**ORAL MICROBIOLOGY
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Oral adhesion and survival of probiotic and other lactobacilli and bifidobacteria *in vitro*

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Introduction: Most probiotic products are consumed orally and hence it is feasible that the bacteria in these products may also attach to oral surfaces; however, the effects of these bacteria on the oral ecosystem are mostly unknown. Our aim was to evaluate the oral colonization potential of different probiotic, dairy, and fecal *Lactobacillus* and *Bifidobacterium* strains *in vitro*.
Methods: The binding of 17 *Lactobacillus* and seven *Bifidobacterium* strains to hydroxyapatite and microtitre wells coated with human saliva was tested. Binding of selected strains to human buccal epithelial cells and co-adherence with *Fusobacterium nucleatum* were also investigated. In addition, the survival in sterilized human whole saliva was examined.
Results: There was a large variation in binding to saliva-coated surfaces and buccal epithelial cells but all strains survived in saliva. The binding pattern of the probiotics did not differ from the binding of the fecal strains. *F. nucleatum* altered the binding of both the low-binding bifidobacteria and the high-binding lactobacilli.
Conclusion: The differences in binding *in vitro* may indicate that there are also differences in the persistence of the different probiotic strains in the oral cavity *in vivo*.

An increasing number of products containing probiotic bacteria are available and used by consumers. Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. The most commonly used strains belong to the genera *Lactobacillus* and *Bifidobacterium* (27), genera that are commonly found in the oral cavity, including caries lesions (10). Numerous studies have been performed with different probiotic bacteria in the intestine. As the same *Lactobacillus* species are found on both rectal and oral mucosa (2) and most of the probiotic products are consumed orally, it is feasible that the consumed probiotic bacteria also attach to oral surfaces.

If these bacteria transiently colonize the oral cavity, their effects are difficult to predict. On the one hand, the efficient production of organic acids, which is a common characteristic of both lactobacilli and bifidobacteria, may be harmful in the mouth, as has been shown in animal studies for the probiotic *Lactobacillus salivarius* LS1952R (20). On the other hand, probiotic *Lactobacillus* and *Bifidobacterium* strains have been reported to exert potentially beneficial effects for the mouth: *Lactobacillus rhamnosus* GG (ATCC 53103) produces a growth inhibitory substance against *Streptococcus sobrinus* (21) and it has been proposed to reduce the risk for caries in 3–4-year-old children (24). In addition, *Lactobacillus reuteri* SD2112 (ATCC 55730) inhibits the growth of *Streptococcus mutans* and consumption of yoghurt containing this bacterium slightly reduced the oral carriage of *S. mutans* (25). Similar results have also been obtained with a *Bifidobacterium* strain (7). However, contradictory results have also been reported: Montalto et al. (23) did not find any effect on salivary *S. mutans* counts when probiotic treatment using a probiotic *Lactobacillus* mixture was administered. Whether the obtained effects relate to the colonization of the bacteria in the oral cavity or some other factors is not clear. Thus, the effects of prolonged persistence of different probiotic bacteria need to be further studied.

There are only a few studies showing that probiotic strains can persist in the oral cavity as they do in the intestine, but here again the results are contradictory. The probiotic strain *L. rhamnosus* GG has been

shown to persist in the mouth for from 1–5 days up to a few weeks after termination of the consumption of a product containing this bacterium (22, 33). On the other hand, no lactobacilli were found in saliva samples from volunteers 1 week after consumption of a bioyoghurt containing two different strains of lactobacilli and a *Bifidobacterium bifidum* strain (6). From previous studies we know that there are huge variations in adhesion characteristics (17) as well as in other biological activities of different probiotic bacteria, even in strains within the same species. Such variations may explain the contradictory results in the studies of the oral effects of probiotic bacteria. To understand or estimate the possible oral effects of a certain probiotic strain, its behavior in oral environment must be characterized.

In this study our aim was to investigate *in vitro* the oral colonization potential of different commercially available probiotic and dairy *Lactobacillus* and *Bifidobacterium* strains together with fecal isolates with potential probiotic characteristics (3, 14,

15, 19). This potential was investigated by studying their survival in human saliva, adherence to saliva-coated surfaces and on human buccal epithelial cells, as well as their co-adhesion with *Fusobacterium nucleatum.*

Materials and methods
Bacteria and growth conditions

This study included 17 *Lactobacillus* strains and seven *Bifidobacterium* strains comprising 10 strains with known probiotic properties, three common dairy strains, and 11 fecal isolates with potential probiotic characteristics taken from healthy infants and adults (Table 1). Two of the fecal *L. rhamnosus* isolates tested in pulsed-field gel electrophoresis (PFGE) analyses made with restriction enzymes *Asc*I, *Fse*I, *Not*I, and *Sfi*I were indistinguishable from *L. rhamnosus* GG (not shown). *Lactobacillus* strains were grown in de Man, Rogosa, and Sharpe medium (MRS medium; Difco™, Difco Laboratories, Sparks, MD) for 16–18 h and

Bifidobacterium strains were grown in reinforced clostridial medium (RCM; LabM, Bury, Lancashire, UK) from 18 to 24 h to late logarithmic or early stationary phase. After survival assays, the bacteria were plated on MRS (Difco) or M17 (Oxoid) agar plates. The *F. nucleatum* ATCC 10953 was cultivated on *Brucella*-agar plates [43 g *Brucella* agar (Difco) and 70 ml horse blood per 1 l of medium] for 2 days. All bifidobacteria and lactobacilli for adhesion experiments on hydroxyapatite (HA) were cultivated in an anaerobic atmosphere (10% CO_2, 80% N_2, 10% H_2), lactobacilli for the adhesion experiments on microtiter wells were cultivated in air/ 5% CO_2 atmosphere at 37°C.

Saliva preparations and buffers

Four different saliva pools were collected for the experiments. Paraffin-stimulated whole saliva was collected from at least nine healthy adult volunteers, who were asked to avoid smoking, eating, drinking, and use of oral hygiene products for 1 h

Table 1. Binding of different lactobacilli and bifidobacteria to saliva-coated surfaces and their viability after 24-h incubation in saliva

Strain	Binding			Survival in saliva[4]	Origin	Source
	S-MTW[1]	S-HA[2]	B-HA[3]			
High-binding						
Lactobacillus						
L. rhamnosus GG	16.2	61.9[f]	26.6	+	Probiotic	ATCC 53103
L. rhamnosus 5.3a	17.8	56.9	44.5	+	Fecal[5]	(3)
L. paracasei 8.12a	13.5	56.1	51.9	+	Fecal	(3)
L. rhamnosus 11.4a	35.0	52.5	36.2	+	Fecal	(3)
L. paracasei 8.16b	8.4	52.3	39.9	+	Fecal	(3)
L. rhamnosus 5.1a	15.3	51.6	40.7	+	Fecal[5]	(3)
Low-binding						
Lactobacillus						
L. delbrueckii sp. *bulgaricus* 365	3.8	2.8[6]	0.5	+	Dairy	ATCC 11842
L. acidophilus NFCM	2.7	4.4[6]	21.2	+/−	Probiotic	Danisco, USA
L. reuteri SD 2112	4.7	2.6	1.5	+	Probiotic	DSM 20016, Rela®, Ingman Foods, Finland
L. plantarum 299V	5.3	2.1	1.6	+/−	Probiotic	DSM 9843, ProViva, Probi AB, Sweden
L. casei 921	3.8	1.1[6]	1.1	+	Dairy	ATCC 334
L. rhamnosus LC 705	1.4	0.8[6]	0.8	+	Dairy	Valio Ltd, Finland
L. paracasei F19	3.5	0.6[6]	0.6	+	Probiotic	Arla Ltd, Sweden
Bifidobacterium						
B. breve H-1-10	0.1	1.6[6]	1.3	+	Fecal	(14)
B. breve H-1–3	3.4	0.8[6]	0.7	+	Fecal	(14)
B. lactis Bb12	5.3	0.3[6]	0.3	+	Probiotic	Chr. Hansen, Denmark
B. longum 2C	0.2	0.2[6]	0.3	+	Probiotic	(19)
B. longum 46	0.1	0.2[6]	0.2	+	Probiotic	(19)
B. adplescentis A 16	0.1	0.2[6]	0.1	+	Fecal	(15)
Low in S-MTW, high in S-HA						
L. rhamnosus 5.5a	6.8	46.6	33.3	+	Fecal	(3)
L. johnsonii LA1	5.2	53.4[6]	42.9	+	Probiotic	LC1®, Nestlé Ltd, Switzerland
L. casei Shirota	0.9	29.9[6]	23.0	+	Probiotic	Yakult®, Yakult Honsha, Japan
High in S-MTW, low in S-HA						
L. paracasei 12.11a	10.8	4.6[6]	3.9	+	Fecal	(3)
B. infantis A3	11.2	0.4[6]	0.4	+	Fecal	(15)

[1]S-MTW: binding to saliva-coated microtiter wells.
[2]S-HA: binding to saliva-coated hydroxyapatite beads.
[3]B-HA: binding to BSA-coated hydroxyapatite beads.
[4]+ No decrease in CFU/ml, +/− some decrease in CFU/ml after 24-h incubation in saliva.
[5]Indistinguishable from *L. rhamnosus* GG based on PFGE (see Materials and methods).
[6]Binding to apatite beads was tested with and without BSA (see Materials and methods).

before saliva collection. Saliva samples were pooled, clarified by centrifugation, and stored in aliquots at −70°C. The first saliva pool was used in all the binding experiments presented in columns 1 and 2 of Table 1. The second pool was collected for the adhesion experiments in which the binding of lactobacilli and bifidobacteria to saliva and *F. nucleatum*-coated HA was tested. The third saliva pool was used to test the survival of lactobacilli and bifidobacteria in saliva. As the sensitivity to the activated peroxidase system was tested, fresh, centrifuged, and filtered saliva pooled from nine non-smoking volunteers was used. Fresh saliva samples were also used when individual differences and differences between different salivas were tested. After the collection of unstimulated whole saliva, the stimulated parotid saliva was collected by means of Lashley cups and stimulation by a Salivin® lozenge (Pharmacia Ltd, Vantaa, Finland). The unstimulated saliva was clarified by centrifugation. Informed consent was obtained from 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.

Buffered KCl (50.0 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, was used in all the adhesion experiments on HA and in experiments with buccal epithelial cells. In addition phosphate-buffered saline (PBS; Gibco™, Invitrogen, Paisley, UK) was used in experiments with buccal epithelial cells and in adhesion experiments on microtiter wells. HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; Sigma, St Louis, MO)-buffered Hanks' balanced salt solution was used in washes in adhesion experiments on microtiter plate wells.

Survival of lactobacilli and bifidobacteria in saliva

Before each experiment with lactobacilli, the saliva samples were thawed, transferred to open Petri dishes and sterilized by UV-radiation (254 nm) for 5 min from a distance of 5 cm at 30 W. The bifidobacteria were tested in fresh saliva treated in a similar manner but under anaerobic conditions. The bacteria from overnight cultures were harvested by centrifugation and washed twice with a 0.9% NaCl solution; the optical density at 492 nm (OD_{492}) of the bacterial suspension was set to 0.5 ($\approx 1.4 \times 10^8$ bacteria/ml). Then, 200-μl aliquots of the bacterial suspensions were

inoculated into 1.8 ml of saliva and incubated at 37°C for 24 h. In a pilot study the OD_{492} was measured at 2-h intervals for 24 h. Because OD_{492} did not increase during the incubation period it was measured only at the beginning of the incubation and after 24 h. Samples were taken before and after 24 h incubation, diluted and cultured on MRS agar and the colonies were counted after 2 days of incubation. As a negative control, the tests were repeated using 0.9% NaCl instead of saliva. All the tests were performed in duplicate and repeated at least once.

To test the sensitivity of the bacteria to the activated peroxidase systems in saliva, *L. rhamnosus* GG and *B. lactis* Bb12 were selected as representative strains because of their wide commercial use. Bacteria were harvested from overnight cultures by centrifugation, washed twice with PBS and the OD_{600} was set to 5.0 [$\approx 10^9$ colony-forming units (CFU)/ml]. A 100-μl bacterial suspension subsample was added to 850 μl saliva supplemented with 50 μl PBS or 1 mM H_2O_2. After 1 h of incubation at 37°C, 100 μl aliquots were taken, diluted, and plated. The colonies were counted after 2 days of incubation. The amount of hypothiocyanite ($OSCN^-$) generated from potassium thiocyanate (KSCN) and H_2O_2 by peroxidases in saliva was quantified by the oxidation reaction of yellow 5-thio-2-nitrobenzoic acid (Nbs) to colorless Nbs_2 by $OSCN^-$ as described by Aune & Thomas (5) and modified by Pruitt et al. (28).

Binding of lactobacilli and bifidobacteria on saliva-coated surfaces

Two commonly used adhesion assays, adhesion on microtiter wells (MTW) and adhesion on HA were used.

Binding of lactobacilli and bifidobacteria on saliva-coated MTW

The bacteria were labeled by adding 10 μl (10 μCi) methyl-1,2[³H]thymidine (Amersham BioSciences, Amersham, UK) in 1 ml growth medium. Bacteria from overnight cultures were harvested by centrifugation and washed twice with PBS and the OD_{570} of the bacterial suspensions was adjusted to 0.5. The salivary proteins were passively immobilized to polystyrene Maxisorp microtiter plate wells (Nunc, Roskilde, Denmark) by incubating saliva on plates and the adhesion experiment was performed as described earlier (17). Briefly, labeled bacteria were added to the wells and allowed to adhere for 60 min.

After washes to remove the unbound bacteria the bacteria were released and lysed and the amount of bound bacteria was measured with a scintillation counter. All experiments were made in triplicate and repeated at least twice.

Binding of lactobacilli and bifidobacteria on saliva or bovine-serum-albumin-coated HA

The bacteria were labeled by adding 5 or 10 μl (50 or 100 μCi) of [³⁵S]methionine (Amersham Biosciences) in 5 ml of growth medium. The labeling efficiency differed between the strains studied; in our experiments the total activities of the bacterial suspensions were at least 1 count/min/10^3 CFU. The bacteria from overnight cultures were washed and suspended in buffered KCl with 0.5% bovine serum albumin (BSA; Sigma Chemical Co., St Louis, MO) to give an OD_{600} of 0.5 ($\approx 10^8$ CFU/ml) and the adhesion experiments were performed as described previously for *Streptococcus* species (11, 13). Briefly, 5 mg of the HA beads were first coated with saliva, after which any possible free HA surfaces were blocked with BSA. A 125-μl sample of the bacterial suspension with labeled bacteria was added and the bacteria were allowed to adhere for 60 min after which the unbound bacteria were washed away. Binding was expressed as the percentage of bound bacteria from the added bacteria. The binding of selected strains (marked in Table 1) was tested also without BSA. All experiments were made in triplicate.

To test differences between whole saliva and pure parotid saliva secretion as well as individual differences, simultaneous experiments were carried out with parotid and whole unstimulated saliva with and without BSA. The saliva samples of five individuals were tested with *L. rhamnosus* GG. Experiments were carried out in duplicate and the experiments with the salivas of two individuals were repeated on two different occasions.

Binding of *Lactobacillus* and *Bifidobacterium* species on saliva and *Fusobacterium-nucleatum*-coated HA

Lactobacillus and *Bifidobacterium* strains (five lactobacilli and two bifidobacteria) with different binding behaviors in saliva-coated MTW or HA were chosen for the tests presented in Table 2. *F. nucleatum* ATCC 10953 was labeled by growing on *Brucella*-agar plates in the presence of 10 μl (100 μCi) [³⁵S]methionine (Amersham). For each experiment, *F. nucleatum*

Table 2. Binding of different lactobacilli and bifidobacteria to saliva-coated HA, saliva and *Fusobacterium nucleatum*-coated HA and to human buccal epithelial cells and comparison with the binding to intestinal mucus

Strain	Binding to saliva coated surfaces[1]	Binding			
		Saliva[2]	Saliva and *F. nucleatum*[3]	BEC[4]	Intestinal mucus[5]
L. rhamnosus GG	H/H	44.3 (3.0)	18.9 (4.0)[8]	14.3 (4.4)[9]	17.73 (2.03)[6]
L. rhamnosus 5.1a	H/H	45.1 (10.8)	18.4 (4.0)[8]	15.3 (6.9)[10]	21.26 (2.00)[6]
L. delbrueckii sp. bulgaricus 365	L/L	4.3 (0.8)	9.9 (2.9)	2.6 (1.5)	21.10 (3.53)[6]
B. lactis Bb12	L/L	2.4 (0.4)	26.0 (13.4)[8]	1.7 (1.0)	6.70 (1.38)[6]
L. paracasei 12.11a	H/L	5.0 (2.3)	7.3 (3.5)	2.7 (2.6)	18.07 (0.35)[6]
B. infantis A3	H/L	4.4 (2.2)	21.4 (7.6)[8]	4.1 (3.5)	3.48 (3.1)[7]
L. casei str. Shirota	L/H	42.9 (1.2)	29.9 (11.1)	3.5 (3.1)	0.58 (0.08)[6]

[1]From Table 1; binding to saliva-coated microtiter wells/binding to saliva-coated HA. H, high binding; L, low binding.
[2]Binding to saliva-coated HA (% of added bacteria; mean ± SD).
[3]Binding to saliva and *F. nucleatum*-coated HA (% of added bacteria; mean ± SD).
[4]Binding to human buccal epithelial cells (bacteria/cell – bacteria/cell in control; mean ± SD).
[5]Bacterial binding to human intestinal mucus, obtained and adapted from [6](3) and [7](15) (% of added bacteria; mean ± SD).
[8]Significant difference when compared to binding on saliva-coated apatite; $P < 0.05$ (Student's paired two-tailed *t*-test).
[9]Significant difference when compared to binding of all other tested strains except *L. rhamnosus* 5.1a and *B. infantis* A3.
[10]Significant difference when compared to all other tested strains except *L. rhamnosus* GG; $P < 0.05$ (Tukey HSD-test).

was cultivated with and without label. The bacteria were collected in buffered KCl and washed once, then bacterial suspensions (with and without label) with $OD_{600} = 1.0$ ($\approx 10^8$ CFU/ml) were made. The binding of *F. nucleatum* to saliva-coated HA was tested with the labeled *F. nucleatum* suspension. The binding of *Lactobacillus* and *Bifidobacterium* strains was tested as described above for binding on saliva-coated HA with the following differences. After coating HA beads with saliva (or buffered KCl) the beads were incubated for 1 h with 125 µl of suspension made from unlabeled *F. nucleatum*. The beads were washed and incubated for an additional 15 min with saliva. Then, after three washes, the *Lactobacillus* or *Bifidobacterium* suspension (without BSA) was added over the *F. nucleatum*. After 1 h of incubation the beads were washed and the amount of bound bacteria was counted. At each step, some of the parallel samples were left untreated as a control, so that at the end there were the following parallel experiments: (i) binding of *F. nucleatum* to HA and to saliva-coated HA, (ii) binding of lactobacilli or bifidobacteria to HA and to saliva-coated HA, (iii) binding of lactobacilli or bifidobacteria to saliva- and *F. nucleatum*-coated HA and (iv) binding of lactobacilli or bifidobacteria to HA coated with saliva, *F. nucleatum* and with saliva again. The experiments were made in triplicate and repeated twice.

Adherence of *Lactobacillus* and *Bifidobacterium* species to buccal epithelial cells

The assay was made as described previously (16), briefly as follows: buccal epithelial cells (BEC) were collected from one healthy female volunteer. Cells were washed and suspended in buffered KCl to give an OD_{600} of 0.500. Bacterial suspensions were made from overnight cultures in buffered KCl to give an OD_{600} of 0.1 ($\approx 2 \times 10^7$ CFU/ml) and equal amounts of BEC and bacterial suspensions were incubated for 60 min. Control cells were treated with buffer only. After washing, the cells with attached bacteria were stained with crystal violet. All bacteria (both indigenous ones and the added lactobacilli or bifidobacteria) bound to BEC were counted under light microscope. Thirty cells were counted from each sample. All bacterial strains were tested in parallel and the experiments were repeated twice.

Statistics

Means and standard deviations of independent experiments were counted from medians of duplicate or triplicate experiments. Spearman correlation coefficients were calculated to assess the possible connection between binding to saliva-coated MTW and HA. To compare the differences in binding between probiotic

and fecal strains to saliva-coated HA or MTW Mann–Whitney *U*-test was performed. As *L. rhamnosus* strains GG, 5.1a and 5.3a were indistinguishable, the test was performed first by treating them as a single probiotic organism (5.1a and 5.3a were left out) and then as three independent organisms (as shown in Table 1). The conclusions drawn from the Mann–Whitney *U*-tests were the same in both cases. In Table 2 the binding on saliva-coated and on saliva and *F. nucleatum*-coated HA were compared with Student's paired two-tailed *t*-test. To compare the differences between attachments on buccal epithelial cells the data were first subjected to one-way analysis of variance and subsequent pairwise comparisons were made with a Tukey HSD test. The level of statistical significance was set at 0.05.

Results
Survival of lactobacilli and bifidobacteria in saliva and in saliva with activated peroxidase system

The *Lactobacillus* and *Bifidobacterium* strains survived in saliva for the 24 h tested (Table 1). None of the tested strains grew in saliva and only two strains showed decreased CFU counts after 24 h of incubation. Addition of 50 µM hydrogen peroxide to saliva resulted in 48–50 µM $OSCN^-$. This amount of $OSCN^-$ did not affect the viability of *L. rhamnosus* GG or *B. lactis* Bb12 (not shown).

Binding of lactobacilli and bifidobacteria to saliva-coated MTW and to saliva- or BSA-coated HA

The results from two different adhesion assays are shown in Table 1. In both assays large variations were observed in the binding of the bacteria to saliva-coated surfaces, but there was no difference between the probiotics and fecal isolates ($P = 0.604$ in the HA assay and $P = 0.243$ in the MTW assay, when *L. rhamnosus* strains GG, 5.1a and 5.3a were treated as a single probiotic organism). In the HA assay the tested strains formed two groups. Out of the 17 *Lactobacillus* strains nine strains bound well (over 20% of the added bacteria were bound) while the binding of the remaining eight *Lactobacillus* strains and all seven *Bifidobacterium* strains was poor (less than 5% were bound). The binding to saliva-coated MTW did not separate the strains as clearly. To allow comparisons between the two assays, mean binding to saliva-coated MTW (7.3%) was used as a

foundation for the groups in Table 1. When the percentage of the bound bacteria was greater than the mean, the binding was considered high. Of the 24 tested *Lactobacillus* and *Bifidobacterium* strains, seven *Lactobacillus* strains bound well to both saliva-coated MTW and saliva-coated HA. All of these strains also showed high binding to BSA-coated HA. When selected strains were tested on BSA-coated MTW, similar results were obtained (not shown). Seven *Lactobacillus* and six *Bifidobacterium* strains exhibited low binding to saliva-coated MTW and to saliva-coated HA. One of these strains (*L. acidophilus* NCFM) bound well to BSA-coated HA.

Most of the strains studied behaved similarly on both saliva-coated surfaces (Spearman correlation 0.700, *P* < 0.001). According to the created grouping only five of the 24 strains behaved differently on saliva-coated MTW and on saliva-coated HA. The probiotic lactobacilli *Lactobacillus johnsonii* and *Lactobacillus casei* Shirota, as well as the fecal strain *L. rhamnosus* 5.5a, bound well to saliva-coated HA but did not bind to saliva-coated MTW. Reverse behavior was seen with the fecal strains *Lactobacillus paracasei* 12.11a and *Bifidobacterium infantis* A3. These strains bound well to saliva-coated MTW but not to HA. The *B. infantis* A3 strain was the only *Bifidobacterium* strain that bound to at least one of the tested surfaces. The two fecal strains which were genetically indistinguishable from *L. rhamnosus* GG and the *L. rhamnosus* GG itself behaved similarly (Table 1).

Since BSA was used as a blocking agent in assays with saliva-coated HA and many of the strains bound to BSA-coated surfaces, the binding of selected strains to HA was tested with and without BSA coating on saliva-coated HA and with and without BSA in buffer (strains are marked in Table 1). The use of BSA did not affect the binding of the high-binding group lactobacilli to the HA that were coated with whole saliva but they did bind to BSA-coated HA in somewhat lesser amounts than to naked apatite beads (not shown). The use of BSA did not affect the binding to HA of the lactobacilli or bifidobacteria in the low-binding groups.

The differences in bacterial binding, both individual differences and those between parotid and unstimulated whole saliva samples, were tested with *L. rhamnosus* GG (Fig. 1). There were clear differences between the salivas of different individuals. Still in every case *L. rhamnosus* GG adhered well to saliva-coated HA. These experiments were performed with and without BSA as a blocking agent. The use of BSA seemed to decrease the difference between parotid saliva-coated and whole saliva-coated HA by slightly bettering the adherence on parotid saliva-coated HA (not shown). Data in Fig. 1 are from experiments without BSA.

Binding to saliva- and *F. nucleatum*-coated HA

Five *Lactobacillus* strains and two *Bifidobacterium* strains with different binding properties in the two adhesion assays (Table 1) were selected for further experiments with *F. nucleatum* and BEC (Table 2). Coating with *F. nucleatum* clearly diminished the binding of the high-binding strains *L. rhamnosus* GG and *L. rhamnosus* 5.1a; also the binding of *L. casei* strain Shirota appeared to be slightly lower to *F. nucleatum*-coated HA than to saliva-coated HA (Table 2). The opposite happened with both bifidobacteria strains. The *F. nucleatum* coating significantly enhanced their binding to HA. The binding of the two low-binding lactobacilli remained almost unaffected (Table 2). The additional saliva coating after *F. nucleatum* coating did not affect these results (not shown).

F. nucleatum bound well to saliva-coated HA; 38.4 ± 1.9% (mean ± SD) of added bacteria were detected on HA after 1 h of incubation. A different saliva pool from that used in Table 1 was used in these experiments. Although there were some differences in the bacterial binding to HA-coated with different saliva pools, the binding pattern did not change: high-binding strains were high binding in both cases and low-binding strains stayed in the low-binding group (Tables 1 and 2).

Binding of lactobacilli and bifidobacteria to BEC

Approximately five indigenous bacteria/cell attached to washed and untreated BEC (not shown). The *L. rhamnosus* strains GG and 5.1a bound well to BEC, approxi-
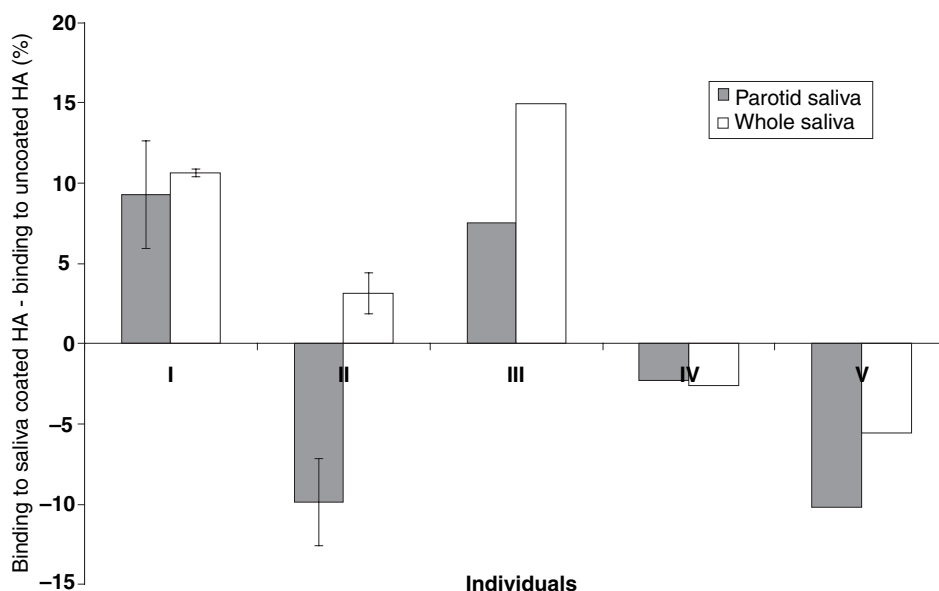

Fig. 1. Binding of *Lactobacillus rhamnosus* GG to apatite beads coated with parotid and unstimulated whole saliva collected from five different individuals (I–V). Binding is expressed as a difference compared to bacterial binding to uncoated apatite beads. The experiments were performed on three different occasions with individuals I and II, mean and SD are shown in the figure.

mately 15 bacteria/cell more than in the controls were detected. There was no difference in levels of attachment between these strains. The binding of the other lactobacilli or bifidobacteria tested was significantly lower, although few bacteria were seen attached to BEC (Table 2).

Discussion

To be able to colonize, microorganisms have to survive in the environment in question and be able to adhere to a surface in it or to grow faster than the dilution rate. In the mouth, bacteria have to resist the defense factors of saliva and unless they adhere to oral surfaces the bacteria are rapidly swallowed. Bacteria can attach to immobilized salivary proteins (i.e. to acquired pellicle), attach to epithelial cells, or (co)aggregate with other bacteria already there.

To measure the *in vitro* binding of probiotic, dairy, and fecal *Lactobacillus* and *Bifidobacterium* strains to salivary proteins we used two different methods: an MTW assay, which is commonly used in studies of bacterial adhesion of probiotic and other intestinal bacteria, and an HA assay, which mimics human dental enamel. Although there are some differences in pellicle composition *in vivo* and on HA *in vitro* (8, 32), HA is a good model for oral bacterial adhesion. Though similar results were obtained for most strains, a few of the strains studied behaved differently on different surfaces. One reason might be that the different surfaces, the more hydrophobic polystyrene and the hydrophilic HA, attract different bacteria or, more likely, are coated with different salivary proteins. For example, the biofilm formation of a *gtfC*-deletion mutant of *S. mutans* is different on saliva-coated HA and polystyrene (31). We did not study the mechanism of adhesion in detail and, therefore, the reason for the observed differences is not clear.

Good attachment ability of a strain to the intestinal mucus or epithelial cells is one of the main selection criteria for a probiotic microorganism (27) and one of the selection criteria of the fecal *Lactobacillus* and *Bifidobacterium* strains for this study was their good adherence to intestinal mucus (3, 14, 15). In our assays, large variations were observed in the binding of the bacteria to saliva-coated surfaces and thus, the binding to intestinal mucus did not correspond to the binding to salivary proteins. The probiotic strains did not stand out as high (or low) binders among the strains studied. Though all strains that

were high binders on both saliva-coated MTW and HA had a tendency to bind to all tested surfaces – to saliva- and BSA-coated surfaces and to BEC in addition to intestinal mucus [Tables 1 and 2 (3)] – the binding to saliva-coated HA was higher than to BSA-coated HA. This indicates at least partial specificity to salivary proteins. This is also supported by the fact that different saliva secretions mediated different bacterial binding. The protein composition of unstimulated whole saliva and pure parotid secretions is different and they can mediate oral bacterial adhesion differently (9). Albumin, for example, is regarded as a serum filtrate to the mouth and therefore, parotid saliva does not normally contain it (30). Interestingly, we found that addition of albumin to parotid saliva diminished the difference between the parotid and whole salivas (not shown).

We could demonstrate clear differences in the binding of *L. rhamnosus* GG on HA coated with saliva samples from different individuals. This is not surprising because there can be large individual variations in saliva composition. Individual differences are often neglected when adhesion of bacteria is studied. For example, when Busscher et al. (6) could not detect lactobacilli in the oral cavities of volunteers after consumption for 1 week of yoghurt containing *Lactobacillus acidophilus*, *L. casei*, and *B. bifidum* strains, which were previously shown to adhere to enamel chips; it might be that the inability of the lactobacilli to colonize was a characteristic for the hosts. The volunteers were, after all, selected on the basis that they did not have oral lactobacilli before the survey. There can also be many other explanations for the finding, but in general the individual characteristics should not be overlooked when evaluating the adhesive potential of different bacteria.

Altogether lactobacilli adhered better to saliva-coated surfaces than the bifidobacteria. In general the *in vitro* adherence of the bifidobacteria to host surfaces is poor when compared to that of lactobacilli (3, 15). They are, however, part of the normal healthy intestinal flora and are also detected in saliva (29), dental plaque (18), and dental caries lesions (12). It is possible that the expression of some genes that are important to the *Bifidobacterium* adherence is downregulated *in vitro*. *F. nucleatum* coating on HA significantly enhanced the binding of the bifidobacteria that did not bind to saliva-coated HA. This is in accordance with the suggestion that other bacteria influence the adherence of bifidobacteria in the intestine. For example,

in vitro L rhamnosus GG and *Lactobacillus delbrueckii* subsp. *bulgaricus* significantly enhanced the adherence of *B. lactis* Bb12 on intestinal mucus (26). In addition consumption of *L. rhamnosus* GG increased the number of bifidobacteria in the feces of healthy test subjects (4). Interestingly *F. nucleatum* coating diminished the adherence of high-binding lactobacilli to the hydroxyapatite, suggesting that these strains may compete for the same binding sites on saliva-coated hydroxyapatite. It is also possible that the *F. nucleatum* cells, being long, sickle-shaped bacteria, simply blocked the binding physically. It can thus be assumed that other bacteria, both in the intestine and in the oral cavity, influence the colonization potential of probiotic bacteria in these sites.

In addition to binding, a strain has to resist the defense factors in saliva, to be able to persist or even act as a probiotic in the mouth. The tested strains survived in saliva and *L. rhamnosus* GG and *Bifidobacterium lactis* Bb12 also resisted the activated peroxidase system, an innate defense system that generates $OSCN^-$ from salivary thiocyanate and hydrogen peroxide. The amount of $OSCN^-$ produced in our experiments is equivalent to *in vivo* concentrations in human whole saliva (28). Hence, there are potential colonizers of the oral cavity among the lactobacillus strains we studied in this paper. Among the probiotic strains only *L. rhamnosus* GG bound well to both the surfaces tested. Together with the fact that *L. rhamnosus* GG also bound well to BEC, it can be considered a potential oral colonizer. Indeed, it has already been recognized as a potential probiotic in the mouth (1, 24).

In summary, there were differences between the oral colonization potential, as tested by bacterial binding, of the strains studied. None of the common dairy strains adhered to saliva-coated surfaces. Of the commercial probiotic strains only one bound well to both saliva-coated surfaces tested and two of the 10 tested strains bound well to saliva-coated HA while the binding of others was low. Similar discrepancies were seen among the fecal isolates. As oral colonization potential is evaluated, it is important to realize that the good adhesion to intestinal mucus does not correlate with the good adherence to oral surfaces. In addition, the bifidobacteria that did not bind to saliva-coated surfaces, bound well to *F. nucleatum*-coated HA, implicating the importance of the other oral bacteria in modulating the colonization potential of the strains tested.

References

1. Ahola AJ, Yli-Knuuttila H, Suomalainen T, Poussa T, Ahlstrom A, Meurman JH, Korpela R. Short-term consumption of probiotic-containing cheese and its effect on dental caries risk factors. Arch Oral Biol 2002: **47**: 799–804.
2. Ahrne S, Nobaek S, Jeppsson B, Adlerberth I, Wold AE, Molin G. The normal *Lactobacillus* flora of healthy human rectal and oral mucosa. J Appl Microbiol 1998: **85**: 88–94.
3. Apostolou E, Kirjavainen PV, Saxelin M, Rautelin H, Valtonen V, Salminen SJ, Ouwehand AC. Good adhesion properties of probiotics: a potential risk for bacteremia? FEMS Immunol Med Microbiol 2001: **31**: 35–39.
4. Apostolou E, Pelto L, Kirjavainen PV, Isolauri E, Salminen SJ, Gibson GR. Differences in the gut bacterial flora of healthy and milk-hypersensitive adults, as measured by fluorescence in situ hybridization. FEMS Immunol Med Microbiol 2001: **30**: 217–221.
5. Aune M, Thomas EL. Accumulation of hypothiocyanite ion during peroxidase-catalyzed oxidation of thiocyanate ion. Eur J Biochem 1977: **80**: 209–214.
6. Busscher HJ, Mulder AF, van der Mei HC. *In vitro* adhesion to enamel and in vivo colonization of tooth surfaces by lactobacilli from a bio-yoghurt. Caries Res 1999: **33**: 403–404.
7. Caglar E, Sandalli N, Twetman S, Kavaloglu S, Ergeneli S, Selvi S. Effect of yoghurt with *Bifidobacterium* DN-173 010 on salivary mutans streptococci and lactobacilli in young adults. Acta Odontol Scand 2005: **63**: 317–320.
8. Carlen A, Borjesson AC, Nikdel K, Olsson J. Composition of pellicles formed *in vivo* on tooth surfaces in different parts of the dentition, and *in vitro* on hydroxyapatite. Caries Res 1998: **32**: 447–455.
9. Carlen A, Rudiger SG, Loggner I, Olsson J. Bacteria-binding plasma proteins in pellicles formed on hydroxyapatite *in vitro* and on teeth *in vivo*. Oral Microbiol Immunol 2003: **18**: 203–207.
10. Chhour KL, Nadkarni MA, Byun R, Martin FE, Jacques NA, Hunter N. Molecular analysis of microbial diversity in advanced caries. J Clin Microbiol 2005: **43**: 843–849.
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. Crociani F, Biavati B, Alessandrini A, Chiarini C, Scardovi V. *Bifidobacterium inopinatum* sp. nov. and *Bifidobacterium denticolens* sp. nov., two new species isolated from human dental caries. Int J Syst Bacteriol 1996: **46**: 564–571.
13. Gibbons RJ, Etherden I. Albumin as a blocking agent in studies of streptococcal adsorption to experimental salivary pellicles. Infect Immun 1985: **50**: 592–594.
14. He F, Ouwehand AC, Isolauri E, Hashimoto H, Benno Y, Salminen S. Comparison of mucosal adhesion and species identification of bifidobacteria isolated from healthy and allergic infants. FEMS Immunol Med Microbiol 2001: **30**: 43–47.
15. He F, Ouwehand AC, Isolauri E, Hosoda M, Benno Y, Salminen S. Differences in composition and mucosal adhesion of bifidobacteria isolated from healthy adults and healthy seniors. Curr Microbiol 2001: **43**: 351–354.
16. Johansson I, Bratt P, Hay DI, Schluckebier S, Strömberg N. Adhesion of *Candida albicans*, but not *Candida krusei*, to salivary statherin and mimicking host molecules. Oral Microbiol Immunol 2000: **15**: 112–118.
17. Kirjavainen PV, Ouwehand AC, Isolauri E, Salminen SJ. The ability of probiotic bacteria to bind to human intestinal mucus. FEMS Microbiol Lett 1998: **167**: 185–189.
18. Kolenbrander PE, Williams BL. Lactose-reversible coaggregation between oral *Actinomycetes* and *Streptococcus sanguis*. Infect Immun 1981: **33**: 95–102.
19. Mäkeläinen H, Tahvonen R, Salminen S, Ouwehand AC. *In vivo* safety assessment of two *Bifidobacterium longum* strains. Microbiol Immunol 2003: **47**: 911–914.
20. Matsumoto M, Tsuji M, Sasaki H, Fujita K, Nomura R, Nakano K, Shintani S, Ooshima T. Cariogenicity of the probiotic bacterium *Lactobacillus salivarius* in rats. Caries Res 2005: **39**: 479–483.
21. 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.
22. Meurman JH, Antila H, Salminen S. Recovery of *Lactobacillus* strain GG (ATCC 53103) from saliva of healthy volunteers after consumption of yoghurt prepared with the bacterium. Microb Ecol Health Dis 1994: **7**: 295–298.
23. Montalto M, Vastola M, Marigo L, Covino M, Graziosetto R, Curigliano V, Santoro L, Cuoco L, Manna R, Gasbarrini G. Probiotic treatment increases salivary counts of lactobacilli: a double-blind, randomized, controlled study. Digestion 2004: **69**: 53–56.
24. Näse L, Hatakka K, Savilahti E, Saxelin M, Ponka A, Poussa T, Korpela R, Meurman JH. 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.
25. Nikawa H, Makihira S, Fukushima H, Nishimura H, Ozaki Y, Ishida K, Darmawan S, Hamada T, Hara K, Matsumoto A, Takemoto T, Aimi R. *Lactobacillus reuteri* in bovine milk fermented decreases the oral carriage of mutans streptococci. Int J Food Microbiol 2004: **95**: 219–223.
26. Ouwehand AC, Isolauri E, Kirjavainen PV, Tolkko S, Salminen SJ. The mucus binding of *Bifidobacterium lactis* Bb12 is enhanced in the presence of *Lactobacillus* GG and *Lact. delbrueckii* subsp. *bulgaricus*. Lett Appl Microbiol 2000: **30**: 10–13.
27. Ouwehand AC, Salminen S, Isolauri E. Probiotics: an overview of beneficial effects. Antonie Van Leeuwenhoek 2002: **82**: 279–289.
28. Pruitt KM, Månsson-Rahemtulla B, Tenovuo J. Detection of the hypothiocyanite (OSCN⁻) ion in human parotid saliva and the effect of pH on OSCN⁻ generation in the salivary peroxidase antimicrobial system. Arch Oral Biol 1983: **28**: 517–525.
29. Sanyal B, Russell C. Nonsporing, anaerobic, gram-positive rods in saliva and the gingival crevice of humans. Appl Environ Microbiol 1978: **35**: 670–678.
30. Schenkels LC, Veerman EC, Nieuw Amerongen AV. Biochemical composition of human saliva in relation to other mucosal fluids. Crit Rev Oral Biol Med 1995: **6**: 161–175.
31. Van Der Ploeg JR, Guggenheim B. Deletion of *gtfC* of *Streptococcus mutans* has no influence on the composition of a mixed-species *in vitro* biofilm model of supragingival plaque. Eur J Oral Sci 2004: **112**: 433–438.
32. Yao Y, Grogan J, Zehnder M, Lendenmann U, Nam B, Wu Z, Costello CE, Oppenheim FG. Compositional analysis of human acquired enamel pellicle by mass spectrometry. Arch Oral Biol 2001: **46**: 293–303.
33. 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.