Oral Microbiology Immunology 2008: 23: 336–343
Printed in Singapore. All rights reserved

ORAL MICROBIOLOGY
AND IMMUNOLOGY

Probiotic bacteria affect the composition of salivary pellicle and streptococcal adhesion *in vitro*

A. Haukioja[1], V. Loimaranta[2], J. Tenovuo[3,4]

[1]Postgraduate School of Oral Health Sciences, Institute of Dentistry, [2]Department of Medical Biochemistry and Molecular Biology, Institute of Biomedicine, [3]Department of Cariology, Institute of Dentistry, University of Turku, Turku, Finland, [4]Department of Oral Diseases, Turku University Central Hospital, Turku, Finland

Haukioja A, Loimaranta V, Tenovuo J. Probiotic bacteria affect the composition of salivary pellicle and streptococcal adhesion in vitro.
Oral Microbiol Immunol 2008: 23: 336–343. © 2008 The Authors. Journal compilation © 2008 Blackwell Munksgaard.

Introduction: The use of probiotic bacteria is increasing worldwide and at least some of them can transiently colonize the oral cavity. Several studies have shown that probiotic bacteria, which are often thought of in relation only to intestinal health, can also affect the oral ecology, but the mechanisms for this are largely unknown. The aim of this study was to investigate *in vitro* if the probiotic bacteria used in commercial products affect the protein composition of the salivary pellicle and the adherence of other oral bacteria.
Methods: Salivary pellicle on hydroxyapatite and the adhesion of two oral streptococci, *Streptococcus mutans* and *Streptococcus gordonii*, were used as a model.
Results: Probiotic bacteria that bound to saliva-coated hydroxyapatite reduced the adhesion of *S. mutans* but the inhibitory effect on the adherence of *S. gordonii* was weaker. Salivary pellicle protein composition was modified by all the strains tested. The modifications in the pellicle affected the adherence of *S. mutans* but not of *S. gordonii*. Two of the proteins missing from the pellicles made of saliva-treated with the probiotic bacteria were identified as salivary agglutinin gp340 and salivary peroxidase. All bacterial strains bound salivary agglutinin gp340. The ability of the probiotic bacteria to degrade peroxidase was demonstrated with purified bovine lactoperoxidase and two of the probiotic strains.
Conclusion: This *in vitro* study showed that probiotic strains used in commercial products may affect the oral ecology by specifically preventing the adherence of other bacteria and by modifying the protein composition of the salivary pellicle.

Key words: bacterial adhesion; probiotics; saliva; salivary pellicle; streptococci

Anna Haukioja, Lemminkäisenkatu 2, Turku, FIN-20520, Finland
Tel.: + 358 44 011 0234;
fax: + 358 2 333 8356;
e-mail: anna.haukioja@utu.fi
Accepted for publication December 12, 2007

An increasing number of products containing probiotic bacteria are available, and these products are consumed orally. The most commonly used probiotic strains belong to the genera of *Lactobacillus* and *Bifidobacterium* (36). Although some species of these genera are associated with the progression of dental caries (4, 6), the main focus in the studies of potential oral probiotics has been on caries prevention, especially on the possibility of reducing the number of mutans streptococci when products containing certain probiotic strains are used (8, 9, 25, 27). Other possible applications, like the reduction of the number of oral *Candida* (15) or of microflora associated with oral malodor (7), have also been investigated.

The probiotic strains most studied in relation to the colonization of mutans streptococci are *Lactobacillus rhamnosus* GG and *Lactobacillus reuteri* SD2112 (also designed as ATCC 55730) (2, 8, 16, 23, 25, 27). The use of products containing these bacteria reduces the amount of oral mutans streptococci (8, 25, 27). *L. rhamnosus* GG can be found in the oral cavities of volunteers who are using products containing this bacterium (43). Colonization by *L. reuteri* SD2112 has not been studied, but the products containing it do not increase the lactobacillus counts in saliva (8). Other probiotic strains have also been studied but with contradictory results: for example, a product containing *Bifidobacterium* DN-173010 seems to decrease

the number of mutans streptococci in saliva (9), but Montalto et al. (24) did not find any effect of probiotic treatment on salivary *Streptococcus mutans* counts.

Although several studies have shown that some probiotic bacteria may affect oral ecology, the mechanisms are largely unknown. One of the assumed mechanisms used by probiotics in the urogenital tract and the intestine is the competitive exclusion of pathogens, either by specific competition for the receptors of adhesion, or by unspecific steric hindrance (26, 30, 32). Such effects have not been studied in the oral environment. Tooth surfaces are rapidly coated with a salivary film, the pellicle, which provides binding receptors for a number of bacteria. Modifications induced by probiotic bacteria in the composition of this pellicle would alter its microbial binding preferences.

The aim of this study was to investigate *in vitro* if the probiotic strains used in commercial products could affect the oral ecology (i) by preventing the adherence of other bacteria or (ii) by modifying the pellicle protein composition (Fig. 1). Probiotic strains with different affinities on saliva-coated surfaces were selected for this study (17). As a model we used salivary pellicle on hydroxyapatite (HA) and the adhesion of two oral streptococci, *S. mutans*, associated with dental caries, and *Streptococcus gordonii*, which is often considered as a commensal.

Materials and methods

Bacteria and growth conditions

Four probiotic bacterial strains with different adhesion properties on saliva-coated surfaces (17) were selected for this study: *L. rhamnosus* GG (ATCC 53103), *Lactobacillus casei* Shirota (Yakult®, Yakult Honsha, Japan), *L. reuteri* SD2112 (DSM 20016, Rela®, Ingman Foods, Finland, also known as ATCC 55730), and *Bifidobacterium lactis* Bb12 (Chr. Hansen, Denmark). In addition *L. casei* ATCC 11578, originally isolated from saliva, and *Lactococcus* (*Lc.*) *lactis* MG1363 (13) were used as the controls. They were all grown in de Man, Rogosa and Sharpe medium (Scharlau Microbiology, Scharlau Chemie, Barcelona, Spain) for 16–18 h to the late logarithmic or the early stationary phase. The streptococci – *S. mutans* MT 8148, *S. mutans* str. Ingbritt, and *S. gordonii* DL1 – were grown in brain-heart infusion medium (Difco Laboratories, Sparks, MD) first overnight and then for 3–4 h to the mid-logarithmic phase. *Lc. lactis* MG 1363 was grown at 30°C, all other bacteria at 37°C.

Buffer and salivas

Buffered KCl (50 mM KCl, 0.35 mM K_2HPO_4, 0.65 mM KH_2PO_4, 1.0 mM $CaCl_2$, 0.1 mM $MgCl_2$), pH 6.5 (11), was used in all experiments except in the peroxidase labeling and digestion.

As the preliminary experiments showed that only *S. gordonii*, but not *S. mutans*, adhered on clarified whole-saliva-coated HA, parotid saliva was used in all experiments.

Stimulated parotid saliva was collected on ice each morning before the experiments by means of Lashley cups and stimulation by a Salivin® lozenge (Pharmacia Ltd, Vantaa, Finland). Fresh, unstimulated whole saliva used in the preliminary experiments was clarified by centrifugation. Saliva was diluted in buffered KCl at 1 : 1 or 1 : 3 depending on the experiment. Saliva from four female donors (age 26–40 years) was used; all the donors were asked to avoid smoking, eating, drinking, and use of oral hygiene products for 1 h before saliva collection. Informed consent was obtained from the volunteers donating saliva. Permission to collect saliva samples was granted by the Joint Ethical Committee of the Turku University and the Turku University Central Hospital.

Adhesion experiments

The basis for all adhesion experiments was the same, as described earlier for *Streptococcus*-species (11, 14). Briefly, the bacteria were labeled by adding 5 µl (50 µCi) of ^{35}S-labeled methionine (Amersham Biosciences, Little Chalfont, UK) in 5 ml of growth medium. After growth the bacteria were washed and suspended in buffered KCl to approximately 10^8 colony-forming units (CFU)/ml (optical densities of 0.5 at 600 nm for lactobacilli and bifidobacteria and of 0.3 at 560 nm for streptococci). The total activities of the bacterial suspensions were at least 10^4 counts/min in the 125-µl samples (≈ 10 counts/min/10^4 CFU). Five milligrams of HA beads (BDH Chemicals Ltd, Poole, UK) were first coated with parotid saliva diluted 1 : 1 in buffered KCl for 60 min at room temperature (20–23°C). Bacterial suspension (125 µl) with labeled bacteria was added and the bacteria were allowed to adhere for 60 min, after which the unbound bacteria were washed away with 125 µl of buffer three times. Binding was expressed as the percentage of bound bacteria from the amount of added bacteria. All experiments were made in triplicate and repeated at least twice. All adhesion experiments made with *S. mutans* MT 8148 were repeated at least once with the *S. mutans* str. Ingbritt; there was no difference between the results obtained with these two strains.

To test the adhesion of streptococci and probiotic bacteria together, three sets of experiments were performed (Fig. 1). First, the probiotic strains (or control strains) were allowed to adhere before the streptococci. After coating HA with parotid saliva the HA beads were coated with bacterial suspension of probiotics or the control strains made in buffered KCl with 0.5% bovine serum albumin (BSA, Sigma Chemicals Co., St Louis, MO), or with 0.5% BSA alone for 30 min. After washings the labeled streptococci were allowed to adhere as described above. In the second set of experiments the streptococci and probiotic or control strains were

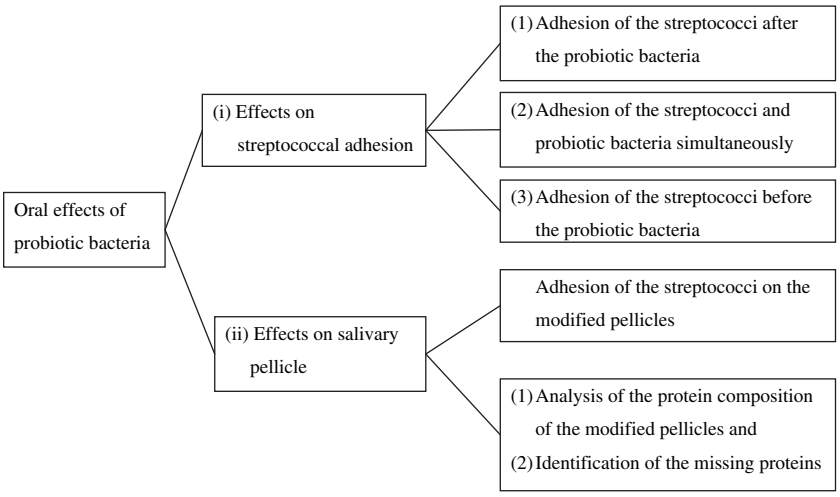

Fig. 1. Experiments made to study the oral effects of probiotic bacteria.

allowed to adhere simultaneously for 60 min. In the last set of experiments the labeled streptococci were allowed to adhere first for 60 min on saliva-coated HA and then the probiotics, the control strains, or buffered KCl were added for 60 min.

Agglutination experiments

Washed bacteria were suspended in buffered KCl to form a suspension with 10^9 CFU/ml. Each of the bacterial suspensions was mixed 1 : 1 with parotid saliva or with buffered KCl and the formation of visible aggregates was followed for 2 min.

Effects of the probiotic strains on the on salivary pellicle composition

Effects of alterations in the salivary pellicle on streptococcal adherence

Parotid saliva was incubated for 60 or 180 min with buffered KCl (1 : 3) or with bacterial suspensions (10^8 CFU/ml or 2×10^9 CFU/ml). The bacteria were then removed by centrifugation (1525 ***g***, 10 min) and the supernatant was used to coat the HA. Control pellicles for adhesion experiments were made by first coating HA with parotid saliva and after that with a supernatant of a bacterial suspension in buffer. The adhesion experiments on these modified pellicles were performed in the same way as the adhesion experiments described above.

Effects on protein composition

To analyze the pellicle protein composition 100 mg of HA was coated for 60 min with 1.25 ml of the parotid saliva first incubated for 180 min with the probiotic bacteria, control strains, or buffer (control). After three washes, the proteins were released by vigorous vortexing with 1 ml of 0.1% sodium dodecyl sulfate (SDS) and the samples were concentrated by freeze drying. The proteins were suspended in SDS sample buffer with reduced amount of SDS and without any reducing agent. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in a BioRad Mini-protean II cell using 4–15% gradient Tris–HCl gels (BioRad Laboratories, Hercules, CA). The gels were stained with silver stain.

Protein identification

The presence (and removal) of salivary agglutinin in the pellicles was ascertained using Western blot analysis (see below) and the salivary peroxidase was identified in the proteomics service at the Turku Centre for Biotechnology (Turku, Finland) as follows. Proteins were in-gel digested according to Shevchenko et al. (37) and the tryptic peptides were analyzed using a nanoflow liquid chromatograph (Famos, SwitchosII, and Ultimate; Dionex Corporation, Sunnyvale, CA) coupled to a QSTAR Pulsar i ESI-hybrid Q-TOF tandem mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada). Data from liquid chromatograph-mass spectrometry/mass spectrometry runs were converted to peak list files with the ANALYST QS software (version 1.1) and searched using an in-house MASCOT search engine (version 2.1, Matrix Sciences, Boston, MA).

Binding of agglutinin to bacterial cells

The binding of agglutinin to bacterial cells was tested by slightly modifying the method described by Prakobphol et al. (29). Briefly, 0.5-ml bacterial suspensions (10^8 CFU/ml or 2×10^9 CFU/ml) of the probiotic or the control strains were incubated with an equal volume of parotid saliva diluted 1 : 1 in buffered KCl. Binding took place at room temperature for 0, 15, 30, or 60 min. The cells were then pelleted by centrifugation for 10 min, a sample was taken from supernatant and the pellet was washed twice with buffered KCl. To release the bound proteins, the bacterial pellet was suspended in 200 µl of SDS–PAGE loading buffer without any reducing agent. After 30 min, the cells were removed. An aliquot of the supernatant as well as the samples taken from saliva incubated with the bacteria (20 µl) was subjected to SDS–PAGE (7.5%) and immunoblotting with anti-gp340 (mAb213-6).

After SDS–PAGE the proteins were transferred onto nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) with Trans-Blot SD semi-Dry transfer cell (BioRad). Membrane was blocked over night in 3% BSA in Tris-buffered saline with 0.05% Tween (TTBS), incubated for 60 min with 0.02 µg/ml of the primary antibody anti-glycoprotein-340 (anti-gp340, HYB213-06; Antibodyshop, Gentofte, Denmark), followed by washings and incubation for 60 min with the secondary antibody, anti-mouse immunoglobulin conjugated horseradish peroxidase (HRP; Dako, Glostrup, Denmark). Detection used ECL™ Western Blotting Detection Reagents (Amersham Biosciences) according to the manufacturer's instructions.

Peroxidase labeling and digestion

Bovine lactoperoxidase (Sigma) was labeled with EZ-Link® Sulfo-NHS-LC biotin (Pierce, Rockford). One milligram of lactoperoxidase was dissolved in phosphate buffer (10 mM, pH 7.4) and mixed with 160 µg biotin in a total volume of 2.0 ml. After 30 min incubation at room temperature, labeled peroxidase was separated from free biotin with a PD-10 column (Pharmacia, Uppsala, Sweden). To test the degradation of peroxidase, the labeled or unlabeled protein (40 µg/ml) was mixed with 2×10^9 CFU/ml of washed, overnight-grown *L. rhamnosus* GG or *L. casei* str. Shirota cells in phosphate buffer (pH 6.8) supplemented with 1 mM $CaCl_2$. In control experiments the bacteria or lactoperoxidase were mixed with buffer alone. After 60 or 180 min incubation at room temperature the bacteria were removed by centrifugation (10,000 ***g***, 5 min) and the supernatants were mixed with SDS-loading buffer, heated for 5 min at 95°C and applied to SDS–PAGE gels. Two identical gels were run, one of which was silver-stained and from the other the proteins were blotted on polyvinylidene fluoride membrane. The free binding sites on the membrane were blocked by 3% BSA, and the membrane was incubated for 60 min with streptavidin-conjugated HRP (0.01 µg/ml). The biotinylated proteins were detected with ECL™ as described above. For comparison, biotinylated lactoperoxidase was incubated and analyzed in an identical way with *S. mutans*.

Statistics and presentation of data

Means and standard deviations of independent experiments were counted from medians of triplicate experiments. As parotid saliva donated by different persons was used in the experiments, there were discrepancies in the adhesion percentages and in the number of bound bacteria on control pellicles. The adhesion of *S. mutans* varied between 7 and 25%, corresponding to approximately 9×10^5 to 3×10^6 CFU per 5 mg HA, and that of *S. gordonii* varied from 16 to 47% (3×10^6 to 6×10^6 CFU per 5 mg HA); for this reason the adherence of *S. mutans* and *S. gordonii* was expressed as a percentage of control in the tables. The control and test adherences were always measured with saliva from the same person in one experimental set up. To compare the effects of different probiotic strains with the effect of the control (*Lc. lactis*) on streptococcal adherence (Table 1) or when

Table 1. Adherence of *Streptococcus mutans* and *Streptococcus gordonii* on parotid saliva-coated hydroxyapatite together with *Lactobacillus (Lb)*, *Bifidobacterium* (*Bb)*, or *Lactococcus (Lc)* [% of control, mean ± SD (*P*-value[1]), $n = 3$ or $n = 4$]

	After *Lb*, *Bb*, or *Lc*		In the presence of *Lb*, *Bb*, or *Lc*		Before the *Lb*, *Bb*, or *Lc*	
	S. mutans	*S. gordonii*	*S. mutans*	*S. gordonii*	*S. mutans*	*S. gordonii*
L. rhamnosus GG	38.2 ± 6.8 (<0.001)	72.8 ± 2.2 (0.004)	96.2 ± 17.07 (ns)	109.1 ± 10.2 (ns)	85.9 ± 4.6 (ns)	122.8 ± 23.6 (ns)
L. casei str. Shirota	49.5 ± 2.9 (<0.001)	89.5 ± 16.5 (ns)	104.4 ± 8.4 (ns)	111.7 ± 15.3 (ns)	95.2 ± 4.7 (ns)	103.7 ± 6.5 (ns)
L. reuteri SD2112	102.3 ± 11.7 (ns)	102.9 ± 3.7 (ns)	94.9 ± 15.0 (ns)	124.6 ± 14.5 (ns)	109.0 ± 11.1 (ns)	129.8 ± 17.0 (ns)
L. casei ATCC 11578	96.8 ± 19.8 (ns)	97.6 ± 5.0 (ns)	61.0 ± 10.9 (0.004)	78.9 ± 28.2 (ns)	23.5 ± 18.3 (<0.001)	58.0 ± 38.8 (0.011)
B. lactis Bb12	98.0 ± 6.9 (ns)	97.6 ± 4.9 (ns)	95.1 ± 5.8 (ns)	104.8 ± 16.8 (ns)	100.6 ± 13.7 (ns)	105.7 ± 14.9 (ns)
Lc. lactis MG 1363	112.1 ± 12.3	100.1 ± 3.7	97.3 ± 10.9	112.2 ± 8.8	89.7 ± 16.9	122.9 ± 10.0

[1]Dunnet's two-sided *t*-test, adherence together with *Lactococcus lactis* MG 1363 was considered as a control.
ns, the difference is not significant.

the adherences of the streptococci on different pellicles were compared with the adherence onto control pellicle (Table 2), the data were first subjected to one-way analysis of variance and subsequent pairwise comparisons were made with Dunnet's two-sided *t*-test. The level of statistical significance was set at 0.05.

Results

Adherence and aggregation of *Lactobacillus*, *Bifidobacterium*, and *Lactococcus* strains

L. rhamnosus GG and *L. casei* str. Shirota adhered well on parotid-saliva-coated HA whereas the other strains tested showed only low or no binding (Table 3). All strains except *L. reuteri* SD2112 formed clear visible aggregates in a mixture of parotid saliva and adhesion buffer (Table 3). *L. rhamnosus* GG, *L. casei* str. Shirota, and *L. casei* ATCC 11578 formed visible aggregates in buffer, the aggregates of *L. casei* ATCC 11578 were very pronounced.

Adherence of probiotic bacteria and streptococci together

The streptococci did not affect the adherence of the lactobacilli, *B. lactis* Bb12 or *Lc. lactis* MG1363 (Table 3). Those probiotic strains that adhered on parotid-saliva-coated HA (*L. rhamnosus* GG and *L. casei* str. Shirota) diminished the adherence of *S. mutans* when they were allowed to adhere before the streptococci. Only *L. rhamnosus* GG reduced the adherence of *S. gordonii* and the inhibitory effect was weaker (Table 1). When the probiotic strains, or the control strains, and the streptococci were allowed to adhere simultaneously, or when the streptococci were allowed to adhere before the probiotics or the control strains, only *L. casei* ATCC 11578 affected the adherence of the streptococci: it slightly inhibited the adherence of *S. mutans* and it could even release the already bound streptococci from the HA (Table 1).

Table 2. Adherence of *Streptococcus mutans* and *Streptococcus gordonii* on hydroxyapatite coated with parotid saliva, which was first incubated with *Lactobacillus*, *Bifidobacterium*, or *Lactococcus*

	S. mutans	*S. gordonii*
Saliva treated with 10^8 CFU/ml of		
Lactobacillus rhamnosus GG	45.1 ± 7.6 (0.043)	95.5 ± 2.6
L. casei str. Shirota	47.2 ± 5.7 (0.046)	103.4 ± 10.1
L. reuteri SD2112	62.4 ± 12.4 (ns)	102.1 ± 3.9
L. casei ATCC 11578	41.5 ± 3.8 (0.021)	91.9 ± 13.3
Bifidobacterium lactis Bb12	24.4 ± 5.3 (0.003)	90.1 ± 10.5
Lactococcus lactis MG 1363	100.3 ± 41.7 (ns)	79.2 ± 9.0
Saliva treated with 2×10^9 CFU/ml of		
Lactobacillus rhamnosus GG	4.9 ± 1.2 (0.001)	86.7 ± 3.3
L. casei str. Shirota	5.2 ± 0.9 (0.001)	87.3 ± 4.1
L. reuteri SD2112	8.2 ± 0.2 (0.001)	97.6 ± 21.5
L. casei ATCC 11578	5.5 ± 1.5 (0.001)	92.1 ± 4.2
Bifidobacterium lactis Bb12	10.6 ± 0.7 (0.001)	99.8 ± 2.8
Lactococcus lactis MG 1363	45.3 ± 26.6 (ns)	95.5 ± 0.2

Percentage of control, mean ± SD with *P*-value in parentheses, $n = 3$. Dunnet's two-sided *t*-test, adherence on pellicle made of untreated saliva was considered as a control.
ns = the difference was not significant.

Table 3. Adherence of *Lactobacillus*, *Bifidobacterium*, or *Lactococcus* on parotid-saliva-coated hydroxyapatite and their aggregation in parotid saliva

	Adherence (mean ± SD, $n = 3$)			
		In the presence of *S. mutans*	In the presence of *S. gordonii*	Aggregation
L. rhamnosus GG	53.7 ± 7.4	56.1 ± 5.8	55.3 ± 4.7	+
L. casei Shirota	26.7 ± 1.2	29.0 ± 1.0	31.9 ± 1.4	+
L. reuteri SD2112	1.8 ± 0.2	1.7 ± 0.5	2.3 ± 1.1	−
L. casei ATCC 11578	7.3 ± 5.4	15.6 ± 18.2	16.9 ± 20.3	+
B. lactis Bb12	6.1 ± 2.7	5.4 ± 2.0	5.9 ± 1.8	+
Lc. lactis MG 1363	1.9 ± 1.0	2.0 ± 1.6	3.2 ± 0.8	+

+, visible aggregates; −, no visible aggregates.

Effect of alterations in the salivary pellicle on streptococcal adherence

Incubation of parotid saliva with the *Lactobacillus* or *Bifidobacterium* strains, before the pellicle was formed, significantly decreased the adherence of *S. mutans* but the adherence of *S. gordonii* remained unaffected (Table 2). This inhibitory effect could be seen already after 1 h of incubation (not shown), but it was more prominent after 3 h of incubation. The inhibition was also dose-dependent: 10^7 CFU/ml of *Lactobacillus* or *Bifidobacterium* strains did not have an effect on the adherence of *S. mutans* (not shown) but a 10 times higher concentration clearly inhibited the adherence of *S. mutans* and with 2×10^9 CFU/ml the adherence of *S. mutans* was almost abolished (Table 2). *Lc. lactis* MG1363 showed a clearly weaker effect on the adherence of *S. mutans* although with a large amount of bacteria and long incubation time it also seemed to have some effect on the adherence of *S. mutans* (Table 2). Control pellicles for these adherence assays were made by first coating HA with parotid saliva and then

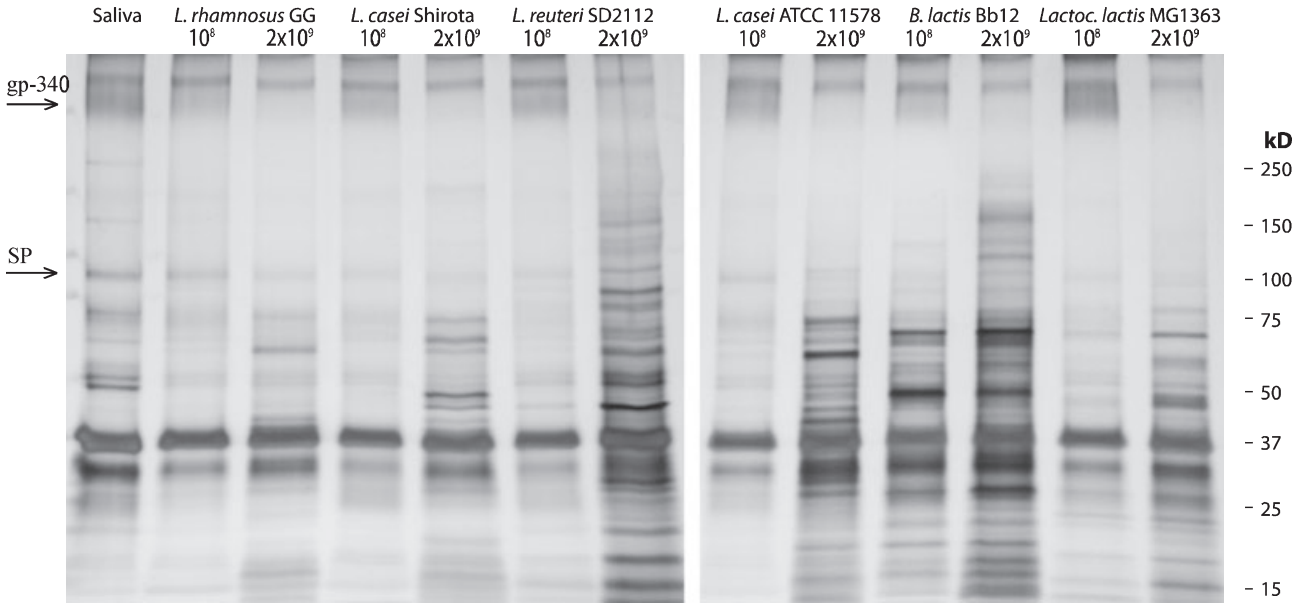

Fig. 2. Silver-stained proteins recovered from hydroxyapatite. Parotid saliva was incubated with 10^8 or 2×10^9 CFU/ml of bacteria for 180 min. The positions of molecular mass standards are shown on the right and the bands of salivary agglutinin (gp340) and salivary peroxidase (SP) are indicated with arrows.

with a supernatant of a bacterial suspension in buffer. There was no difference in the adherence of *S. mutans* or *S. gordonii* on the control pellicles (not shown).

Effect of the *Lactobacillus*, *Bifidobacterium*, and *Lactococcus* strains on the pellicle composition

All strains studied had a clear effect on the protein composition of the salivary pellicle on HA (Fig. 2). After saliva was incubated with high amounts of bacteria, especially with *L. reuterii* SD2112 and *B. lactis* Bb12, new protein bands were visible on the gels. New protein bands were seen also on silver-stained gels with control samples in which the bacteria were incubated in buffer alone (not shown). Incubation of the saliva with any of the strains removed a heavy molecular weight protein band that contained salivary agglutinin gp340 as analyzed in Western blots (Fig. 2). With strains other than *Lc. lactis* MG1363 the weakening of the protein band containing salivary agglutinin was obvious already with a lower amount of bacteria (10^8 CFU/ml). Another protein of which the amount was reduced in the pellicles made of parotid saliva incubated with the probiotic bacteria or the controls was salivary peroxidase (Fig. 2).

Interactions of probiotic bacteria with salivary agglutinin and peroxidase

All bacterial strains used bound salivary agglutinin (Fig. 2) and the bound protein could be released from the bacteria. In Fig. 3 the release of salivary agglutinin from *L. reuterii* SD2112 is shown. The binding of salivary agglutinin took place in less than 10 min (time of centrifugation, see Materials and methods) with the strains *L. rhamnosus* GG, *L. casei* Shirota, *B. lactis* Bb12, and *L. casei* ATCC 11578; also the amount of salivary agglutinin released from the bacteria was approximately the same regardless of the incubation time. *L. reuterii* SD2112 and *Lb. lactis* bound salivary agglutinin somewhat slower, all the binding had occurred in approximately 30 min (as shown for *L. reuterii* SD2112 in Fig. 3).

The ability of the *L. rhamnosus* GG and *L. casei* str. Shirota to degrade peroxidase was tested with purified bovine lactoperoxidase. After 60 or 180 min incubation of biotinylated lactoperoxidase with the probiotic lactobacilli most of the peroxidase was still present in the supernatant. After 180 min of incubation with the probiotic bacteria, but not with the buffer alone, a novel protein band of approximately 40 kDa was also visible in the silver-stained gels (Fig. 4A). This band also gave a clear signal in streptavidin–HRP-stained Western blots, indicating that it originates from the biotinylated peroxidase (Fig. 4B). No such protein was visible in the control samples where the bacteria were incubated with the buffer alone (not shown). When non-biotinylated peroxidase was used, the same 40-kDa band was seen in the silver-stained gels (Fig. 4C), but not in Western blots (Fig. 4D). ECL™ detection is based on the peroxidase reaction so lactoperoxidase itself can react with the ECL™ reagents. For this reason, the unlabeled peroxidase was also visible in the Western blots. The signal produced was clearly weaker than the signal from biotinylated protein stained with streptavidin–HRP, and the 40-kDa protein did not produce any signal. When lactoperoxidase was incubated with *S. mutans*, no such novel protein bands were detected (Fig. 4A and B).

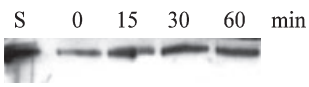

Fig. 3. Release of salivary agglutinin from *Lactobacillus reuterii* SD2112. Bacteria were incubated for 0, 15, 30, or 60 min with parotid saliva and the bound agglutinin was released from the bacteria with SDS-sample buffer. After SDS–PAGE the proteins were transferred to the nitrocellulose membrane and the salivary agglutinin was detected with mAb213-6 in Western blot analysis. Parotid saliva (S) was used as the control.

Discussion

This *in vitro* study describes two new possible mechanisms of probiotic action in the oral cavity. Our results show that *Lactobacillus* and *Bifidobacterium* strains used in commercial probiotic products may affect the oral ecology by specifi-

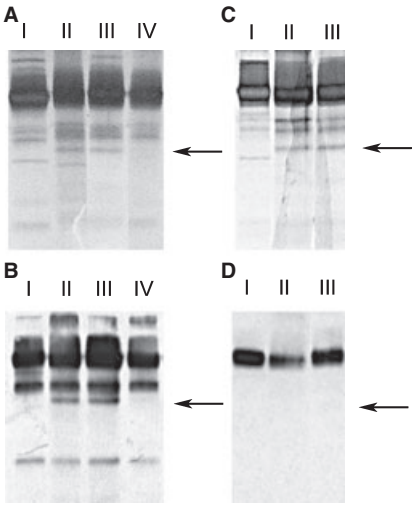

Fig. 4. Degradation of purified lactoperoxidase (LP) by *Lactobacillus casei* str. Shirota and *Lactobacillus rhamnosus* GG. After 180 min of incubation of LP with the bacteria, the bacterial cells were removed by centrifugation and the supernatant was applied on the SDS–PAGE gel. Gels were either silver-stained (A and C) or transferred to a polyvinylidene fluoride membrane for Western blot detection (B and D). Peroxidase was either biotinylated (A and B) or not (C and D) before incubation with the bacteria. Lane I, LP incubated in buffer; lane II, LP with *L. casei* str. Shirota; lane III, LP with *L. rhamnosus* GG; lane IV, LP with *Streptococcus mutans*. Arrows indicate the place of the novel protein bands, which were visible after incubation of LP with *L. casei* Shirota and *L. rhamnosus* GG, and stained with streptavidin-HRP when the LP was biotinylated.

cally preventing the adherence of other bacteria and by modifying the protein composition of the salivary pellicle. The latter seems to occur by the binding and degrading of proteins important for bacterial adherence and involved in oral defense systems.

Only those probiotic strains that adhered on parotid-saliva-coated HA prevented the *in vitro* adhesion of other bacteria. The binding of *S. mutans* was reduced significantly, while the adherence of *S. gordonii* was less affected. The inhibition was therefore apparently not only the result of unspecific steric hindrance of binding. Although it is possible that part of the obtained difference is the result of the better adherence of *S. gordonii*, it seems likely that while the lactobacilli occupy specific salivary receptors of *S. mutans* there are still binding receptors left for *S. gordonii*. In fact, *S. gordonii* has a wider pattern of receptors on salivary pellicles than *S. mutans* (1, 34).

The adhesion of streptococci was inhibited only when the probiotic bacteria were added before them. The competition or exclusion of binding is found to be more effective than displacement of the pathogens also in a model mimicking the intestine (19). Interestingly, milk fermented with *L. rhamnosus* GG prevents the *in vitro* adhesion of *S. mutans* (42) and milk containing *L. rhamnosus* GG seems to decrease the caries risk in children (25), known to be related to the number of adhered *S. mutans* cells (3, 41). Hence, it can be hypothesized that the inhibition of adhesion by probiotics could affect the initial colonization of *S. mutans* also *in vivo*.

By using the adherence of streptococci as a biological indicator, we could show that all probiotic strains tested modified the composition of saliva and, thereafter, the formed artificial salivary pellicle on HA. Again, the treatment of saliva affected only the adherence of *S. mutans* whereas the adherence of *S. gordonii* remained unaffected. Our results show that the probiotic bacteria can modify the protein composition of the pellicle by at least two different methods: binding and degradation of salivary proteins. Salivary agglutinin gp340 is the main receptor for *S. mutans* in pellicle (10, 12, 18). In addition to interaction with *S. mutans*, salivary agglutinin gp340 mediates aggregation and adhesion of other *Streptococcus* species and, for example, *Actinomyces* and *Helicobacter pylori* (20, 29). All our strains bound salivary agglutinin gp340 and removed it from saliva, which correlates well with the observed low adhesion of *S. mutans* after probiotic treatment of saliva.

All the strains tested, regardless of their aggregation (agglutination) or adhesion properties, bound salivary agglutinin gp340. Previously a non-agglutinating *L. casei* strain has been shown to bind salivary agglutinin(s) active for other strains (35). In addition, fluid and surface-bound forms of salivary agglutinin gp340 mediate the aggregation and adhesion of oral streptococci differently even between strains within the same species (20). Salivary agglutinin gp340 seems to mediate the interactions between bacteria differently when bound by different bacterial species. The binding of salivary agglutinin took place in minutes with strains other than *L. reuteri* SD2112 and *Lc. lactis* MG1363, but further incubation of saliva with the probiotic bacteria amplified the effect on *S. mutans* adherence, predicting that other modifications also occurred in saliva during the longer incubation time.

The amount of another protein, the salivary peroxidase, was also reduced after incubation of saliva with the probiotic bacteria. It is possible that part of the salivary peroxidase missing from the pellicles was bound by bacteria because peroxidase is shown to bind, for example, to streptococcal surfaces (31). However, *L. rhamnosus* GG and *L. casei* str. Shirota also degraded bovine lactoperoxidase; this glycoprotein closely resembles human salivary peroxidase (21). Salivary peroxidase is not a receptor for *S. mutans* or *S. gordonii* adherence [(33), unpublished observations], but this protein might have an important role in oral ecology because it is part of the antimicrobial salivary peroxidase system, one of the innate defense factors found in human saliva (40). Degradation of an innate immune system protein by a probiotic strain was surprising and has not, to our knowledge, been reported earlier. On the other hand, the proteolytic activity of *L. rhamnosus* GG (39) and also of other lactic acid bacteria (28) is well acknowledged and has been used for example to generate bioactive peptides from milk proteins. Also in saliva antimicrobial peptides can be released by cleavage from proteins that may have only limited antimicrobial activity by themselves (5). Although the degradation of the lactoperoxidase was slow in our experimental conditions, the finding cannot be overlooked and it evidently calls for further studies to find out the effects and the exact nature of this proteolytic activity.

It seems likely that different probiotic strains have different mechanisms of action in the oral cavity. In addition, previously reported probiotic properties do not necessarily correlate with the probiotic activity in the oral cavity. Only two out of six strains tested were effective in the blocking of *S. mutans* binding sites. On the other hand, the ability to modify the pellicle composition by binding salivary agglutinin gp340 was not specific to the probiotic strains but was common to all *Lactobacillus* strains tested and even to the *Bifidobacterium* and *Lactococcus* strains used in this study. Another type of mechanism by which the oral probiotics can affect the oral microbiota is the inhibition of the growth of the mutans streptococci (23, 27, 38). It also cannot be ruled out that other, possibly systemic, mechanisms are involved. For example, *L. reuterii* SD2112 decreases the number of mutans streptococci in saliva (8, 27), but both *in vitro* and *in vivo* observations predict that it does not colonize the oral cavity (8, 17). It is also notable that the acid-producing

probiotic bacteria do not necessarily have only positive effects in the mouth. This is indicated not only by caries induction in rats (22) but also by the ability of these bacteria to degrade a protein belonging to the human innate defense system, as shown in this study. As the use of probiotic bacteria is increasing worldwide, it is most important that we understand also their functions in the oral cavity.

Acknowledgments

Dr Arthur Ouwehand and Prof. Seppo Salminen are acknowledged for providing the probiotic strains. Ms Mariia Henttinen and Ms Katja Sampalahti are thanked for their skilful technical assistance. This study was supported by Finnish Dental Society Apollonia and Turku University Central Hospital.

References

1. Ahn SJ, Kho HS, Kim KK, Nahm DS. Adhesion of oral streptococci to experimental bracket pellicles from glandular saliva. Am J Orthod Dentofacial Orthop 2003: **124**: 198–205.
2. 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.
3. Alaluusua S, Renkonen OV. *Streptococcus mutans* establishment and dental caries experience in children from 2 to 4 years old. Scand J Dent Res 1983: **91**: 453–457.
4. Becker MR, Paster BJ, Leys EJ et al. Molecular analysis of bacterial species associated with childhood caries. J Clin Microbiol 2002: **40**: 1001–1009.
5. Bellamy W, Takase M, Yamauchi K, Wakabayashi H, Kawase K, Tomita M. Identification of the bactericidal domain of lactoferrin. Biochim Biophys Acta 1992: **1121**: 130–136.
6. Boyar RM, Bowden GH. The microflora associated with the progression of incipient carious lesions of children living in a water-fluoridated area. Caries Res 1985: **19**: 298–306.
7. Burton JP, Chilcott CN, Moore CJ, Speiser G, Tagg JR. A preliminary study of the effect of probiotic *Streptococcus salivarius* K12 on oral malodour parameters. J Appl Microbiol 2006: **100**: 754–764.
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. Caglar E, Sandalli N, Twetman S et al. Effect of yoghurt with *Bifidobacterium* DN-173 010 on salivary mutans streptococci and lactobacilli in young adults. Acta Odont Scand 2005: **63**: 317–320.
10. Carlen A, Olsson J. Monoclonal antibodies against a high-molecular-weight agglutinin block adherence to experimental pellicles on hydroxyapatite and aggregation of *Streptococcus mutans*. J Dent Res 1995: **74**: 1040–1047.
11. Clark WB, Bammann LL, Gibbons RJ. Comparative estimates of bacterial affinities and adsorption sites on hydroxyapatite surfaces. Infect Immun 1978: **19**: 846–853.
12. Ericson T, Rundegren J. Characterization of a salivary agglutinin reacting with a serotype c strain of *Streptococcus mutans*. Eur J Biochem 1983: **133**: 255–261.
13. Gasson MJ. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. J Bacteriol 1983: **154**: 1–9.
14. Gibbons RJ, Etherden I. Albumin as a blocking agent in studies of streptococcal adsorption to experimental salivary pellicles. Infect Immun 1985: **50**: 592–594.
15. Hatakka K, Ahola AJ, Yli-Knuuttila H et al. Probiotics reduce the prevalence of oral *Candida* in the elderly – a randomized controlled trial. J Dent Res 2007: **86**: 125–130.
16. Hatakka K, Savilahti E, Ponka A et al. Effect of long term consumption of probiotic milk on infections in children attending day care centres: double blind, randomised trial. BMJ 2001: **322**: 1327.
17. Haukioja A, Yli-Knuuttila H, Loimaranta V et al. Oral adhesion and survival of probiotic and other lactobacilli and bifidobacteria *in vitro*. Oral Microbiol Immunol 2006: **21**: 326–332.
18. Kishimoto E, Hay DI, Gibbons RJ. A human salivary protein which promotes adhesion of *Streptococcus mutans* serotype c strains to hydroxyapatite. Infect Immun 1989: **57**: 3702–3707.
19. Lee YK, Puong KY, Ouwehand AC, Salminen S. Displacement of bacterial pathogens from mucus and caco-2 cell surface by lactobacilli. J Med Microbiol 2003: **52**: 925–930.
20. Loimaranta V, Jakubovics NS, Hytönen J, Finne J, Jenkinson HF, Strömberg N. Fluid- or surface-phase human salivary scavenger protein gp340 exposes different bacterial recognition properties. Infect Immun 2005: **73**: 2245–2252.
21. Månsson-Rahemtulla B, Rahemtulla F, Baldone DC, Pruitt KM, Hjerpe A. Purification and characterization of human salivary peroxidase. Biochemistry 1988: **27**: 233–239.
22. Matsumoto M, Tsuji M, Sasaki H et al. Cariogenicity of the probiotic bacterium *Lactobacillus salivarius* in rats. Caries Res 2005: **39**: 479–483.
23. Meurman JH, Antila H, Korhonen A, Salminen S. Effect of *Lactobacillus rhamnosus* strain GG (ATCC 53103) on the growth of *Streptococcus sobrinus in vitro*. Eur J Oral Sci 1995: **103**: 253–258.
24. Montalto M, Vastola M, Marigo L et al. Probiotic treatment increases salivary counts of lactobacilli: a double-blind, randomized, controlled study. Digestion 2004: **69**: 53–56.
25. 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.
26. Neeser JR, Granato D, Rouvet M, Servin A, Teneberg S, Karlsson KA. *Lactobacillus johnsonii* La1 shares carbohydrate-binding specificities with several enteropathogenic bacteria. Glycobiology 2000: **10**: 1193–1199.
27. Nikawa H, Makihira S, Fukushima H et al. *Lactobacillus reuteri* in bovine milk fermented decreases the oral carriage of mutans streptococci. Int J Food Microbiol 2004: **95**: 219–223.
28. Pihlanto A, Korhonen H. Bioactive peptides and proteins. Adv Food Nutr Res 2003: **47**: 175–276.
29. Prakobphol A, Xu F, Hoang VM et al. Salivary agglutinin, which binds *Streptococcus mutans* and *Helicobacter pylori*, is the lung scavenger receptor cysteine-rich protein gp-340. J Biol Chem 2000: **275**: 39860–39866.
30. Pretzer G, Snel J, Molenaar D et al. Biodiversity-based identification and functional characterization of the mannose-specific adhesin of *Lactobacillus plantarum*. J Bacteriol 2005: **187**: 6128–6136.
31. Pruitt KM, Adamson M, Arnold R. Lactoperoxidase binding to streptococci. Infect Immun 1979: **25**: 304–309.
32. Reid G., Burton J. Use of *Lactobacillus* to prevent infection by pathogenic bacteria. Microbes Infect 2002: **4**: 319–324.
33. Roger V, Tenovuo J, Lenander-Lumikari M, Söderling E, Vilja P. Lysozyme and lactoperoxidase inhibit the adherence of *Streptococcus mutans* NCTC 10449 (serotype c) to saliva-treated hydroxyapatite *in vitro*. Caries Res 1994: **28**: 421–428.
34. Rosan B, Lamont RJ. Dental plaque formation. Microbes Infect 2000: **2**: 1599–1607.
35. Rundegren J. Calcium-dependent salivary agglutinin with reactivity to various oral bacterial species. Infect Immun 1986: **53**: 173–178.
36. Saxelin M, Tynkkynen S, Mattila-Sandholm T, de Vos WM. Probiotic and other functional microbes: from markets to mechanisms. Curr Opin Biotechnol 2005: **16**: 204–211.
37. Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 1996: **68**: 850–858.
38. Simark-Mattsson C, Emilson CG, Hakansson EG, Jacobsson C, Roos K, Holm S. *Lactobacillus*-mediated interference of mutans streptococci in caries-free vs. caries-active subjects. Eur J Oral Sci 2007: **115**: 308–314.
39. Sütas Y, Soppi E, Korhonen H et al. Suppression of lymphocyte proliferation *in vitro* by bovine caseins hydrolyzed with *Lactobacillus casei* GG-derived enzymes. J Allergy Clin Immunol 1996: **98**: 216–224.
40. Tenovuo J. Antimicrobial function of human saliva – how important is it for oral health? Acta Odontol Scand 1998: **56**: 250–256.
41. Tenovuo J, Häkkinen P, Paunio P, Emilson CG. Effects of chlorhexidine-fluoride gel treatments in mothers on the establishment of mutans streptococci in primary teeth and

the development of dental caries in children. Caries Res 1992: **26**: 275–280.
42. Wei H, Loimaranta V, Tenovuo J et al. Stability and activity of specific antibodies against *Streptococcus mutans* and *Streptococcus sobrinus* in bovine milk fermented with *Lactobacillus rhamnosus* strain GG or treated at ultra-high temperature. Oral Microbiol Immunol 2002: **17**: 9–15.
43. Yli-Knuuttila H., Snäll J, Kari K, Meurman JH. Colonization of *Lactobacillus rhamnosus* GG in the oral cavity. Oral Microbiol Immunol 2006: **21**: 129–131.