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Oral lactobacilli in chronic periodontitis and periodontal health: species composition and antimicrobial activity

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Background/aims: Lactobacilli are known to play an important role in the maintenance of health by stimulating natural immunity and contributing to the balance of microflora. However, their role in chronic periodontitis is unclear. We aimed to identify oral lactobacilli in chronic periodontitis and periodontally healthy subjects, and to determine their antimicrobial activity against putative oral pathogens.
Methods: A total of 238 *Lactobacillus* isolates from the saliva and subgingival sites of 20 chronic periodontitis and 15 healthy subjects were collected. In all, 115 strains were identified using rapid amplified ribosomal DNA restriction analysis. Antimicrobial activity against *Streptococcus mutans*, *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Prevotella intermedia* was assessed.
Results: Lactobacilli belonging to 10 species were identified. The most prevalent strains in healthy persons were *Lactobacillus gasseri* and *Lactobacillus fermentum* and in chronic periodontitis patients, *Lactobacillus plantarum*. Obligately homofermentatives, particularly *L. gasseri*, were less prevalent in chronic periodontitis patients compared with healthy subjects (8% vs. 64% for *L. gasseri*, $P < 0.01$). Sixty-nine percent of tested lactobacilli inhibited *S. mutans*, 88% *A. actinomycetemcomitans*, 82% *P. gingivalis* and 65% *P. intermedia*. The strongest antimicrobial activity was associated with *Lactobacillus paracasei*, *L. plantarum*, *Lactobacillus rhamnosus*, and *Lactobacillus salivarius*. The strains from periodontally healthy patients showed a lower antimicrobial activity against *S. mutans* than the strains from chronic periodontitis patients.
Conclusion: The composition of oral lactoflora in chronic periodontitis and healthy subjects differs, with a higher prevalence of homofermentative lactobacilli, particularly *L. gasseri*, in the latter group. Both homo- and heterofermentative oral lactobacilli suppress the growth of periodontal pathogens, but the antimicrobial properties are strain, species and origin specific.

Key words: antimicrobial activity; chronic periodontitis; lactobacilli; periodontal health

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Lactobacilli are innocuous commensals living in close association with the human organism (4). They play an important role in the maintenance of health by stimulating the natural immunity as well as contributing to the balance of microflora by interacting with the other members of the flora (23, 24, 29). The ability of lactobacilli to inhibit the growth of various infectious agents in gut is well reviewed, although the mechanisms are not entirely understood (2, 12).

Lactobacilli make up approximately 1% of the cultivable oral microflora (21). The most common species are heterofermentative lactobacilli such as *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus casei*, and *Lactobacillus fermentum*, and the homofermentative *Lactobacillus salivarius* (1, 8, 19). Several other species that have been isolated from the mouth include *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus oris*, *Lactobacillus paracasei*, *Lactobacillus delbrueckii*, *Lactobacillus acidophilus*, *Lactobacillus jensenii*, *Lactobacillus gasseri*, and *Lactobacillus agilis* (1, 8, 35).

However, it has not been assessed whether the species composition of oral lactobacilli is related to oral health.

Chronic periodontitis is one of the most common infectious diseases of the oral cavity largely associated with the imbalance of indigenous microflora (21), resulting in overgrowth of periodontal pathogens such as *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Actinobacillus actinomycetemcomitans*. Recently, we have found that destruction and inflammation at periodontal sites were closely associated with the decreased level of certain lactic acid bacteria such as streptococci and aerobic coryneforms (15). Moreover, *in vitro* it has been shown that some lactobacilli and streptococci possess antimicrobial activity against periodontal pathogens such as *P. gingivalis* and *P. intermedia* (10, 36).

To our knowledge there are no data about the species composition and antimicrobial activity of oral lactobacilli originating from chronic periodontitis patients. It is unclear whether either homo- or heterofermentative species of lactobacilli control the overgrowth of periodontal pathogens in the oral cavity. The metabolism of these biochemical groups of lactobacilli is quite different as the former produce mainly lactic acid and the latter, various organic acids and even small amounts of ethanol (2, 13). A more profound knowledge about oral lactobacilli could help to understand the ecologic imbalance in periodontitis and might provide future measures for successful control of the disease.

The aim of the present study was to identify the *Lactobacillus* species isolated from the oral cavity of chronic periodontitis patients and periodontally healthy individuals. In addition, we aimed to characterize the *Lactobacillus* isolates for their antagonistic properties against putative oral pathogens.

Material and methods

Subjects

The study group comprised 20 chronic periodontitis patients (mean age 46.4 ± 10.4 years) and 15 periodontally healthy subjects (mean age 37.5 ± 10.4 years), both groups with no history of systemic disease or antibiotic therapy within the 6 months prior to sampling. Chronic periodontitis patients were diagnosed as having chronic periodontitis based on gingival inflammation, periodontal breakdown with pocket depth ≥ 5 mm and radiographic evidence of bone loss. Healthy individuals were defined as having no radiographic or clinical evidence of attachment loss. There were no statistically significant differences in dental caries status between chronic periodontitis and healthy individuals. Informed consent was obtained from all subjects, in accordance with the procedures of the Ethics Review Committee on Human Research of the University of Tartu.

Clinical examination

The baseline examination included registration of dental plaque as plaque index (31), gingival inflammation as gingival index (17) at four sites (distal, mesial, lingual and buccal sites), and periodontal probing depth (PPD) with Williams periodontal probe at six sites (distal, mid and mesial aspects for both buccal and lingual sites) of each tooth, excluding third molars. The mean PPD across all sites of one patient formed the periodontal probing depth of the individual, designated as PPD_{all}. Sites with a probing depth of ≥ 5 mm were defined as diseased sites (DS), and the mean PPD of diseased sites was designated as PPD_{ds}. The same examiner performed all clinical measurements.

Sampling of saliva and subgingival sites

All subjects were investigated for the presence of lactobacilli in saliva and two subgingival sites. Salivary lactobacilli were obtained by using the Dentocult®LB dip-slide (Orion Diagnostica, Espoo, Finland) method (5). Paraffin-stimulated whole saliva was collected and a 1-ml aliquot was transferred to a selective dip-slide. After 3 days of incubation in a 10% CO_2 environment at 37°C, the number of lactobacilli was estimated by comparing the slides with a density chart provided by the manufacturer. The results were expressed as missing, low (≤ 10^3 CFU/ml), medium (10^4 CFU/ml), high (10^5 CFU/ml), and very high (> 10^6 CFU/ml) counts of lactobacilli.

Subgingival microbial samples were collected from two diseased sites in each chronic periodontitis patient and from two sites in each healthy subject by means of a gingival crevice lavage method as described by Boström et al. (6). Prior to sampling, the area was isolated with cotton rolls and the supragingival region of the tooth surface to be sampled was cleaned and dried with sterile cotton pellets. A reduced transport fluid RTF was used as sampling fluid. Small volumes (10–20 μl) were ejected from a glass ampoule with a cannula into the periodontal pocket about 1 mm from the bottom and aspirated into the ampoule. The total volume of sampling fluid was 250 μl. The ejection and aspiration procedure was repeated four times. Four drops (about 40 μl) were transferred from the ampoule to a vial containing anaerobic transport medium VMGA III. Following rapid transport to the lab, the samples were tenfold serially diluted in prereduced peptone water (Oxoid, Unipath, Basingstoke, UK) and 100 μl of appropriate dilutions were plated onto freshly prepared de Man-Rogosa-Sharpe (MRS) agar (Oxoid) and incubated for 72 h in microaerobic atmosphere with 10% of CO_2 at 37°C. In addition to the isolation of lactobacilli, subgingival samples were analyzed for the growth of putative periodontal pathogens *A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia*/*Prevotella nigrescens*. Tryptone soya agar (Oxoid), supplemented with yeast extract (0.1%), horse serum (10%), bacitracin (75 μg/ml) and vancomycin (5 μg/ml) (TSBV) for isolation of *A. actinomycetemcomitans* (33) and Brucella agar (Oxoid), supplemented with 5% defibrinated horse blood and menadione (2.5 μg/ml) (34) for isolation of anaerobic bacteria were used. After incubation of TSBV plates in microaerobic atmosphere (10% CO_2) for 72 h and Brucella plates in anaerobic glove box (Sheldon Manufacturing Inc. (Shel LAB, Cornelius, OR) with a gas mixture of 5% H_2, 5% CO_2, 90% N_2) for 5–6 days (up to 2 weeks in negative cases), colonies with different morphology were gram stained and the microorganisms were identified using standard methods (27).

Isolation and provisional identification of lactobacilli

Colonies with different morphology (at least 2–3 colonies from each morphologic type) were picked from Dentocult®LB dip-slides and MRS plates into MRS broth and incubated in a 10% CO_2 environment for 24–48 h. Thereafter, a series of MRS plates were repeatedly streaked to purify the culture. On each step of purification, colony as well as cell morphology of an isolate was checked. Provisional identification was based on the ability of the isolate to grow in the MRS broth, and also on a gram-positive rod-shaped nonsporing cell morphology and negative catalase reaction (13). A total of 238 isolates were provisionally identified as lactobacilli (112 isolates from 18 chronic periodontitis patients and 126 isolates from 15 healthy subjects) and were further analyzed for fermentation

type according to their physiological properties.

The ability of isolates to grow in MRS broth for 24 h in a 10% CO_2 environment at 15°C and 37°C and to produce gas in MRS agar containing 1% glucose was also assessed. The fermentation of glucose without gas, growth at 37°C and no growth at 15°C identifies obligately homofermentative, growth both at 15°C and 37°C without gas production is characteristic of facultatively heterofermentative, and gas production at 37°C and variable growth at 15°C are characteristic of obligately heterofermentative *Lactobacillus* species. To verify the fermentation type, the isolates were further analyzed for sugar fermentation pattern (sorbitol, tagatose, melezitose) and arginine hydrolysis (13). The prevalence of obligately homofermentative, facultatively heterofermentative and obligately heterofermentative strains among chronic periodontitis patients and periodontally healthy individuals were compared.

Molecular identification of lactobacilli

For further studies, 12 chronic periodontitis and 11 healthy persons were randomly selected. Based on the data of provisional identification, all strains with different characteristics from each particular individual, in total 115 strains, were subjected to species identification. Of these, 108 strains were isolated from saliva and seven strains from subgingival samples.

The species of the lactobacilli were identified using rapid amplified ribosomal DNA restriction analysis (ARDRA) (41). Firstly, the strains were grouped according to their phenotypic features (fermentation of 0.5% ribose, resistance to 12 µl/ml vancomycin and production of CO_2 in the presence of glucose), followed by amplification and restriction analysis of the 16S-rRNA gene.

The genomic DNA was obtained by the phenol/chloroform extraction. PCR amplification of the 16S-rRNA gene (1.5 kb) with primers P0 (5′-GAGAGTTTGATCCTGGCTCAG-3′) and P6 (5′-CTACGGCTACCTTGTTACGA-3′) was carried out using 2.5 U of *Taq* DNA polymerase in a total volume of 25 µl in buffer containing 100 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$, 500 mM KCl, 0.2 mM (each) deoxynucleoside triphosphates, and 1 µl of template DNA. Each sample was subjected to 30 cycles (denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and primer extension at 72°C for 2 min) in an automated thermal cycler (Perkin Elmer). Three to five PCR reactions were performed for each strain. The PCR product was checked in an 0.8% (v/w) agarose gel and digested with a set of four restriction enzymes (*Sau*3AI, *Hinf*I, *Hinc*II and *Dra*I) provided by Promega (Madison, WI). Digested DNA fragments were separated by electrophoresis in a 2.5% (v/w) agarose gel with 50 bp DNA Ladder (GibcoBRL®, Gaithersburg, MD) as a molecular weight marker, stained with ethidium bromide and photographed under UV light. The different species were identified by comparison of restriction patterns with type strains described previously (41).

Partial sequencing of the 16S-rDNA fragment was performed for strains with uncertain identity by using ABI PRISM® BigDye™ Terminator Cycle Sequencing (Applied Biosystems, Foster City, CA). The determined 16S-rDNA sequences were compared with the GeneBank database (http://www.ncbi.nlm.nih.gov/BLAST/).

Antimicrobial activity testing

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

Antimicrobial activity against target bacteria was tested after growth of lactobacilli under anaerobic environmental conditions by two methods, depending on the target bacteria used.

Antimicrobial activity against S. mutans *and* A. actinomycetemcomitans

The antimicrobial activity of lactobacilli (in total 115 strains: 48 from chronic periodontitis patients and 67 from healthy subjects) against the target bacteria *S. mutans* and *A. actinomycetemcomitans* was assessed by the deferred antagonism method (26). The media used as bottom agar (1.4%) were MRS agar (Merck, Darmstadt, Germany) for *S. mutans* and MRS agar without tri-ammonium-citrate and sodium-acetate (pH = 7.1) for *A. actinomycetemcomitans* (2). The media used as top agar (0.7%) were brain-heart infusion agar (BHIA) (Merck) for *S. mutans* and BHIA enriched with 0.5% yeast extract (Merck) and 0.0005% hemin (Sigma, Steinheim, Germany) for *A. actinomycetemcomitans*. 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 bacteria were precultivated in their appropriate media and suspensions of cells were adjusted to a predetermined optical density (OD 0.10–0.25 at 600 nm depending on target bacteria used) to yield confluent growth in the top agar. Thereafter, the melted (and cooled to 42°C) top agar was seeded with the precultivated target bacterial suspension and poured over the macrocolonies of lactobacilli. The plates were incubated anaerobically (BBL®*GasPakPlus*™) for *S. mutans* and under microaerophilic conditions (BBL®*CampyPakPlus*™, BBL Microbiology Systems) for *A. actinomycetemcomitans* 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 *Lactobacillus* strain to the margin of the inhibition zone.

Antimicrobial activity against P. gingivalis *and* P. intermedia

The antimicrobial activity of lactobacilli (in total 63 strains: 21 from chronic periodontitis patients and 42 from healthy subjects) against the target bacteria *P. gingivalis* and *P. intermedia* was assessed using a streak line procedure (2) on Wilkins-Chalgren blood agar plates (Oxoid). A single line of lactobacilli culture, grown in MRS broth for 48 h, was seeded in the middle of the agar plate. *Lactobacillus* strains were then cultivated for 48 h at 37°C in an anaerobic glove chamber (Sheldon Manufacturing, Inc. Shel LAB) with a gas mixture of CO_2/H_2/N_2: 5/5/90%. Target bacteria were cultured in Wilkins-Chalgren broth for 48 h at 37°C in anaerobic conditions and seeded in triplicate perpendicular to the streak line of lactobacilli. Following incubation of the plates for 72 h at 37°C in anaerobic conditions, the width of the zone of inhibition (mm) of the target bacteria extending from the culture line of lactobacilli was measured.

Statistical methods

Statistical analyses were performed using SIGMASTAT (Jandel Scientific, San Rafael, CA) and EXCEL (Microsoft Corp., Redmond, WA) programs. The following tests were employed: Fisher exact test, *t*-test and

Mann–Whitney rank sum test (comparison of different study and bacterial groups) and Pearson Product Moment Correlation (correlating the presence of various fermentation groups of lactobacilli to the subgingival presence of periodontal pathogens and to various clinical parameters). The differences were considered significant when $P < 0.05$.

Results

Clinical characteristics

The clinical data of chronic periodontitis patients and periodontally healthy individuals are shown in Table 1. The mean number of teeth was significantly lower in the chronic periodontitis patients. A marked increase in the amount of dental plaque and gingival inflammation, with higher mean periodontal probing depth, was observed in the chronic periodontitis patients as compared to the healthy group ($P < 0.001$).

Prevalence and distribution of oral lactobacilli

All healthy subjects ($n = 15$) and 18 of 20 chronic periodontitis patients harbored salivary lactobacilli as determined on Dentocult®LB dip-slides. The counts of salivary lactobacilli were similar in both groups (very high, high, and medium counts in 80% of healthy and 70% of chronic periodontitis patients, and missing and low counts in 20% vs. 30%, respectively). Of two *Lactobacillus* negative patients, one had only streptococci growing on a dip-slide and the second had no growth.

In subgingival samples lactobacilli were rare – two out of 15 healthy subjects, but none of the chronic periodontitis patients was colonized. The counts of subgingival lactobacilli in two *Lactobacillus* positive sites were 4.5 and 3.5 $\log_{10}$ CFU/ml.

The prevalence of different fermentation types of lactobacilli in healthy subjects was quite even (obligately homofermentative lactobacilli in 67% of subjects, facultatively heterofermentative in 67% and obligately heterofermentative in 73%), whereas a twofold decrease of obligately homofermentative and a predominance of facultatively heterofermentative type was seen in chronic periodontitis patients (Table 2).

Identification and composition of *Lactobacillus* species

Strain level

In total, 115 lactobacilli isolates were identified by molecular methods, and of those, 113 were identified by ARDRA as *L. acidophilus*, *Lactobacillus crispatus*, *L. gasseri*, *L. salivarius*, *L. casei*, *L. plantarum*, *L. rhamnosus*, and *L. fermentum*. Thirty strains out of 113 (26%) were later reassigned based on results from the 16S-rRNA gene sequencing (first 500 bases of the 16S-rRNA gene). The latter included all strains of *L. casei* to *L. paracasei* ssp. *paracasei* and 14 strains of *L. fermentum* to *L. oris*. Two strains which showed an unknown restriction pattern by ARDRA were identified following sequencing of the 16S-rRNA gene as *L. delbrueckii* and *L. fermentum*.

Of the seven subgingival *Lactobacillus* strains isolated from two periodontally healthy sites, three strains were *L. gasseri*, three were *L. oris* and one was *L. paracasei*.

Patient level

The distribution of different *Lactobacillus* species among chronic periodontitis and healthy patients is shown in Table 2, with *L. gasseri* and *L. fermentum* being the most prevalent (both 64%) in healthy persons. In comparison with healthy subjects, in chronic periodontitis patients obligately homofermentative lactobacilli, particularly *L. gasseri,* were less prevalent (64% vs. 8% for *L. gasseri,* $P < 0.01$). *L. plantarum*, the most prevalent strain in chronic periodontitis patients, was found in about one-third of healthy subjects. Healthy subjects were colonized by somewhat higher number of species than diseased ones (mean 3.2, range 1–6 species vs. mean 2.1, range 1–4 species), however, this difference was not statistically significant.

Table 1. Clinical parameters of chronic periodontitis patients and periodontally healthy individuals

	Periodontitis patients (n = 20) Mean ± SD	Healthy subjects (n = 15) Mean ± SD	*P*-value
No. of teeth, n	21.7 ± 4.3	25.5 ± 2.8	< 0.01
Plaque index (PI)	1.4 ± 0.4	0.3 ± 0.2	< 0.001
Gingival index (GI)	1.1 ± 0.3	0.2 ± 0.1	< 0.001
PPD, mm			
PPD_{ds}	5.7 ± 0.8	Nd	< 0.001
PPD_{all}	3.6 ± 1.0	1.5 ± 0.2	< 0.001

PPD, periodontal probing depth. Nd, not detected.

Table 2. Composition of oral lactoflora with respect to periodontal health: prevalence (%) of various fermentation groups and *Lactobacillus* species in chronic periodontitis patients and periodontally healthy subjects harboring lactobacilli

Lactobacilli	Colonized periodontitis patients		Colonized healthy subjects	
Fermentation type/species	n	%	n	%
Identified by biochemical methods	18		15	
Obligately homofermentative	6	33	10	67
Facultatively heterofermentative	16	89	10	67
Obligately heterofermentative	9	50	11	73
Identified additionally by molecular methods	12		11	
Obligately homofermentative	2*	17	8*	73
L. acidophilus	0	0	1	9
L. crispatus	0	0	1	9
L. delbrueckii	0	0	1	9
L. gasseri	1**	8	7**	64
L. salivarius	2	17	3	27
Facultatively heterofermentative	11	92	7	64
L. paracasei	5	42	4	36
L. plantarum	7	58	4	36
L. rhamnosus	3	25	2	18
Obligately heterofermentative	5	42	9	82
L. fermentum	5	42	7	64
L. oris	2	17	5	45

Difference in prevalence: *$P < 0.05$, **$P < 0.01$.

Antimicrobial activity

Fermentation type and species level

The majority of *Lactobacillus* strains suppressed growth of *A. actinomycetemcomitans* (88% of tested strains), *P. gingivalis* (82%), *P. intermedia* (65%), and *S. mutans* (69%). The antimicrobial activity was mainly species specific; however, some strain specific differences were observed, particularly among strains of *L. fermentum*, *L. oris*, and *L. gasseri*. The strongest antimicrobial activity was associated with facultatively heterofermentative lactobacilli and homofermentative *L. salivarius* (Table 3). In addition, homofermentative *L. crispatus* and *L. gasseri* had quite high activity against anaerobic *P. gingivalis* and *P. intermedia*, whereas *L. fermentum* (obligately heterofermentative) inhibited neither of these anaerobic bacteria. Lactobacilli from all fermentation types had stronger antimicrobial activity against *P. gingivalis* than against *P. intermedia* (inhibition zone 18.6 ± 5.4 vs. 7.4 ± 3.9 mm, $P < 0.001$, in obligately homofermentative group; 22.0 ± 3.3 vs. 10.5 ± 1.7 mm, $P < 0.001$, in facultatively heterofermentative group; 6.4 ± 7.4 vs. 0 mm, $P < 0.05$, in 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 (data not shown).

Patient level

The antimicrobial activity of heterofermentative lactobacilli isolated from healthy subjects and chronic periodontitis patients was similar (Table 3). Obligately homofermentative *L. gasseri* isolated from healthy subjects showed a higher antimicrobial activity against *A. actinomycetemcomitans* ($P < 0.05$) and a lower activity against *S. mutans* ($P < 0.05$) than strains isolated from chronic periodontitis patients. As a whole, the strains from periodontally healthy patients showed a lower antimicrobial activity against *S. mutans* than the strains from chronic periodontitis patients (mean 1.2 ± 1.4 mm vs. 2.3 ± 1.4 mm, $P < 0.001$).

Relations between lactobacilli, subgingival periodontal pathogens and clinical parameters

The periodontal pathogens *A. actinomycetemcomitans* and *P. gingivalis* were only found in chronic periodontitis individuals (40% and 30% of the patients, respectively). *P. intermedia/nigrescens* was isolated more frequently from diseased (80%) than from healthy individuals (47%). The mean counts of *A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia/nigrescens* in chronic periodontitis patients were 5.0 ± 5.4, 6.4 ± 7.0, and 6.6 ± 6.9 $\log_{10}$ CFU/ml, respectively. The mean count of *P. intermedia/nigrescens* in healthy subjects was 4.1 ± 4.5 $\log_{10}$ CFU/ml.

The presence of the obligately homofermentative group of lactobacilli and especially *L. gasseri* was inversely associated with chronic periodontitis-related clinical parameters (Table 4). Their inverse associations were also seen with subgingival colonization of periodontal pathogens *A. actinomycetemcomitans*, *P. intermedia/nigrescens*, and *P. gingivalis* as well as with coinfection with at least two of the aforenamed pathogens. No significant correlations were found for the other fermentation groups.

Discussion

In the present study we show for the first time that *Lactobacillus* species composition in the oral cavity differs in respect of periodontal health. Most oral lactobacilli were able to inhibit the growth of both periodontitis- and caries-related pathogens *in vitro*.

The *Lactobacillus* strains in this study originated both from subgingival sites and from saliva, though saliva was significantly more often inhabited. No differences in salivary lactobacilli counts were observed between chronic periodontitis and healthy patients. The methods used in this study seem to be reliable for isolation of oral lactobacilli, though the Dentocult®LB dip-slide method gives only a rough estimation of *Lactobacillus* count.

Table 3. Antimicrobial activity of oral lactobacilli originating from chronic periodontitis patients and periodontally healthy subjects, expressed as inhibition zone values (mm)

Study group	Lactobacilli fermentation type/species	Inhibition of target bacteria: zone values (mm), mean ± SD			
		S. mutans	*A. actinomycetemcomitans*	*P. gingivalis*	*P. intermedia*
Healthy subjects	Obligately homofermentative, n =	22	22	18	18
	L. acidophilus	0	2.5 ± 0	12.0 ± 0	0
	L. crispatus	0	1.0 ± 0.7	26.7 ± 0	9.5 ± 0
	L. delbrueckii	1.5 ± 0	4.3 ± 0	11.3 ± 0	0
	L. gasseri	0.1 ± 0.2*	1.6 ± 0.8†	17.1 ± 4.2	6.1 ± 2.7
	L. salivarius	2.7 ± 2.1	4.2 ± 0.8	24.4 ± 4.2	11.2 ± 1.9
	Facultatively heterofermentative, n =	18	18	10	10
	L. paracasei	2.2 ± 1.4	4.0 ± 1.3	23.7 ± 1.6	11.4 ± 1.7
	L. plantarum	3.0 ± 0.8	6.1 ± 0.7	21.7 ± 5.4	9.6 ± 1.3
	L. rhamnosus	2.0 ± 0.5	4.4 ± 1.0	22.1 ± 1.2	11.2 ± 0.3
	Obligately heterofermentative, n =	27	27	14	14
	L. fermentum	1.3 ± 0.8	3.1 ± 2.4	0	0
	L. oris	0.1 ± 0.3	2.0 ± 1.7	11.0 ± 6.5	0
Periodontitis patients	Obligately homofermentative, n =	5	5	5	5
	L. gasseri	1.5 ± 0.7*	0.5 ± 0.5†	16.7 ± 1.9	8.5 ± 0.1
	L. salivarius	4.4 ± 1.2	4.0 ± 1.1	25.0 ± 5.6	13.5 ± 1.8
	Facultatively heterofermentative, n =	27	27	10	10
	L. paracasei	2.0 ± 1.4	3.9 ± 0.9	19.9 ± 3.3	9.2 ± 1.9
	L. plantarum	3.7 ± 1.1	5.5 ± 1.8	21.1 ± 2.2	9.7 ± 0.9
	L. rhamnosus	2.3 ± 0.5	3.2 ± 0.8	24.2 ± 4.4	12.4 ± 1.9
	Obligately heterofermentative, n =	16	16	6	6
	L. fermentum	1.6 ± 0.6	2.0 ± 2.4	0	0
	L. oris	0	2.1 ± 0.6	14.0 ± 4.2	0

Difference in the antimicrobial activity: *, † $P < 0.05$.

Table 4. Correlations between the presence of certain fermentation types of lactobacilli, and clinical parameters and presence of subgingival periodontal pathogens (Pearson correlation coefficient R_p)

Periodontal status	Fermentation type[a]/ species[b] of lactobacilli	Correlation coefficient R_p	*P*-value
Plaque index	vs. obligately homofermentative	− 0.38†	0.032
	vs. *L. gasseri*	− 0.44†	0.045
	vs. facultatively heterofermentative	0.09	0.633
	vs. obligately heterofermentative	− 0.26	0.155
Gingival index	vs. obligately homofermentative	− 0.40†	0.023
	vs. *L. gasseri*	− 0.49†	0.025
	vs. facultatively heterofermentative	0.11	0.536
	vs. obligately heterofermentative	− 0.30	0.095
Periodontal probing depth	vs. obligately homofermentative	− 0.40†	0.027
	vs. *L. gasseri*	− 0.46†	0.039
	vs. facultatively heterofermentative	0.17	0.362
	vs. obligately heterofermentative	− 0.13	0.497
Periodontal pathogens			
A. actinomycetemcomitans	vs. obligately homofermentative	− 0.35†	0.040
	vs. *L. gasseri*	− 0.43†	0.038
	vs. facultatively heterofermentative	0.18	0.320
	vs. obligately heterofermentative	− 0.10	0.576
P. intermedia/nigrescens	vs. obligately homofermentative	− 0.40†	0.019
	vs. *L. gasseri*	− 0.40	0.060
	vs. facultatively heterofermentative	0.01	0.944
	vs. obligately heterofermentative	− 0.19	0.268
P. gingivalis	vs. obligately homofermentative	− 0.26	0.144
	vs. *L. gasseri*	− 0.38	0.069
	vs. facultatively heterofermentative	0.10	0.563
	vs. obligately heterofermentative	− 0.08	0.641
At least two pathogens	vs. obligately homofermentative	− 0.35†	0.040
	vs. *L. gasseri*	− 0.48†	0.019
	vs. facultatively heterofermentative	0.18	0.320
	vs. obligately heterofermentative	− 0.10	0.576

[a]Data given for all subjects.
[b]Data given for 23 subjects.
†Statistically significant correlation.

The composition of lactoflora in saliva was rich, with most patients colonized by several different species. Altogether, we were able to identify 10 different species: heterofermentative *L. fermentum*, *L. oris*, *L. plantarum*, *L. paracasei*, and *L. rhamnosus*, and homofermentative *L. gasseri*, *L. salivarius*, *L. acidophilus*, *L. crispatus*, and *L. delbrueckii*. The species most frequently present in saliva of healthy individuals were *L. gasseri* and *L. fermentum*. A similar diversity in oral lactoflora was observed by Ahrne et al. (1); however, the species they most frequently recovered from the tongue mucosa of healthy humans were *L. plantarum* and *L. rhamnosus*. Colloca et al. (8) found *L. fermentum*, *L. plantarum*, *L. salivarius* and *L. rhamnosus* to be the predominant species in healthy human mouth (teeth, tongue, saliva, and gums).

The subgingival sites investigated here (total 70) were rarely colonized by lactobacilli (two sites, both in healthy persons). The finding that only two persons in periodontally healthy group and none in periodontitis group harbored lactobacilli in their subgingival sites, although there was evident colonization in saliva, indicates that the subgingival region is not a common habitat for lactobacilli. The isolated strains belonged to three species: *L. gasseri*, *L. oris*, and *L. paracasei*. Very scarce knowledge about subgingival lactobacilli exists, and *Lactobacillus uli* and *Lactobacillus rimae*, isolated from human subgingival sites (25), have been reclassified as *Olsenella uli* and *Atopobium rimae* (7, 9).

A variety of anatomic and physiologic factors as well as beneficial and antagonistic interactions among microbes influence the homeostasis of oral microbiota (20). We found that most of the oral *Lactobacillus* strains isolated from healthy and diseased patients showed antimicrobial activity against putative oral pathogens. Facultatively heterofermentative lactobacilli (*L. plantarum, L. paracasei*, *L. rhamnosus*) and homofermentative *L. salivarius* expressed the strongest antimicrobial activity. *L. gasseri* and *L. fermentum*, being the most prevalent species in saliva of healthy persons, differed in the ability to suppress the growth of different target bacteria. *L. fermentum* was more active against microaerophiles and *L. gasseri* against anaerobes.

Good antimicrobial activity of oral *L. paracasei* and *L. rhamnosus* against periodontal pathogen *P. gingivalis* has previously been shown by Sookkhee et al. (36). However, Testa et al. (39) found no antagonistic interactions between oral lactobacilli (*L. casei*, *L. rhamnosus*, *L. plantarum*, and *L. salivarius*) and the anaerobes *P. intermedia* and *Fusobacterium nucleatum*, contradicting our results. These conflicting results could be strain-related from both sides (lactobacilli and target bacteria) as few strains were tested in that study, and we have observed strain-specific differences in our investigation. Differences in methodology (different growth conditions, as well as using cells vs. supernatants) might also influence the outcome of the antagonism tests. We have seen in our lab that the antimicrobial properties of intestinal lactobacilli depend on their growth conditions (microaerobic vs. anaerobic) and are principally related to the production of organic acids such as lactic and acetic acid upon fermentation of glucose with concomitant decrease in pH (2). While testing antimicrobial activity in this study, we observed that the antimicrobial activity of strains of *L. salivarius*, *L. plantarum*, *L. rhamnosus*, and *L. fermentum* against *S. mutans* and *A. actinomycetemcomitans* was lost when lactobacilli were cultured on media with low glucose content (2% vs. 0.2% of glucose) (data not shown). Similarly, Doran et al. (10) showed that oral streptococci inhibited anaerobic bacteria (*P. intermedia*, *F. nucleatum*, *Veillonella dispar*, *P. gingivalis*, and *Peptostreptococcus micros*) on media with added glucose (1%) more often than on media without glucose, indicating that the availability of substrate for fermentation seems to be one of the essential factors for the expression of antimicrobial activity. In addition, the activity could also be related to the production of other antimicrobial factors, like hydrogen peroxide and bacteriocins, by lactobacilli (22, 43). Also, the target microorganism should be sensitive to particular antimicrobial factors. In the present study we observed that *P. gingivalis* was more sensitive than *P. intermedia*, which may be caused partly by the inability of *P. gingivalis* to grow at a pH below 6.5, whereas *P. intermedia* can grow at a pH as low as 5.0 (37).

Investigating the pathogenesis of chronic periodontitis, it is generally accepted that the disease is caused by bacteria in dental plaque, with evidence that specific periodontal pathogens are responsible for the development of the disease. However,

some individuals harbor these periodontal pathogens, but do not appear to show clinical evidence of disease (11, 40). These data suggest that the virulence of particular strains might be low or their numbers are kept below the detrimental threshold for the host by the other members of the resident microflora.

In the present study, using both phenotypic and genotypic identification methods, we found a significantly higher prevalence of obligately homofermentative lactobacilli, especially *L. gasseri*, among healthy persons. The presence of *L. gasseri* was associated with less dental plaque and less gingival inflammation. In addition, *L. gasseri* from healthy persons had a higher antimicrobial activity against the periodontal pathogen *A. actinomycetemcomitans* than did the strains from patients with chronic periodontitis. As a whole, the homofermentative lactobacilli were associated with the absence of subgingival putative periodontal pathogens, confirming our *in vitro* results in which homofermentatives expressed significant antimicrobial activity against periodontal pathogens.

Health-promoting activity of homofermentative lactobacilli has been noted in previous studies. Sakamoto et al. (30) reported the effectiveness of *L. gasseri* OLL 2761 in both suppressing *Helicobacter pylori* and reducing the gastric mucosal inflammation. Characterizing intestinal lactobacilli, Annuk et al. (2) found that the strains in obligately homofermentative group, in addition to antimicrobial activity, had the highest values for production of hydrogen peroxide and for total antioxidative activity, which was suggested to be useful for the host in reducing oxidative damage of human cells. The high antioxidative ability of homofermentative lactobacilli has also been shown by Lin & Yen (16). Recently, Kitazawa et al. (14) revealed a novel immunostimulating aspect of homofermentative lactobacilli as *L. acidophilus* and *L. gasseri* induced significant chemotaxis of macrophages. Thus, the presence of lactobacilli with antimicrobial activity as well as with good antioxidative and immunostimultative properties could be one of the factors regulating the presence and the number of periodontal pathogens.

Most periodontal pathogens colonize several niches within the mouth (e.g. the sub- and supragingival plaque, the saliva, the tongue, and the other mucosal surfaces) and the oral soft tissues are considered an important reservoir of periodontal pathogens for colonization or reinfection of subgingival sites (3, 38, 42). We have shown that prevalence of lactobacilli varies in different biotopes within the mouth, being high in saliva and low in gingival crevices. Mager et al. (18) showed that the microbiota of saliva resembled that of the dorsum and lateral surfaces of the tongue and lactobacilli could thus mainly be expected to control the growth of putative periodontal pathogens colonizing the tongue, consequently diminishing the colonization of subgingival sites by these periodontal pathogens.

In general, lactobacilli in the oral cavity are considered to be cariogenic bacteria. In the present study we observed that lactobacilli were able to inhibit *S. mutans in vitro* and Näse et al. (28) have shown that long-term consumption of milk containing *L. rhamnosus* GG reduced the risk of dental caries in children, suggesting that in spite of the cariogenic potential of lactobacilli, some of them may be associated with oral health and need further investigation. Interestingly, we also found that as a whole the *Lactobacillus* strains isolated from chronic periodontitis patients were more active against *S. mutans* and our finding may at least partly explain an inverse association between dental caries and periodontal diseases observed by Sioson et al. (32). It may also be an indication of complex interactions not only between lactic acid bacteria and putative periodontal pathogens but also between different species of microorganisms within the lactic acid producing group of bacteria.

In summary, the composition of oral lactoflora between healthy subjects and patients with chronic periodontitis differs, with a higher prevalence of homofermentative lactobacilli, particularly *L. gasseri*, in the former group. Both homo- and heterofermentative oral lactobacilli suppress the growth of periodontal pathogens, yet the antimicrobial properties are strain, species and origin specific. These findings indicate that lactobacilli may play a crucial role in the maintenance of the microecologic balance in the oral cavity.

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