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ORIGINAL ARTICLE

Oral microbial ecology in chronic periodontitis and periodontal health

PIRET KÕLL-KLAIS[1,2], REET MÄNDAR[2], EDVITAR LEIBUR[1] & MARIKA MIKELSAAR[2]

[1]*Department of Stomatology and* [2]*Department of Microbiology, Faculty of Medicine, University of Tartu, Tartu, Estonia*

Abstract
Our aim was to find out differences between chronic periodontitis (CP) and periodontally healthy subjects with respect to oral lactic acid bacteria (LAB) as well as subgingival microbial relations. Clinical data, salivary levels of lactobacilli and mutans streptococci, and subgingival microbial samples were obtained from 26 CP and 15 periodontally healthy subjects. Antimicrobial activity of LAB against periodontal pathogens was assessed. We found *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Prevotella intermedia/nigrescens* in 54%, 23% and 73% of CP patients, respectively; the latter was also found in 47% of healthy subjects. The mean proportion of streptococci (27.0 vs 15.2%), particularly *S. mutans* group (5.7 vs 0.8%) and *S. mitis* group (18.1 vs 5.0%), as well as aerobic coryneforms (24.7 vs 11.9%) was higher in healthy persons ($p < 0.05$). An inverse relationship of subgingival streptococci and aerobic coryneforms with periodontal pathogens and deteriorated clinical parameters were seen. Salivary counts of mutans streptococci were higher in healthy persons. Inhibition of periodontal pathogens by LAB was observed. In conclusion, the proportions of oral LAB were significantly lower in CP than in healthy subjects, whilst being important antagonists against periodontal pathogens. These oral commensals may play an important role in the suppression of periodontal pathogens and maintenance of microecological balance in the oral cavity.

Key words: *Chronic periodontitis, periodontal health, lactic acid bacteria, subgingival microflora, antimicrobial activity, microbial ecology*

Introduction

Periodontitis is a chronic inflammation of the periodontium that results in periodontal tissue destruction and alveolar bone loss. Tissue destruction occurs as a consequence of the host's attempt to eliminate bacteria from the gingival sulcus by evoking an immunoinflammatory response. A report from the World Workshop in Periodontics in 1996 emphasized the polymicrobial nature of chronic periodontitis, but most frequently it has been related to the black-pigmented gram-negative anaerobic rods *Porphyromonas gingivalis* and *Prevotella intermedia*, accompanied by the gram-negative microaerobic coccobacillus *Actinobacillus actinomycetemcomitans* (1).

Although there have been several studies on putative periodontal pathogens, less is known about the microorganisms that might participate in the maintenance of periodontal health. There are numerous microbiotopes in the mouth that are colonized by a variety of microorganisms playing an active role in the maintenance of local oral health as well as influencing each other through various synergistic and antagonistic interactions. Some oral commensals such as lactic acid bacteria (LAB), including among others the genera *Streptococcus* and *Lactobacillus*, may prohibit periodontal pathogens. Hillman et al. (2) showed that the presence of the putative periodontal pathogens *P. gingivalis*, *P. intermedia* and *A. actinomycetemcomitans* in subgingival plaque of periodontitis patients was correlated with the absence of certain streptococci (e.g. *Streptococcus sanguis*). Investigating mutans streptococci in the subgingival plaque of periodontitis patients at different stages of periodontal therapy, van der Reijden et al. (3) found their proportions to increase following therapy, as well as a negative correlation between mutans streptococci and *P. gingivalis*. However, they did not relate the microbiological data to the clinical measurements.

Recent studies have also described the ability of streptococci and lactobacilli to exert antibacterial

Correspondence: Reet Mändar, MD, PhD, Department of Microbiology, University of Tartu, Ravila 19, 50411 Tartu, Estonia. Fax: +372 7 374172. E-mail: Reet.Mandar@ut.ee

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activity against various pathogens, including *P. gingivalis* and *P. intermedia* (4,5). Thus, we may suppose that colonization of the oral cavity by LAB may aid in suppression of periodontal pathogens and hence reduce the incidence of chronic periodontitis (CP). However, the existing data do not provide a clear picture as to whether colonization with LAB differs with respect to periodontal health and how the microecological relations between LAB and other microorganisms are changed in CP patients and periodontally healthy subjects.

The objective of the present study was to find out differences in the colonization of LAB in saliva and periodontal sites in CP and periodontally healthy subjects. In addition, we aimed to investigate subgingival microbial relations with respect to different clinical parameters.

Materials and methods

Study group

The study group included 26 patients with CP (16 female, 10 male, mean age 47.2 ± 11.3 years) and 15 periodontally healthy subjects (7 female, 8 male, mean age 37.5 ± 10.4 years); both groups had no history of systemic disease or antibiotic therapy within the 6 months prior to sampling. The CP patients were diagnosed as having CP based on gingival inflammation, periodontal breakdown with pocket depth ≥ 5 mm and radiographic evidence of bone loss (6). They were consecutively drawn from the waiting list of patients who were referred to the Department of Oral Surgery at the Clinic of Stomatology of Tartu University for diagnosis and treatment of periodontitis during the years 1999–2001. Healthy individuals were defined as having no radiographic or clinical evidence of attachment loss. The smoking history of the subjects was checked according to a questionnaire. In the periodontitis group 9 patients were non-smokers, 2 were current smokers and 15 were former smokers who had stopped smoking on average 16.1 (± 7.5) years ago. In the healthy group 13 subjects were non-smokers and 2 were former smokers who had stopped smoking on average 3.0 (± 1.4) years ago.

All patients were screened for their suitability and selection of sampling sites a day prior to collection of microbiological samples.

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 the registration of dental plaque, gingival inflammation and presence of suppuration on probing at four sites (distal, mesial, lingual and buccal sites), and periodontal probing depth and attachment level at six sites (distal, mid and mesial aspects for both buccal and lingual sites) of each tooth, excluding third molars.

The plaque status of an individual was given as the plaque index (PI) according to Silness and Löe (7) and as the frequency of plaque-positive surfaces expressed as a percentage of the total number of surfaces. Gingival inflammation was given as a modified gingival index (GI: 0, healthy gingiva with no bleeding on probing; 1, 'pinprick' bleeding on probing; 2, immediate and overt bleeding on probing; 3, spontaneous bleeding) (8,9) and as the frequency of bleeding sites on probing, expressed as a percentage of all sites. Suppuration on probing was recorded as present or absent and the results were given as the frequency of pus-positive sites expressed as a percentage of all sites. Periodontal probing depth (PPD) was measured to the nearest millimetre from the gingival margin to the bottom of the gingival sulcus/pocket and periodontal attachment level (PAL) from the cemento-enamel junction to the bottom of the periodontal pocket with a Williams periodontal probe. The mean across all sites formed the PPD and attachment level of the patient, designated as PPD_{all} and PAL_{all}, respectively. Sites with a probing depth of ≥ 5 mm were defined as diseased sites (DS). The frequency of DS was expressed as percentage of the total number of sites. The mean PPD and attachment level of diseased sites was designated as PPD_{ds}, and PAL_{ds}, respectively. In addition, dental caries was registered in accordance with the WHO criteria (10). The same examiner performed all clinical measurements.

Salivary levels of lactobacilli and mutans streptococci

Salivary lactobacilli were investigated in 20 CP patients and 15 healthy subjects by the Dentocult®LB dip-slide method (Orion Diagnostica, Espoo, Finland), and mutans streptococci in 14 CP and 14 healthy patients by the Dentocult®SM strip method (Orion Diagnostica) in paraffin-stimulated saliva (11). After incubation for 3 days at 37°C the number of lactobacilli and mutans streptococci per ml of saliva was estimated by comparing the slides with a density chart provided by the manufacturer. Dentocult®LB test results were expressed as missing, low ($\leq 10^3$ CFU/ml), medium

(10^4 CFU/ml), high (10^5 CFU/ml) and very high ($>10^6$ CFU/ml) counts of lactobacilli. Dentocult®SM test results were given as missing, low ($<10^5$ CFU/ml), medium (10^5–10^6 CFU/ml) and high ($>10^6$ CFU/ml) counts of mutans streptococci.

Subgingival samples

In periodontitis patients the two deepest periodontal pockets with inflammation, one in the upper and one in the lower jaw, were selected for sampling (e.g. the first sample was taken from the deepest pocket with inflammation in the first quadrant and the second sample was taken from the deepest pocket with inflammation in the third quadrant). In total, 54% of sampled sites in periodontitis patients were located in the molar/premolar region and 46% in the canine/incisor region. In healthy subjects two clinically healthy gingival sulci, one in the molar/premolar region and the other in the canine/incisor region in opposite jaws, were selected for sampling. The samples were obtained by means of a gingival crevice lavage method as described by Boström et al. (12). 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 the 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. The samples were processed within 2 h.

Microbiological analysis

After vortex mixing for 30 s, samples were 10-fold serially diluted in pre-reduced peptone water (Oxoid, Unipath, Basingstoke, UK) and 100 µl of appropriate dilutions were plated onto agar media. The following media were used: Brucella agar (Oxoid), supplemented with 5% defibrinated horse blood and menadion (2.5 µg/ml) (13) for enumeration of anaerobic and facultative anaerobic bacteria; tryptone soya agar (Oxoid), supplemented with yeast extract (0.1%), horse serum (10%), bacitracin (75 µg/ml) and vancomycin (5 µg/ml) (TSBV) for identification of *A. actinomycetemcomitan*s (14); and MRS agar (Oxoid) for lactobacilli and streptococci. Brucella plates were incubated in an anaerobic glove box (Sheldon Manufacturing, Inc., Shel LAB, Cornelius, OR, USA) with a gas mixture of 5% H_2, 5% CO_2, 90% N_2 for 5–6 days. TSBV and MRS plates were incubated in a microaerophilic atmosphere (10% CO_2) for 72 h.

Colonies with different morphology were Gram stained and examined microscopically. The microorganisms were identified mostly to the genus level by standard methods (15). Streptococci and enterococci were identified by the absence of catalase production and differentiated by the fermentation of esculin in the presence of bile. Viridans streptococci were distinguished from *Streptococcus pneumoniae* by the optochin susceptibility test and from *Streptococcus bovis* by the bile esculin test, and were further grouped by hydrolysis of arginine and production of acetoin (Voges-Proskauer test). Gram-positive rod-shaped non-spore-forming cells expressing a negative catalase reaction and multiplying in microaerobic conditions on MRS medium were considered as *Lactobacillus* species and were further identified by molecular methods (16). *A. actinomycetemcomitans* was differentiated by colony morphology (star-like inner structure) on selective medium, cell morphology and catalase production, inability to ferment sucrose and absence of beta-glucuronidase activity using medium containing MUG supplement (Oxoid).

The anaerobes were identified by their colony and cellular morphology and Gram stain reaction and some rapid tests and diagnostic disks (catalase, oxidase, spot indole, fluorescence, oxgall, brilliant green, bile esculin, colistin, vancomycin, kanamycin). Gram-positive anaerobic non-spore-forming rods with negative catalase and indole reaction were identified as *Eubacterium* spp. and spore-forming bacteria as *Clostridium* spp. Gram-positive irregularly shaped non-spore-forming anaerobic or facultatively anaerobic rods were classified respectively as 'anaerobic coryneforms' and 'aerobic coryneforms' and included species of *Actinomyces*, *Corynebacterium*, *Propionibacterium* and *Bifidobacterium*. Gram-negative anaerobic rods that formed black-pigmented colonies, were vancomycin-resistant, colistin-sensitive, indole-positive and showed brick-red fluorescence under UV light, were presumptively identified as *P. intermedia/nigrescens,* and black-pigmented colonies with vancomycin sensitivity, colistin resistance, indole production and no fluorescence as *P. gingivalis*. All anaerobic microorganisms were tested for absence of growth under aerobic and microaerophilic conditions on freshly prepared chocolate agar and blood agar plates.

In vitro antimicrobial activity testing

Antimicrobial activity of *Streptococcus mutans* NG8 (wild type) against target bacteria *A. actinomycetemcomitans* 31-1-1A (wild type), *A. actinomycetemcomitans* 31-2-1A (wild type), *P. gingivalis* ATCC 49417, *P. gingivalis* W83 and *P. intermedia* ATCC 25611 was assessed using a streak line procedure (17) on Wilkins-Chalgren blood agar plates (Oxoid). The same method was used to test antimicrobial activity of subgingival lactobacilli (three strains of *L. gasseri*, two of *L. oris* and one of *L. paracasei*) against *P. gingivalis* ATCC 49417 and *P. intermedia* ATCC 25611. A single line of *S. mutans* or lactobacilli culture (grown in MRS broth for 48 h at 37°C in a 10% CO_2 environment) was seeded in the middle of the agar plate, and cultivated for 48 h at 37°C in an anaerobic glove box (Sheldon Manufacturing, Inc.) with a gas mixture of $CO_2/H_2/N_2$ at 5%/5%/90%, respectively, for testing against anaerobic bacteria (*P. gingivalis*, *P. intermedia*) and in 10% CO_2 for testing against *A. actinomycetemcomitans*. Target bacteria *P. gingivalis* and *P. intermedia* were cultured in Wilkins-Chalgren broth for 48 h at 37°C in anaerobic conditions and *A. actinomycetemcomitans* in tryptone soya broth, supplemented with yeast extract, in microaerophilic conditions for 48 h at 37°C. Thereafter, a 10 µl aliquot of target bacterial culture (2.0 on the McFarland turbidity scale) was streaked in duplicate perpendicular to the streak line of *S. mutans* or lactobacilli. Following incubation of the plates for 72 h at 37°C in anaerobic or microaerophilic conditions depending on the target bacteria used, the width of the zone of inhibition (mm) of the target bacteria extending from the culture line of *S. mutans* or lactobacilli was measured.

The streak line procedure (17) was also used to assess the antimicrobial activity of the above-described periodontal pathogens against *S. mutans* NG8 and lactobacilli. The periodontal pathogens were precultivated in their appropriate media and incubation conditions for 48 h at 37°C, streaked in the middle of the Wilkins-Chalgren blood agar plate and incubated for 48 h at 37°C in anaerobic (*P. gingivalis*, *P. intermedia*) or microaerophilic (*A. actinomycetemcomitans*) conditions. The target bacterial strains *S. mutans* and lactobacilli were cultured on MRS agar for 48 h and 24 h, respectively, in microaerophilic conditions, suspended in saline solution (1.0 on the McFarland turbidity scale) and streaked (10 µl) in duplicate perpendicular to the streak line of test bacteria. Following incubation of the plates for 72 h at 37°C in anaerobic or microaerophilic conditions depending on the periodontal pathogens used, the width of the zone of inhibition (mm) of the target bacteria extending from the culture line of periodontal pathogens was measured.

Statistical methods

The total count ($\log_{10}$ CFU/ml – colony forming units per millilitre of crevicular fluid) of microorganisms and the counts of various genera and species were calculated for each patient. The detection level of the various microorganisms was 3 $\log_{10}$ CFU/ml. A proportion (%) of the particular microbe in the total count was estimated. Statistical analyses were performed using SigmaStat (Jandel Scientific) and Excel (Microsoft Corp.). The following tests were employed: Fisher exact test, *t* test and Mann-Whitney rank sum test (comparison of different study and bacterial groups), Pearson product moment correlation (measuring correlations between the proportion (%) of various subgingival bacteria and the clinical indices) and Spearman rank order correlation (measuring correlations between the proportions of various microbes).

Results

Clinical characteristics

The clinical parameters of the subjects are shown in Table I. The mean number of teeth present was significantly lower in the periodontitis group. We could observe much higher occurrence of dental plaque, a significant increase in all measured inflammatory parameters and much higher mean PPD in the CP patients as compared with the healthy group. Approximately a quarter of all measured periodontal sites in CP patients were equal to or deeper than 5 mm. With respect to the experience of dental caries, no statistically significant differences were observed between the two groups.

Salivary levels of lactobacilli and mutans streptococci

All healthy subjects and 18 of 20 CP patients harboured salivary lactobacilli. Their counts were similar – very high, high and medium count in 80% of healthy and 70% of CP patients, and missing and low count in 20% vs 30%, respectively.

Mutans streptococci were isolated from all subjects in both groups; however, a trend for an inverse relationship was observed: the counts were low in 64% of CP patients and 36% of healthy subjects, while medium and high counts were seen in 36% of CP and 64% of healthy subjects.

Table I. Clinical parameters of chronic periodontitis (CP) patients and periodontally healthy individuals.

	CP patients ($n = 26$)	Healthy subjects ($n = 15$)	
Parameter	Mean ± SD	Mean ± SD	p value
Number of teeth present, *n*	22.0 ± 4.3	25.5 ± 2.8	0.009
Dental caries			
DMFT	15.3 ± 4.8	14.8 ± 5.6	0.797
DFT	9.2 ± 4.6	12.3 ± 5.9	0.094
Plaque			
Sites with plaque (%)	69.9 ± 20.3	22.0 ± 14.9	<0.001
Plaque index (PI)	1.5 ± 0.4	0.3 ± 0.2	<0.001
Inflammation			
Sites with gingival bleeding (%)	55.0 ± 21.6	5.7 ± 3.9	<0.001
Gingival index (GI)	1.1 ± 0.3	0.2 ± 0.1	<0.001
Sites with suppuration (%)	1.3 ± 3.0	ND	<0.001
Diseased sites (%)	26.1 ± 17.2	ND	<0.001
Periodontal probing depth (mm)			
PPD_{ds}	5.8 ± 0.7	ND	<0.001
PPD_{all}	3.7 ± 1.0	1.5 ± 0.2	<0.001
Periodontal attachment level (mm)*		...	...
PAL_{ds}	6.4 ± 1.4	...	...
PAL_{all}	4.7 ± 1.6	...	...

DMFT, decayed, missing and filled teeth; DFT, decayed and filled teeth; PPD_{ds}, periodontal probing depth of diseased sites; PPD_{all}, periodontal probing depth of all sites; PAL_{ds}, periodontal attachment level of diseased sites; PAL_{all}, periodontal attachment level of all sites; ND, not detected.
*Healthy individuals had no evidence of attachment loss.

Subgingival microflora

Mixed aerobic and anaerobic subgingival microflora was seen in all studied subjects (Table II). The total bacterial count in diseased patients was much higher than in healthy individuals. The typical periodontal pathogens *A. actinomycetemcomitans* and *P. gingivalis* were only found in individuals with CP. *P. intermedia/nigrescens* was isolated from both diseased and healthy individuals with higher prevalence and count in the former group. The mean number of periodontal pathogens was significantly higher in CP patients than in healthy subjects (1.50 ± 0.95 vs 0.47 ± 0.52, $p = 0.001$).

The counts of different gram-negative anaerobic rods, *Streptococcus anginosus* group, peptostreptococci and anaerobic coryneforms were significantly higher in diseased than in healthy individuals. The former were also somewhat less often colonized by a gram-positive rod, *Eubacterium* spp., as compared with periodontally healthy subjects.

A strong inverse relationship in the proportion of aerobic and anaerobic as well as gram-positive and gram-negative microorganisms was found in periodontally diseased sites of CP patients and in healthy sites of healthy subjects (Figure 1). The mean proportion of anaerobic bacteria, particularly gram-negative anaerobic rods (29.9 ± 22.4% vs 12.7 ± 11.4%, $p < 0.001$) and gram-positive anaerobic coryneforms (8.7 ± 10.9% vs 2.5 ± 3.7%, $p < 0.001$) was significantly higher in diseased sites, while the proportion of facultative anaerobic gram-positive bacteria – particularly streptococci (27.0 ± 23.4% vs 15.2 ± 19.2%, $p = 0.012$) and aerobic coryneforms (24.7 ± 27.5% vs 11.9 ± 14.7%, $p = 0.028$) – was significantly higher in healthy sites. Within the group of streptococci the mean proportion of *S. mutans* group (5.7 ± 9.7% vs 0.8 ± 1.8%, $p = 0.015$) and *S. mitis* group (18.1 ± 18.9% vs 5.0 ± 5.8%, $p = 0.004$) was significantly higher in healthy sites.

Positive correlations between the proportions of subgingival periodontal pathogens with each other and negative correlations with streptococci and aerobic coryneforms are shown in Table III. *A. actinomycetemcomitans* and *P. gingivalis* were in stronger negative association with streptococci, and *P. intermedia/nigrescens* with coryneforms.

Similar tendencies were observed when these microorganisms were correlated with CP-related clinical parameters. Plaque index (PI), gingival index (GI) and periodontal probing depth (PPD) were positively associated with the proportion of subgingival gram-negative anaerobic rods, and negatively with the proportion of subgingival streptococci and aerobic coryneforms (Table III).

Table II. Composition of the subgingival microflora in chronic periodontitis (CP) patients and periodontally healthy subjects.

	CP patients		Healthy subjects	
		Count in subgingival sites		Count in subgingival sites
Microorganism	Subjects colonized (%)	median (quartiles)	Subjects colonized (%)	median (quartiles)
Total prevalence/count	100	7.3 (6.6 −7.8)***	100	6.3 (5.8 −6.5)***
Actinobacillus actinomycetemcomitans	54**	<3.0 (<3.0 −4.2)**	0**	<3.0 (<3.0)**
Porphyromonas gingivalis	23	<3.0 (<3.0)	0	<3.0 (<3.0)
Prevotella intermedia/nigrescens	73	5.5 (<3.0 −6.5)***	47	<3.0 (<3.0 −3.8)***
Gram-positive bacteria				
Streptococcus mutans group[1]	64	<3.0 (<3.0 −4.8)	83	4.7 (<3.0 −5.5)
Streptococcus mitis group[2]	100	5.8 (5.3 −6.2)	100	5.5 (4.2 −5.8)
Streptococcus anginosus group[3]	73	5.0 (<3.0 −5.8)**	67	<3.0 (<3.0 −4.3)**
Streptococcus spp.	100	6.1 (5.4 −6.5)	100	5.8 (4.8 −6.0)
Staphylococcus spp.	31	<3.0 (<3.0)	20	<3.0 (<3.0)
Enterococcus spp.	12	<3.0 (<3.0)	20	<3.0 (<3.0)
Aerobic coryneforms	96	5.8 (4.8 −6.5)	100	5.4 (4.7 −5.8)
Lactobacillus spp.	0	<3.0 (<3.0)	13	<3.0 (<3.0)
Peptostreptococcus spp.	92	5.6 (4.7 −6.8)**	100	4.9 (4.3 −5.5)**
Anaerobic coryneforms	92	5.8 (4.6 −6.7)***	73	3.9 (<3.0 −4.8)***
Eubacterium spp.	62*	<3.0 (<3.0 −5.9)	93*	3.9 (<3.0 −4.5)
Clostridium spp.	8	<3.0 (<3.0)	7	<3.0 (<3.0)
Gram-negative bacteria				
Fam. Neisseriaceae	69	<3.0 (<3.0 −4.0)	60	<3.0 (<3.0 −4.1)
Fam. Enterobacteriaceae	4	<3.0 (<3.0)	7	<3.0 (<3.0)
Haemophilus spp.	62	<3.0(<3.0 −4.5)	67	<3.0 (<3.0 −4.4)
Capnocytophaga spp.	50	<3.0 (<3.0 −5.1)	40	<3.0 (<3.0)
Other Gram-negative facultative rods	35	<3.0 (<3.0)	27	<3.0 (<3.0)
Veillonella spp.	81	4.3 (<3.0 −5.7)	100	4.7 (4.0 −5.3)
Fusobacterium spp.	54	<3.0 (<3.0 −5.5)	40	<3.0 (<3.0 −3.5)
Campylobacter spp.	42	<3.0 (<3.0 −5.4)	47	<3.0 (<3.0)
Other Gram-negative anaerobic rods	100	6.5 (5.8 −7.3)***	100	5.0 (4.5 −5.7)***
Yeasts				
Candida spp.	4	<3.0 (<3.0)	7	<3.0 (<3.0)

The table shows the prevalence of various species (%) among CP patients and healthy subjects, and microbial counts in subgingival sites ($\log_{10}$ CFU/ml; detection level 3.0). The level of significance for differences in prevalence and positive counts between two groups are indicated by ***$p \leq 0.001$, **$p < 0.01$, *$p < 0.05$.

[1]*S. mutans, S. sobrinus*.

[2]*S. mitis, S. oralis, S. sanguis, S. parasanguis, S. gordonii, S. crista*.

[3]*S. anginosus, S. constellatus, S. intermedius*.

In vitro antimicrobial activity between lactic acid bacteria and periodontal pathogens

Inhibition of the periodontal pathogens *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* by *S. mutans* was observed (Table IV), with strongest antimicrobial activity towards *P. gingivalis*. Antimicrobial activity of lactobacilli against anaerobes was species-specific. All lactobacilli inhibited the growth of *P. gingivalis*, whereas only strains of *L. gasseri* and *L. paracasei* were able to inhibit *P. intermedia*.

On the other hand, none of the strains of *A. actinomycetemcomitans* and *P. gingivalis* were able to inhibit the growth of *S. mutans*, and only mild antimicrobial activity was expressed by *P. intermedia* (mean zone of inhibition 2.9 ± 0.2 mm). Neither *P. gingivalis* nor *P. intermedia* inhibited the growth of lactobacilli.

Discussion

In the present study we found that the proportion of LAB was decreased in oral microflora in patients with CP as compared with healthy subjects.

Total counts of subgingival microorganisms

As expected and in agreement with previous reports (1,18), the mean total count of microorganisms was

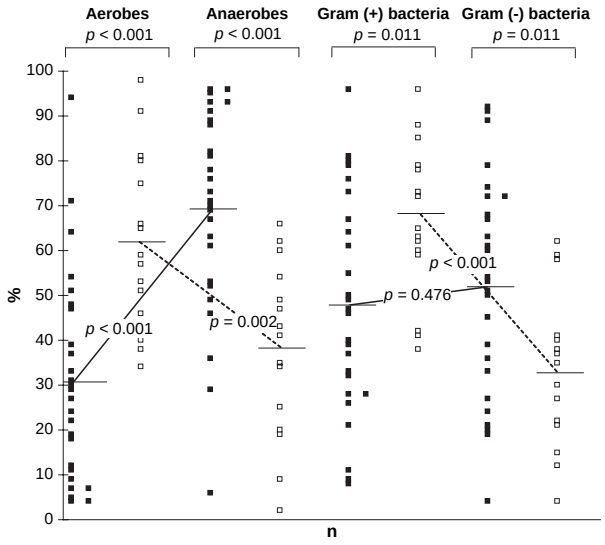

Figure 1. The proportion of aerobic, anaerobic, gram-positive and gram-negative bacteria expressed as a percentage of the total subgingival flora of chronic periodontitis (solid squares, $n = 26$) patients and periodontally healthy (open squares, $n = 15$) subjects. Each symbol represents a mean proportion of a particular microbial group in a subject. Perpendicular lines represent a mean value for each group of microorganisms averaged across subjects. All microaerophilic and facultatively anaerobic bacteria were included in the 'aerobes' and all obligately anaerobic bacteria in the 'anaerobes'.

much higher in periodontitis patients. This could be explained by different subgingival environmental conditions, because as revealed by clinical data, the periodontitis subjects had significantly deeper periodontal sites with obviously larger area for bacterial colonization. In addition, the high level of inflammation in diseased sites probably increased the amount of necessary nutrients for bacterial multiplication. Moreover, the possible mechanisms aiming to keep the bacterial numbers under control might have weakened. Large inter-individual variations in the number of different species and their counts were seen. This clearly supports the data on the individuality of a host's microflora, assessed in different biotopes of gastrointestinal and urogenital tracts (19).

Periodontal pathogens

In our study, along with the increase in the absolute numbers of microbes, the number of suspected periodontal pathogens (*P. gingivalis*, *P. intermedia/nigrescens*, *A. actinomycetemcomitans*) also increased in periodontitis, yet the prevalence of the acknowledged periodontal pathogen *P. gingivalis* remained rather low, contradicting earlier reports (9,20). This could be explained by the differences in methodology (culturing vs molecular techniques), but recently the regional differences in the composition of the subgingival microbiota have been revealed as well (21). We also observed that periodontally healthy persons were never colonized with *A. actinomyctemcomitans* and *P. gingivalis*, while the colonization with *P. intermedia/nigrescens* was seen in half of them. These data suggest that the mere presence of a single periodontal pathogen does not necessarily lead to periodontal destruction. Instead, the possibility of causing inflammation may be dependent on the particular combination of various microorganisms (20,22), as the mixed infection with two or three pathogens was seen in nearly half of the periodontitis patients. Also, the differences in the virulence of a particular strain might influence the outcome of the infection (23).

Relationships between different groups of microorganisms

In addition to the usual analysis of the prevalence and total counts of species and genera of microbes, the proportions (%) of bacteria from the total count of microorganisms in a particular periodontal site were calculated. Due to the large individual variations in the absolute numbers of bacteria in different individuals (19), we considered the proportions of microbes in the total count to be more informative. We found that anaerobic and gram-negative species predominated in the periodontitis patients, while aerobic and gram-positive species predominated in healthy individuals – confirming some earlier findings (18,24). The significant increase in the proportion of different anaerobic gram-negative rods in diseased sites occurred mostly at the expense of certain aerobic gram-positive bacteria, as periodontally healthy patients harboured much higher proportions of streptococci and aerobic coryneforms in their subgingival sites.

Lactic acid bacteria and periodontitis

The data from the present study show that colonization by LAB varies in different biotopes within the mouth. Lactobacilli, although frequently isolated from saliva, were seldom found from subgingival sites. Only 2 of 82 investigated subgingival sites were colonized by lactobacilli, which clearly shows that the subgingival region is not their common habitat. On the other hand, streptococci were frequently found in both saliva and subgingival sites, confirming earlier results (3) and indicating that they could be the guardians aiding in the balance of subgingival microecology.

Streptococci and coryneforms (e.g. *Actinomyces* spp.) have frequently been found in healthy individuals (18,22,24) and their possible beneficial role in subgingival plaque has been emphasized (25). In the

Table III. Correlations of the proportions of periodontal pathogens and clinical parameters with the proportions of streptococci, aerobic coryneforms and anaerobic gram-negative rods at respective subgingival sites.

Periodontal pathogens	Oral microbes	Correlation coefficient, *r*	*p* value
A. actinomycetemcomitans	vs streptococci	−0.21	0.059
	S. mutans group	−0.28	0.046
	S. mitis group	−0.42	0.002
	vs aerobic coryneforms	−0.17	0.127
	vs *P. gingivalis*	0.34	0.002
	vs *P. intermedia/nigrescens*	0.26	0.020
P. gingivalis	vs streptococci	−0.27	0.014
	S. mutans group	−0.28	0.046
	S. mitis group	−0.23	0.104
	vs aerobic coryneforms	−0.08	0.473
	vs *P. intermedia/nigrescens*	0.35	0.002
P. intermedia/nigrescens	vs streptococci	−0.16	0.151
	S. mutans group	−0.11	0.435
	S. mitis group	−0.02	0.902
	vs aerobic coryneforms	−0.24	0.034
Periodontal status			
Plaque index (PI)	vs streptococci	−0.37	0.002
	S. mutans group	−0.30	0.032
	S. mitis group	−0.33	0.016
	vs aerobic coryneforms	−0.40	0.001
	vs anaerobic gram-negative rods	0.45	<0.001
Gingival index (GI)	vs streptococci	−0.44	<0.001
	S. mutans group	−0.31	0.028
	S. mitis group	−0.35	0.011
	vs aerobic coryneforms	−0.30	0.014
	vs anaerobic gram-negative rods	0.44	<0.001
Periodontal probing depth (PPD)	vs streptococci	−0.23	0.034
	S. mutans group	−0.33	0.016
	S. mitis group	−0.39	0.004
	vs aerobic coryneforms	−0.28	0.012
	vs anaerobic gram-negative rods	0.36	<0.001

present study we found a significantly higher proportion of streptococci, particularly *S. mutans* and *S. mitis* groups, as well as aerobic coryneforms among healthy persons. In addition, correlation analysis showed that a high proportion of these bacteria in a periodontal site was inversely associated with that of periodontal pathogens, suggesting antagonistic interactions between LAB and periodontal pathogens. Clinical investigations have shown that periodontal treatment significantly increases the proportions of streptococci (e.g. mutans streptococci) in both subgingival plaque and saliva (3,26), confirming our results where both salivary and subgingival streptococci were in positive association with periodontal health.

Bacterial interactions, including antagonism, are likely to play an important role in the ecology of the microflora found in subgingival areas. Several inhibitory substances have been identified, including hydrogen peroxide, organic fatty acids, lactic acid, antibiotics, enzymes and bacteriocins. Inhibition of the *in vitro* growth of periodontal pathogens by viridans streptococci due to the production of hydrogen peroxide (2) and antimicrobial activity

Table IV. Antimicrobial activity of *S. mutans* and subgingival lactobacilli against putative periodontal pathogens.

	Inhibition zone values (mm) mean ± SD			
Target bacteria	*S. mutans* ($n=1$)	*L. gasseri* ($n=3$)	*L. paracasei* ($n=1$)	*L. oris* ($n=2$)
A. actinomycetemcomitans 31-1-1A	6.2 ± 0.5	ND	ND	ND
A. actinomycetemcomitans 31-2-1A	6.0 ± 0.8	ND	ND	ND
P. gingivalis ATCC 49417	22.0 ± 1.4	17.4 ± 5.1	23.0 ± 2.8	7.4 ± 7.9
P. gingivalis W83	17.0 ± 1.0	ND	ND	ND
P. intermedia ATCC 25611	12.3 ± 0.6	4.5 ± 3.0	10.3 ± 0.6	0 ± 0

ND, not determined.

of *Actinomyces* strains due to the production of various organic acids has been reported (27). Recently, Doran et al. (5) showed that anaerobic bacteria (including *P. intermedia* and *P. gingivalis*) were inhibited by metabolic end products of glucose fermentation by oral streptococci, particularly strains of *S. mutans* and *S. salivarius*. We succeeded in confirming the ability of both *S. mutans* and lactobacilli to inhibit the growth of anaerobic *P. gingivalis* and *P. intermedia* by our *in vitro* antimicrobial activity testing. In addition, *S. mutans* suppressed microaerophilic *A. actinomycetemcomitans*, although to a lesser extent than anaerobic bacteria. As the pH is an important determinant of the microbial composition of plaque, the production of organic acids from carbohydrate fermentation by lactic acid-producing bacteria and concomitant fall in pH can interfere with the growth of surrounding microorganisms, including putative periodontal pathogens (4,5). The inability of *P. gingivalis* to grow at a pH below 6.5 (28) makes its growth dependent on environmental conditions, and conceivably, the ecological determinants that reduce the proportion of LAB may favour the periodontal disease-inducing flora due to insufficient control mechanisms. Thus, the measures against decreasing the proportion of LAB microflora by antiseptics should be supported with evidence-based studies in different clinical situations, e.g. prevention of different oral diseases, not merely dental caries.

In summary, several microecological relations were substantially changed in CP patients as compared with periodontally healthy individuals, the major finding being a significant decrease in the proportions of oral streptococci and aerobic coryneforms in the former group. The data suggest that these oral commensals may play an important role in the suppression of periodontal pathogens and maintenance of microecological balance in the oral cavity. Thus, the excessive use of broad-spectrum antimicrobial agents against gram-positive microflora might shift the balance towards the overgrowth of potentially pathogenic species due to insufficient control mechanisms.

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