	0.38	0.19	≥256	1.0	0.5	16	32	0.75	4	≥32	≥256
27-DLB-2A	*L. salivarius*	0.75	16	1.5	0.75	≥256	12	0.75	1.0	1.5	0.25	2	1.5	≥256
43-DLB-3-A	*L. salivarius*	0.19	1.5	0.75	0.25	≥256	2	0.25	0.25	0.25	0.19	1.5	≥32	≥256
21-DLB-5-B	*L. rhamnosus*	0.5	≥256	6	4	≥256	8	0.38	0.5	1.0	0.38	4	12	≥256
35-DLB-5	*L. rhamnosus*	0.75	≥256	8	6	≥256	12	0.25	0.5	0.5	0.75	3	4	≥256
21-DLB-6-B	*L. paracasei*	0.75	≥256	6	6	≥256	4	0.19	0.75	0.75	0.25	3	6	≥256
37-DLB-1	*L. salivarius*	0.25	6	0.75	0.38	≥256	12	0.38	0.75	1.0	0.19	2	≥32	≥256
21-DLB-7	*L. rhamnosus*	0.75	≥256	8	6	≥256	8	0.25	0.5	0.5	0.5	3	12	≥256
13-DLB-6A	*L. gasseri*	0.38	≥256	3	1.0	1.5	8	0.19	2	3	8	6	≥32	≥256
33-DLB-2	*L. gasseri*	0.5	≥256	1.5	2	1.5	12	0.09	1.5	0.75	8	3	≥32	≥256
13-DLB-4A	*L. fermentum*	0.25	≥256	12	0.5	≥256	1.5	0.12	3	2	0.01	3	≥32	≥256
37-DLB-2B	*L. fermentum*	0.25	128	0.75	0.5	≥256	0.25	0.09	6	6	0.02	3	6	≥256
21-DLB-1B-2	*L. oris*	0.38	≥256	3	1.5	≥256	2	0.19	16	24	0.03	6	≥32	≥256
25-DLB-4A-1	*L. oris*	0.25	≥256	4	1.5	≥256	0.75	0.19	12	16	0.12	4	≥32	≥256
Subgingival strains														
8-2-16A-B	*L. paracasei*	1.0	≥256	8	12	≥256	8	0.25	0.75	0.75	0.25	3	1.0	≥256
8-2-1A	*L. gasseri*	0.19	32	1.5	0.38	1.5	16	0.19	1.5	1.5	48	3	≥32	≥256
37-2-10-A	*L. gasseri*	0.19	4	1.5	0.5	1.5	6	0.12	0.38	0.38	4	2	≥32	≥256
8-2-16B	*L. oris*	0.38	≥256	2	0.38	≥256	0.25	0.12	12	12	0.19	3	≥32	≥256

[*]AC, amoxicillin; FX, cefoxitin; FP, cefprozil; CT, cefotaxime; VA, vancomycin; GM, gentamicin; EM, erythromycin; DC, doxycycline; TC, tetracycline; CM, clindamycin; CL, chloramphenicol; CI, ciprofloxacin; MZ, metronidazole.

subgingival lactobacilli isolates that belonged to 10 different species. Subsequently, the identified *Lactobacillus* strains were subjected to characterization for their functionality and safety.

The main *in vitro* tests currently used for the study of the functionality of probiotic strains of gastrointestinal origin are antimicrobial activity against potentially pathogenic bacteria, and tolerance of gastric acidity and bile salts. To the best of our knowledge there are no specific guidelines for the evaluation of potential probiotic strains originating from the oral cavity. Therefore, the above mentioned tests were

considered as a prerequisite for screening oral lactobacilli strains for their functional properties. We found that most of the oral *Lactobacillus* strains showed antimicrobial activity against putative oral pathogens. Yet, as shown also for intestinal lactobacilli (3), it was largely fermentation group-, species-, and strain-specific. Facultatively heterofermentative lactobacilli (*L. plantarum*, *L. paracasei*, *L. rhamnosus*) and homofermentative *L. salivarius* expressed the strongest antimicrobial activity, which is consistent with their phylogenetic relatedness, both belonging to the *Lactobacillus casei–Pediococcus* group. Good antimicrobial activity of oral *L. paracasei* and *L. rhamnosus* against oral pathogens has previously been shown by Sookkhee et al. (42). In contrast, Testa et al. (44) found no antagonistic interactions between oral lactobacilli (*L. casei*, *L. rhamnosus*, *L. plantarum*, and *L. salivarius*) and the anaerobes *P. intermedia* and *Fusobacterium nucleatum.* In the present study, we also tested the ability of oral lactobacilli to inhibit the growth of *C. albicans*, but found no inhibition. These results are in accordance with data published recently by Strahinic et al. (43). Some anticandidal effect of strains of *L. paracasei* and *L. rhamnosus* was found by Sookkhee et al. (42), but no inhibition of *C. albicans* by lactobacilli was seen when Mastromarino et al. (30) characterized vaginal lactobacilli. These conflicting results regarding antimicrobial activity could be the result of differences in methodology, but could also be related to microbial factors, like the strain-specific antimicrobial activity of lactobacilli as well as variability in the sensitivity of different target strains. Our earlier results have shown that the antimicrobial activity of lactobacilli could be related to the production of organic acids, such as lactic and acetic acid upon fermentation of glucose with a concomitant decrease in pH (3). In addition, some lactobacilli produce hydrogen peroxide and bacteriocins (3, 30, 47).

In the present study, the acid and bile tolerance was higher among heterofermentative *Lactobacillus* strains, yet as seen for the antimicrobial activity, the properties were largely strain- and species-specific. The lowest pH that was resisted by lactobacilli following 4 h of incubation was pH 2.5, with heterofermentative *L. fermentum* and *L. plantarum* being the most stable species. Even the most resistant strains were unable to grow at this pH, confirming the results by Jacobsen et al. (21). In a recent study, two oral lactobacilli strains (*L. salivarius* BGHO1 and *L. gasseri* BGHO89) were shown to survive at pH 2.5, although the percentage of viable cells decreased remarkably following 24 h of incubation (43). In resting dental plaque, the pH has been reported to be around pH 6.5 (ranging between pH 5.6 and pH 7.0), with a drop down to pH 4.5 and pH 4.0 following a sucrose rinse (37). The pH of the stomach may fall as low as pH 1.0, but when food comes into the stomach, the pH may rise to levels of 3.0–4.0 because of the buffering capacity of proteins. Most of the studied strains resisted incubation at pH 3.0 for 4 h and tolerated physiological concentrations of bile (0.5–2% V/V in the small intestine), which makes them good candidates for use as probiotics in the mouth as well as in other parts of the gastrointestinal tract. However, in this case additional tests are needed, such as adherence to human intestinal cells and antimicrobial activity against intestinal pathogens.

Based on the results of antimicrobial activity, and acid and bile tolerance we selected 22 (18 salivary and four subgingival) potential probiotic strains and extended our study to assess the effect of lysozyme on their growth. Lysozyme is an enzyme that is found in saliva at concentrations up to 180 μg/ml (25). It is effective in killing several types of gram-positive bacteria by promoting cell wall disruption and subsequent cell lysis. In the present study, we found that all tested lactobacilli were resistant to lysozyme at a concentration that was 50 times the physiological concentration. These results are in accordance with data published earlier (34) and confirm the potential of oral lactobacilli to be used as probiotics for oral health.

An important safety requirement for probiotic strains is that they should not carry transferable antibiotic resistance genes (15). The spread of such genes among bacterial species through lateral gene transfer may contribute to dissemination of resistance to antibiotics used for therapy (13, 14, 39), and therefore, potential probiotics should be screened for their antibiotic susceptibility pattern. The safety assessment in the present study involved screening the antibiotic susceptibility of the 22 most potentially probiotic lactobacilli for a group of 13 antibiotics, including inhibitors of cell wall, protein, and nucleic acid synthesis. There are no generally accepted standard procedures for MIC determination for lactobacilli and there is a general lack of agreement in the susceptibility/resistance breakpoints for lactobacilli for most antibiotics (14). Therefore, we determined the MICs and breakpoints of oral lactobacilli according to the methods published for intestinal lactobacilli (28). We found that oral lactobacilli, as we have also observed for intestinal lactobacilli (28), did not display uniform susceptibility to antibiotics. Although most of the strains were sensitive to a number of clinically effective antibiotics, high level of resistance to cefoxitin, vancomycin, ciprofloxacin, and metronidazole was found. Similar results have previously been reported (11, 18, 28) and could be interpreted as high natural resistance to these antibiotics. Metronidazole resistance, being considered as *Lactobacillus* genus-specific natural resistance, is linked to the absence of hydrogenase activity in lactobacilli (9). At the same time, susceptibility to vancomycin has been found to be related to *Lactobacillus* species, with all heterofermentative lactobacilli being vancomycin-resistant and susceptible ones belonging to the obligately homofermentative group (11, 12, 16, 28). Natural vancomycin resistance is related to the production of cell wall peptidoglycan precursors terminating in D-alanine-D-lactate, to which vancomycin does not bind (19). Our study results, where all heterofermentative lactobacilli and *L. salivarius* were resistant to vancomycin, and strains of *L. gasseri* were susceptible, are in accordance with the above mentioned studies. Interestingly, we found these vancomycin-susceptible *L. gasseri* strains to be resistant to clindamycin. Similar findings have been published by Danielsen and Wind (11) and Delgado et al. (12) who found clindamycin MICs equal to or above 4 μg/ml to be common for *L. gasseri*, and therefore, clindamycin resistance in *L. gasseri* could be considered as natural resistance.

It is important to confirm whether the antibiotic resistance of the probiotic strain is of intrinsic origin or is carried by highly mobile genetic elements, such as plasmids and transposons (39). Plasmid-encoded erythromycin and tetracycline resistance has been reported in lactobacilli (4, 17). We observed no resistance to erythromycin in the lactobacilli studied. These findings somewhat contradict the results of other authors (11, 12) who have found some resistance to erythromycin. However, a lower number of strains was used in the present study. We also observed that although most of the strains had low MICs to tetracyclines, some resistant strains appeared. These included all four strains of *L. plantarum* and two *L. oris* strains, with MICs up to 32 μg/ml.

According to the guidelines of the Scientific Committee on Animal Nutrition (SCAN), the microbiological breakpoint for tetracycline is 16 μg/ml for *Lactobacillus* species and strains with MICs equal to or higher than the breakpoints are considered resistant (14). However, as pointed out by several authors (11, 16, 46), there is a need for differentiating between *Lactobacillus* species when determining the breakpoints for antibiotics. Results of Danielsen and Wind (11) showed that strains of *L. plantarum*/*pentosus* had relatively high MICs for tetracycline and they proposed 64 μg/ml as a breakpoint for these species. Thus, even though the resistance of the studied *L. plantarum* strains could be of intrinsic origin, the strains of both *L. plantarum* and *L. oris* should be tested further by genetic analysis and by transfer experiments to determine whether they have transferable antibiotic-resistance genes. If not, the moderate natural resistance could be favorable when used in antibiotic/probiotic combination therapies. The concentration of tetracycline/doxycycline achieved in gingival crevicular fluid is between 0.6 and 16 μg/ml and in saliva is between 0.1 and 0.5 μg/ml as indicated in the literature (24, 40).

In summary, the present study demonstrates that several human oral lactobacilli possess good functional probiotic properties like antimicrobial activity against oral pathogens as well as high tolerance of environmental stress factors, which make them suitable for using as potential probiotics for oral health. These beneficial properties are better expressed in facultatively heterofermentative *L. plantarum*, *L. paracasei*, and *L. rhamnosus*, and homofermentative *L. salivarius* strains. At the same time, the strains of *L. plantarum* differ from the natural resistance pattern of lactobacilli and therefore, should be considered non-safe.

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References

1. Ahola AJ, Yli-Knuuttila H, Suomalainen T et al. Short-term consumption of probiotic-containing cheese and its effect on dental caries risk factors. Arch Oral Biol 2002: **47**: 799–804.
2. Ahrne S, Nobaek S, Jeppsson B, Adlerberth I, Wold AE, Molin G. The normal *Lactobacillus* flora of healthy human rectal and oral mucosa. J Appl Microbiol 1998: **85**: 88–94.
3. Annuk H, Shchepetova J, Kullisaar T, Songisepp E, Zilmer M, Mikelsaar M. Characterization of intestinal lactobacilli as putative probiotic candidates. J Appl Microbiol 2003: **94**: 403–412.
4. Axelsson LT, Ahrne SE, Andersson MC, Stahl SR. Identification and cloning of a plasmid-encoded erythromycin resistance determinant from *Lactobacillus reuteri*. Plasmid 1988: **20**: 171–174.
5. Axelsson L. Lactic acid bacteria: classification and physiology. In: Salminen S, von Wright A, Ouwehand A, ed. Lactic acid bacteria: microbiology and functional aspects, 3rd edn. New York: Marcel Dekker, 2004: 1–66.
6. Birkhed D, Edwardsson S, Andersson H. Comparison among a dip-slide test (Dentocult®), plate count, and Snyder test for estimating number of lactobacilli in human saliva. J Dent Res 1981: **60**: 1832–1841.
7. Boström L, Linder LE, Bergström J. Influence of smoking on the outcome of periodontal surgery. A 5-year follow-up. J Clin Periodontol 1998: **25**: 194–201.
8. Caglar E, Cildir SK, Ergeneli S, Sandalli N, Twetman S. Salivary mutans streptococci and lactobacilli levels after ingestion of the probiotic bacterium *Lactobacillus reuteri* ATCC 55730 by straws or tablets. Acta Odontol Scand 2006: **64**: 314–318.
9. Church DL, Bryant RD, Sim V, Laishley EJ. Metronidazole susceptibility and the presence of hydrogenase in pathogenic bacteria. Anaerobe 1996: **2**: 147–153.
10. Colloca ME, Ahumada MC, López ME, Nader-Macías ME. Surface properties of lactobacilli isolated from healthy subjects. Oral Dis 2000: **6**: 227–233.
11. Danielsen M, Wind A. Susceptibility of *Lactobacillus* spp. to antimicrobial agents. Int J Food Microbiol 2003: **82**: 1–11.
12. Delgado S, Flórez AB, Mayo B. Antibiotic susceptibility of *Lactobacillus* and *Bifidobacterium* species from the human gastrointestinal tract. Curr Microbiol 2005: **50**: 202–207.
13. Duncan MJ. Genomics of oral bacteria. Crit Rev Oral Biol Med 2003: **14**: 175–187.
14. European Commission. Opinion of the Scientific Committee on Animal Nutrition on the criteria for assessing the safety of micro-organisms resistant to antibiotics of human clinical and veterinary importance. 2002. [http://ec.europa.eu/food/fs/sc/scan/out64_en.pdf] [accessed on 12 July 2007].
15. FAO/WHO. Guidelines for the Evaluation of Probiotics in Food. Report of a Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food. London, Ontario, Canada, 2002. [ftp://ftp.fao.org/es/esn/food/wgreport2.pdf] [accessed on 12 July 2007].
16. Felten A, Barreau C, Bizet C, Lagrange PH, Philippon A. *Lactobacillus* species identification, H_2O_2 production, and antibiotic resistance and correlation with human clinical status. J Clin Microbiol 1999: **37**: 729–733.
17. Gevers D, Huys G, Swings J. *In vitro* conjugal transfer of tetracycline resistance from *Lactobacillus* isolates to other Gram-positive bacteria. FEMS Microbiol Lett 2003: **8**: 125–130.
18. Hamilton-Miller JM, Shah S. Vancomycin susceptibility as an aid to the identification of lactobacilli. Lett Appl Microbiol 1998: **26**: 153–154.
19. Handwerger S, Pucci MJ, Volk KJ, Liu J, Lee MS. Vancomycin-resistant *Leuconostoc mesenteroides* and *Lactobacillus casei* synthesize cytoplasmic peptidoglycan precursors that terminate in lactate. J Bacteriol 1994: **176**: 260–264.
20. Hojo K, Mizoguchi C, Taketomo N et al. Distribution of salivary *Lactobacillus* and *Bifidobacterium* species in periodontal health and disease. Biosci Biotechnol Biochem 2007: **71**: 152–157.
21. Jacobsen CN, Rosenfeldt Nielsen V, Hayford AE et al. Screening of probiotic activities of forty-seven strains of *Lactobacillus* spp. by *in vitro* techniques and evaluation of the colonization ability of five selected strains in humans. Appl Environ Microbiol 1999: **65**: 4949–4956.
22. Jorgensen JH, Turnidge JD. Susceptibility test methods: dilution and disk diffusion methods. In: Murray PR, Baron EJ, Jorgensen JH, Pfaller MA, Yolken RH, ed. Manual of clinical microbiology, 8th edn. Washington, DC: ASM Press, 2003: 1108–1125.
23. Kandler O, Weiss N. Regular, nonsporing Gram-positive rods. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG, ed. Bergey's manual of systematic bacteriology, Vol. 2. Baltimore, MD: Williams & Wilkins, 1986: 1208–1234.
24. Kim TS, Burklin T, Schacher B et al. Pharmacokinetic profile of a locally administered doxycycline gel in crevicular fluid, blood, and saliva. J Periodontol 2002: **73**: 1285–1291.
25. Koh D, Yang Y, Khoo L, Nyunt SZ, Ng V, Goh CL. Salivary immunoglobulin A and lysozyme in patients with psoriasis. Ann Acad Med Singapore 2004: **33**: 307–310.
26. Kõll-Klais P, Mändar R, Leibur E, Marcotte H, Hammarström L, Mikelsaar M. Oral lactobacilli in chronic periodontitis and periodontal health: species composition and antimicrobial activity. Oral Microbiol Immunol 2005: **20**: 354–361.
27. Krüger C, Hu Y, Pan Q et al. *In situ* delivery of passive immunity by lactobacilli producing single-chain antibodies. Nat Biotechnol 2002: **20**: 702–706.
28. Mändar R, Lõivukene K, Hütt P, Karki T, Mikelsaar M. Antibacterial susceptibility of intestinal lactobacilli of healthy children. Scand J Infect Dis 2001: **33**: 344–349.
29. Marcotte H, Kõll-Klais P, Hultberg A et al. Expression of single-chain antibody against RgpA protease of *Porphyromonas gingivalis* in *Lactobacillus*. J Appl Microbiol 2006: **100**: 256–263.

30. Mastromarino P, Brigidi P, Macchia S et al. Characterization and selection of vaginal *Lactobacillus* strains for the preparation of vaginal tablets. J Appl Microbiol 2002: **93**: 884–893.
31. McFarland LV. Normal flora: diversity and functions. Microb Ecol Health Dis 2000: **12**: 193–207.
32. Meurman JH. Probiotics: do they have a role in oral medicine and dentistry? Eur J Oral Sci 2005: **113**: 188–196.
33. Mikelsaar M, Mändar R, Sepp E, Annuk H. Human lactic acid microflora and its role in the welfare of the host. In: Salminen S, von Wright A, Ouwehand A, ed. Lactic acid bacteria, 3rd edn. New York: Marcel Dekker, 2004: 453–505.
34. Mikelsaar M, Türi M, Lencner H, Kolts K, Kirch R, Lencner A. Interrelations between mucosal and luminal microflora of gastrointestine. Nahrung 1987: **31**: 449–456.
35. Morency H, Mota-Meira M, LaPointe G, Lacroix C, Lavoie MC. Comparison of the activity spectra against pathogens of bacterial strains producing a mutacin or a lantibiotic. Can J Microbiol 2001: **47**: 322–331.
36. Näse L, Hatakka K, Savilahti E et al. Effect of long-term consumption of a probiotic bacterium, *Lactobacillus rhamnosus* GG, in milk on dental caries and caries risk in children. Caries Res 2001: **35**: 412–420.
37. Nyvad B, Fejerskov O. Development, structure and pH of dental plaque. In: Thylstrup A, Fejerskov O, ed. Textbook of clinical cariology, 2nd edn. Copenhagen: Munksgaard, 1994: 89–110.
38. Perdigon G, Fuller R, Raya R. Lactic acid bacteria and their effect on the immune system. Curr Issues Intest Microbiol 2001: **2**: 27–42.
39. Saarela M, Mogensen G, Fonden R, Mätto J, Mattila-Sandholm T. Probiotic bacteria: safety, functional and technological properties. J Biotechnol 2000: **84**: 197–215.
40. Sakellari D, Goodson JM, Kolokotronis A, Konstantinidis A. Concentration of 3 tetracyclines in plasma, gingival crevice fluid and saliva. J Clin Periodontol 2000: **27**: 53–60.
41. Schillinger U, Lücke FK. Antibacterial activity of *Lactobacillus sake* isolated from meat. Appl Environ Microbiol 1989: **55**: 1901–1906.
42. Sookkhee S, Chulasiri M, Prachyabrued W. Lactic acid bacteria from healthy oral cavity of Thai volunteers: inhibition of oral pathogens. J Appl Microbiol 2001: **90**: 172–179.
43. Strahinic I, Busarcevic M, Pavlica D, Milasin J, Golic N, Topisirovic L. Molecular and biochemical characterizations of human oral lactobacilli as putative probiotic candidates. Oral Microbiol Immunol 2007: **22**: 111–117.
44. Testa MM, Ruiz de Valladares R, Benito de Cardenas IL. Antagonistic interactions among *Fusobacterium nucleatum* and *Prevotella intermedia* with oral lactobacilli. Res Microbiol 2003: **154**: 669–675.
45. Ventura M, Casas IA, Morelli L, Callegari ML. Rapid amplified ribosomal DNA restriction analysis (ARDRA) identification of *Lactobacillus* spp. isolated from fecal and vaginal samples. Syst Appl Microbiol 2000: **23**: 504–509.
46. Zarazaga M, Sáenz Y, Portillo A et al. *In vitro* activities of ketolide HMR3647, macrolides, and other antibiotics against *Lactobacillus*, *Leuconostoc* and *Pediococcus* isolates. Antimicrob Agents Chemother 1999: **43**: 3039–3041.
47. Zhu WM, Liu W, Wu DQ. Isolation and characterization of a new bacteriocin from *Lactobacillus gasseri* KT7. J Appl Microbiol 2000: **88**: 877–886.