Oral Microbiology Immunology 2009: 24: 218–223
Printed in Singapore. All rights reserved

ORAL MICROBIOLOGY AND IMMUNOLOGY

In vitro evaluation of yoghurt starter lactobacilli and *Lactobacillus rhamnosus* GG adhesion to saliva-coated surfaces

I. Stamatova[1], K. Kari[2], S. Vladimirov[1], J. H. Meurman[2,3]

[1]Faculty of Dental Medicine, Medical University, Plovdiv, Bulgaria, [2]Institute of Dentistry, University of Helsinki, Helsinki, Finland, [3]Department of Oral and Maxillofacial Diseases, Helsinki University Central Hospital, Helsinki, Finland

Stamatova I, Kari K, Vladimirov S, Meurman JH. In vitro *evaluation of yoghurt starter lactobacilli and* Lactobacillus rhamnosus *GG adhesion to saliva-coated surfaces. Oral Microbiol Immunol 2009: 24: 218–223.* © 2009 John Wiley & Sons A/S.

Aim: The aim of the study was to evaluate the adhesion of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus rhamnosus* strain GG to saliva-coated surfaces *in vitro*.

Methods: Fifteen radiolabeled dairy *L. delbrueckii* subsp. *bulgaricus* strains and *L. rhamnosus* GG were tested for their ability to adhere to saliva-coated hydroxyapatite beads and polystyrene microtiter plates and the radioactivity was measured by liquid scintillation counter. The effects of lysozyme on the adhesion of lactobacilli and of pretreatment with lactobacilli on the adhesion of *Streptococcus sanguinis* were also assessed.

Results: All strains tested adhered to saliva-coated surfaces but with significantly different binding frequencies. The adhesion of the *L. delbrueckii* subsp. *bulgaricus* strains remained lower in comparison to *L. rhamnosus* strain GG. One *L. delbrueckii* subsp. *bulgaricus* strain showed binding frequency comparable to *S. sanguinis*. Lysozyme pretreatment of the samples significantly increased lactobacillus adhesion to saliva-coated surfaces.

Conclusion: The present results showed significant variations in the adhesion capacity of the *Lactobacillus* strains studied. Adhesion to oral surfaces is of primary importance for bacterial colonization in the mouth. Only one of the *L. delbrueckii* subsp. *bulgaricus* dairy starter culture strains investigated had a high adhesion percentage. This strain might then be considered for further investigations in the oral environment.

Key words: bacterial adhesion; saliva; *Lactobacillus delbrueckii* subsp. *bulgaricus*; probiotics

Iva Stamatova, 3 Christo Botev Blvd., 4000 Plovdiv, Bulgaria
Tel.: + 359 32 602225;
fax: +359 32632687;
e-mail: iva.stamatova@abv.bg

Accepted for publication November 14, 2008

For the past 20 years the oral cavity has been considered as an attractive target for probiotic applications. Among commonly studied probiotics, *Lactobacillus rhamnosus* GG has shown favorable results in the reduction of *Streptococcus mutans* counts in the oral cavity after 7-month consumption of probiotic milk (16) and short-term cheese intake (1). Similar decrease in counts of most common caries pathogens has been observed with the administration of *Bifidobacterium* DN-173 000 and *Lactobacillus reuteri* ATCC 55730 given in various vehicles (3–5). Probiotic intervention has also been reported to reduce the risk of high yeast counts and hyposalivation in the elderly (11). The number of probiotic species tends to increase and new sources of beneficial microbes are being investigated. Yet the specific mechanisms whereby probiotic species affect the microbial balance in the oral ecosystem remain evasive.

Recently, the role of *Lactobacillus delbrueckii* subsp. *bulgaricus* with respect to oral health has been addressed (30). This bacterium is the obligatory starter culture in yoghurt production. A strong strain-specific inhibitory activity against different oral streptococci and *Aggregatibacter actinomycetemcomitans* strains has been detected *in vitro* (29).

In preliminary studies evaluating probiotic properties adhesion is of paramount importance. Bacterial attachment is an essential step for colonization in environments that contain surfaces exposed to a fluid flow. The mouth contains several types of surfaces including keratinized and non-keratinized epithelium, and the teeth, all bathed in saliva. Adhesion is required to prevent the organisms from being washed away by oral fluids, and hence to facilitate the expression of their probiotic properties.

A remarkable tropism for colonization of oral surfaces has been observed within the microbial community. Among the commonly used *in vitro* methods studying adhesion of oral species are the adhesion of microorganisms to saliva-coated hydroxyapatite (sHA) or polystyrene surfaces, and attachment to epithelial cells (18, 23, 27).

The aim of the present study was to assess the ability of several dairy *L. delbrueckii* subsp. *bulgaricus* strains to adhere to saliva-coated surfaces and to evaluate whether this species might affect the adhesion of oral streptococci *in vitro*. We hypothesized that differences exist between the dairy strains and commercially available probiotic species in their ability to adhere to saliva-coated surfaces. We also assumed that *L. delbrueckii* subsp. *bulgaricus*, regularly taken with fermented milks, is able to establish itself in oral biofilms, so modifying the microbial composition of the biofilm.

Materials and methods

Bacterial strains and growth conditions

Fifteen *L. delbrueckii* subsp. *bulgaricus* strains kindly provided by LB Lactis (Applied and Environmental Laboratory for Probiotics, Plovdiv, Bulgaria) and a commercial probiotic strain, *L. rhamnosus* GG (Valio Ltd., Helsinki, Finland), were used. Two strains of *Streptococcus sanguinis* and *S. mutans* ATCC 25175 were used as positive controls. Table 1 shows the type and origin of strains used in the study. Stock cultures were stored in 20% skimmed milk at −70°C. In all experiments lactobacilli were grown in De Man, Rogosa and Sharpe (i.e. MRS) broth (Lab M, Ltd., Bury, Lancashire, UK) for 18–20 h at 37°C in 5% CO_2. Streptococci were grown in brain–heart infusion broth (Difco Laboratories, Detroit, MI) at 37°C in 5% CO_2 overnight. For adhesion studies the bacteria were radiolabeled by growing the cells in appropriate broth supplemented with 10 μl/ml of [methyl-1,2-^{3}H]thymidine, 122 Ci/mmol (GE Healthcare, Chalfont St Giles, Buckinghamshire, UK) as previously described (7). After incubation the bacteria were harvested by centrifugation (2000 ***g***, 7 min), washed thrice and suspended in buffered KCl (0.05 M KCl containing 1 mM KH_2PO_4, 1 mM $CaCl_2$ and 0.1 mM $MgCl_2$ at pH 6.5). The absorbance at 492 nm was adjusted to 0.25 ± 0.05 to standardize the number of bacteria (approximately 10^7 colony-forming units/ml). Preliminary studies indicated that this was the best working density with these bacteria (data not shown).

Table 1. Source and origin of *Lactobacillus* strains used in the study

Strain	Source/origin
L. rhamnosus GG (ATCC 53103)	Valio Ltd., Helsinki, Finland
L. delbrueckii subsp. *bulgaricus* LBL-23	Laboratory collection, LB Lactis, Bulgaria
L. delbrueckii subsp. *bulgaricus* LBL-12	Laboratory collection, LB Lactis, Bulgaria
L. delbrueckii subsp. *bulgaricus* LBL-3	Laboratory collection, LB Lactis, Bulgaria
L. delbrueckii subsp. *bulgaricus* LBL-22	Laboratory collection, LB Lactis, Bulgaria
L. delbrueckii subsp. *bulgaricus* LBL-9	Laboratory collection, LB Lactis, Bulgaria
L. delbrueckii subsp. *bulgaricus* LBL-11	Laboratory collection, LB Lactis, Bulgaria
L. delbrueckii subsp. *bulgaricus* LBL-6	Laboratory collection, LB Lactis, Bulgaria
L. delbrueckii subsp. *bulgaricus* LBL-20	Laboratory collection, LB Lactis, Bulgaria
L. delbrueckii subsp. *bulgaricus* LBL-39	Laboratory collection, LB Lactis, Bulgaria
L. delbrueckii subsp. *bulgaricus* LBL-42	Laboratory collection, LB Lactis, Bulgaria
L. delbrueckii subsp. *bulgaricus* LBL-43	Laboratory collection, LB Lactis, Bulgaria
L. delbrueckii subsp. *bulgaricus* LBL-10	Laboratory collection, LB Lactis, Bulgaria
L. delbrueckii subsp. *bulgaricus* LBL-81	Laboratory collection, LB Lactis, Bulgaria
L. delbrueckii subsp. *bulgaricus* LBL-13	Laboratory collection, LB Lactis, Bulgaria
L. delbrueckii subsp. *bulgaricus* LBL-83	Laboratory collection, LB Lactis, Bulgaria
S. sanguinis ATCC 10556	American Type Culture Collection (ATCC)
S. sanguinis, serotype I 972	Clinical isolate
S. mutans ATCC 25175	ATCC

Saliva collection and *in vitro* adhesion assays

Saliva collection

Unstimulated whole saliva was collected from five healthy individuals who were instructed not to eat, drink, smoke, or use chewing gum for an hour before the saliva collection. Informed consent was obtained before the collection began.

The saliva was collected into chilled tubes on ice and clarified by centrifugation (14,000 ***g*** for 20 min at 4°C). The pooled samples were divided into aliquots and frozen at −20°C before the adhesion assays.

Adhesion to sHA beads

Aliquots of 50 mg spheroid HA beads (Macro-Prep Ceramic Hydroxyapatite TYPE II 80 μm, Bio-Rad Laboratories, Hercules, CA) were washed three times in 10 ml buffered KCl (0.05 M KCl containing 1 mM KH_2PO_4, 1 mM $CaCl_2$ and 0.1 mM $MgCl_2$ at pH 6.5), in glass test tubes and equilibrated for 2 h in the same buffer. A 200-μl aliquot of this suspension was added to each well of V-bottom-shaped 96-well plates (2 μg HA/well) (Thermo Scientific, Loughborough, UK) and allowed to settle for 30–60 s. The supernatant was removed by aspiration, and 100 μl saliva was added per well and the mixture was incubated for 1 h at 37°C. After three washings with buffered KCl (200 μl/well) 100 μl radioactive bacterial suspension was added to each well and incubated with shaking (50 r.p.m.) for 1 h. To remove non-adherent bacteria the wells were washed three times with 200 μl KCl and the HA beads were suspended in 200 ml KCl were transferred into scintillation liquid (High Safe 3, Perkin Elmer, Groningen, the Netherlands). The radioactivity was measured by liquid scintillation counter (Winspectral 1414, Wallac, Turku, Finland). Three parallel wells were used for each strain in three independent experiments. The adhesion ratio (%) of bacteria was calculated by comparing the radioactivity of the adhered bacteria to the radioactivity of the added bacteria.

Adhesion to saliva-coated microtiter plates

Adhesion to human saliva was assessed according to the method studying adhesion to intestinal mucus as described earlier by Ouwehand et al. (22). In brief, saliva was

immobilized passively overnight at 4°C in 96-well polystyrene microtiter plates (Maxisorp, Nunc, Roskilde, Denmark; 100 μl/well), followed by two washes with 200 μl/well HEPES–Hanks' buffer (10 mmol/l HEPES; pH 7.4). Bacterial suspensions were added (100 μl/well) and bacteria were allowed to adhere at 37°C for 1 h. Wells were washed thrice with 200 μl HEPES–Hanks' buffer to remove non-adherent bacteria. Bacteria bound to saliva were released and lysed with 1% sodium dodecyl sulfate–0.1 M NaOH by incubation at 60°C for 1 h. The radioactivity was measured as described above. To assess the effect of lysozyme (Chicken Egg White, Sigma Chemicals Co., St Louis, MO) on adhesion of lactobacilli, the radiolabeled bacteria were pretreated with the enzyme [0.05 mg/ml in phosphate-buffered saline (PBS), pH 6.2] for 1 h at 37°C and washed three times with PBS, pH 7.2 (21). Bacterial suspensions incubated with PBS were used as controls. Adhesion of lysozyme-pretreated samples to saliva-coated plates was performed as described above. The effect of the treatment was calculated by comparing the adhesion of the treated plates to the respective buffer control.

Adhesion to solvents

Microbial adhesion to n-hexadecane was measured according to the method of Rosenberg et al. (25). Briefly, bacteria were harvested after 20 h of incubation by centrifugation at 5000 ***g*** for 20 min at room temperature, washed twice with PUM buffer (pH 7.1, 22.2 g $K_2HPO_4.3H_2O$; 7.26 g KH_2PO_4; 1.8 g urea; 0.2 g $MgSO_4.7H_2O$; and distilled water to 1000 ml) and resuspended in the same buffer. The optical density at 492 nm was adjusted to 0.5 (A_0); 600 μl *n*-hexadecane (Sigma Aldrich Chemie GmbH, Buchs, Germany) was added to 1.2 ml bacterial suspension and left for 10 min at 22°C. Each test tube was stirred vigorously for 1 min to allow the two phases to mix. Samples were incubated for 20 min at 22°C and the optical density of the aqueous phase was measured (A_1) (Multiscan Plus, Labsystems, Helsinki, Finland). Adhesion was calculated according to the formula: Adhesion % = $(1 - A_1/A_0) \times 100$.

Adhesion of oral streptococci to saliva-coated microtiter plates after pretreatment with lactobacilli

To study the effect on adhesion of *S. sanguinis* ATCC 10556 after lactobacillus pretreatment of saliva-coated MaxiSorp plates, non-radiolabeled *L. delbrueckii* subsp. *bulgaricus* strains were allowed to adhere to immobilized saliva for 1 h at 37°C. After two washes with HEPES–Hanks' buffer, 100 μl streptococcal suspension was added per well and incubated for 1 h at 37°C and the adhesion experiment was performed as already described.

Statistical analysis

The results from the adhesion experiments are expressed as the average of three independent experiments, and each adhesion assay was performed with three parallels to correct for intra-examiner variation. Student's *t*-test was used to analyse differences between the samples. The significance was set at $P < 0.05$. Spearman correlation coefficients were calculated to assess the possible connection between binding to saliva-coated surfaces and hydrophobicity.

Results

The adhesion was measured quantitatively by applying the radiolabeled bacteria to saliva-coated surfaces. Saliva-coated HA beads have been commonly used as an *in vitro* model to study adhesion because the surface properties are similar to those of tooth enamel (8). Before the experiment three of the strains were incubated with HA beads equilibrated with buffered KCl only to evaluate the effect of saliva on microbial adhesion. Adhesion of these strains was significantly higher to non-saliva-coated HA beads compared to saliva-coated surfaces, providing evidence that saliva modifies adhesion (data not shown).

Lactobacilli strains used in the present study were found to adhere to sHA from 1 to 17%. LBL-39 showed adhesion percentages to sHA comparable to that of the reference strains *S. sanguinis*, as given in Table 2. *S. sanguinis* is the first colonizer on tooth surfaces *in vivo* and its ability to adhere to sHA make it a suitable model for dental adhesion studies. In the present series, we observed that there is variation in adhesion between the strains used as references. The wild-type of *S. sanguinis* adhered better to saliva-coated microtiter plates than the ATCC strain. *S. mutans* adhesion did not differ significantly from lactobacillus adhesion to the two types of saliva-coated surfaces. Generally, the adhesion of most *L. delbrueckii* subsp. *bulgaricus* strains to sHA was low (<5%) under the present experimental conditions. The adhesion to saliva-coated Maxisorp plates ranged between 3 and 22%, with LBL-39 exhibiting the strongest ability to adhere. The commercially available probiotic *L. rhamnosus* GG showed significantly higher levels of adhesion to saliva-coated surfaces compared to the *L. delbrueckii* subsp. *bulgaricus* strains (Table 2). Most of the strains showed similar behavior on both the saliva-coated surfaces (Spearman correlation 0.905, $P < 0.001$).

A significant increase in the adhesive properties was observed when the strains were pretreated with lysozyme ($P < 0.05$); results are shown in Fig. 1.

Table 2. Adhesion[1] to saliva-coated hydroxyapatite and microtiter plates of lactobacilli

Strain	Adhesion to sHA (mean % ± SD)	Adhesion to sMaxisorp (mean % ± SD)
L. rhamnosus GG (ATCC 53103)	9.89 ± 0.11	16.09 ± 0.04
L. delbrueckii subsp. *bulgaricus* LBL-23	5.68 ± 0.06	8.69 ± 0.07
L. delbrueckii subsp. *bulgaricus* LBL-12	2.76 ± 0.003	6.16 ± 0.02
L. delbrueckii subsp. *bulgaricus* LBL-3	4.33 ± 0.05	5.53 ± 0.04
L. delbrueckii subsp. *bulgaricus* LBL-22	2.64 ± 0.01	7.18 ± 0.02
L. delbrueckii subsp. *bulgaricus* LBL-9	2.13 ± 0.01	3.40 ± 0.02
L. delbrueckii subsp. *bulgaricus* LBL-11	2.34 ± 0.01	3.30 ± 0.03
L. delbrueckii subsp. *bulgaricus* LBL-6	1.58 ± 0.01	8.84 ± 0.06
L. delbrueckii subsp. *bulgaricus* LBL-20	1.39 ± 0.004	9.71 ± 0.03
L. delbrueckii subsp. *bulgaricus* LBL-39	17.23 ± 0.05	21.93 ± 0.03
L. delbrueckii subsp. *bulgaricus* LBL-42	2.62 ± 0.01	5.35 ± 0.01
L. delbrueckii subsp. *bulgaricus* LBL-43	1.55 ± 0.002	8.39 ± 0.03
L. delbrueckii subsp. *bulgaricus* LBL-10	1.58 ± 0.01	4.30 ± 0.01
L. delbrueckii subsp. *bulgaricus* LBL-81	1.27 ± 0.01	8.61 ± 0.02
L. delbrueckii subsp. *bulgaricus* LBL-13	2.00 ± 0.02	6.01 ± 0.03
L. delbrueckii subsp. *bulgaricus* LBL-83	1.26 ± 0.01	4.68 ± 0.03
S. sanguinis ATCC 10556	18.89 ± 0.08	14.39 ± 0.04
S. sanguinis, serotype I 972	11.18 ± 0.05	25.55 ± 0.08
S. mutans ATCC 25175	2.99 ± 0.01	5.86 ± 0.02

[1]Adhesion was calculated based on results from three independent experiments in which each strain was tested in triplicate.
ATCC, American Type Culture Collection; sHA, saliva-coated hydroxyapatite.

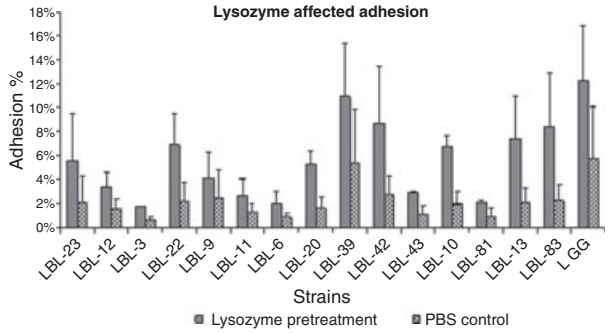

Fig. 1. Adhesion of the lactobacilli strains to saliva-coated Maxisorp plates after lysozyme pretreatment. For abbreviations, see Table 1.

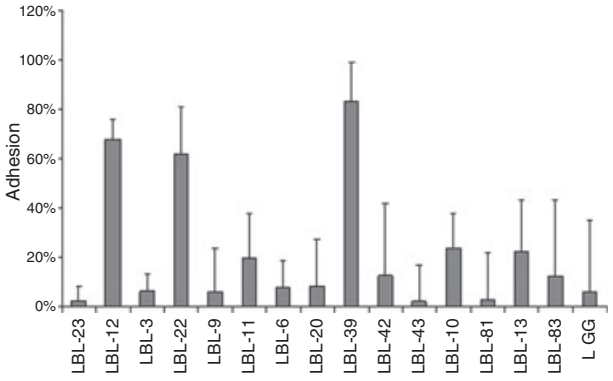

Fig. 2. Adhesion to *n*-hexadecane of the lactobacilli strains. For abbreviations, see Table 1.

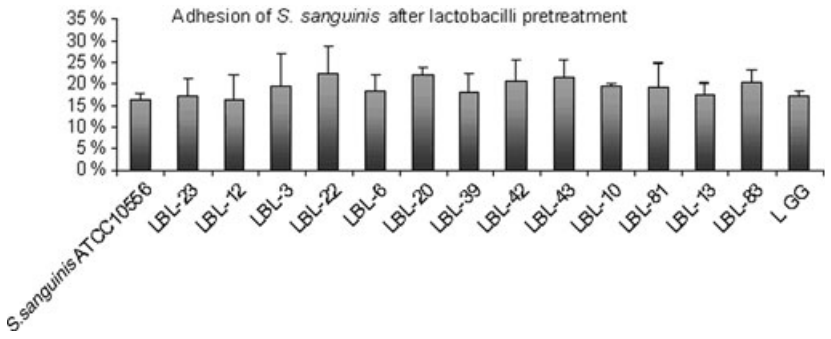

Fig. 3. Adhesion of *Streptococcus sanguinis* ATCC 10556 to saliva-coated microtiter plates after pretreatment with the lactobacilli studied. For abbreviations, see Table 1.

Cell surface hydrophobicity has been considered a valuable reference when evaluating the adhesive properties of microorganisms, and high hydrophobicity correlated with marked adhesion (34). By measuring adhesion to *n*-hexadecane we observed that the strains investigated showed various patterns of interaction with the organic solvent, as shown in Fig. 2. *Lactobacillus* strain LBL-39, which had shown the most pronounced adhesive properties to saliva-coated surfaces, again displayed the strongest adhesive potential. A moderately strong relationship between adhesion to sHA beads and hydrophobicity was observed (correlation coefficient 0.575, $P < 0.05$), whereas the relationship between adhesion to saliva-coated microtiter plates and hydrophobicity was weak (correlation coefficient 0.447, $P < 0.05$).

As *S. sanguinis* and lactobacilli were able to adhere to saliva-coated surfaces we hypothesized that these two species may compete when present together. To study the influence of adhered lactobacilli, the *Lactobacillus* strains were allowed to bind to immobilized saliva on Maxisorp plates and *S. sanguinis* ATCC 10556 was incubated subsequently. The adhesion of *S. sanguinis* was not significantly affected by the pretreatment of the wells with any of the lactobacillus strains, as shown in Fig. 3.

Discussion

Adhesion of bacteria to host surfaces is regarded as of major importance in contributing to permanent, or even transient, establishment of probiotic species in any environmental niche. In the present study we focused on the bacterial adhesion to human saliva that is the main fluid overlying oral surfaces. Saliva is the first biological constituent in the contact between microorganisms and the host and it forms a protein pellicle on all oral tissue surfaces exposed in the mouth. Presumably probiotic bacteria that express good binding ability to salivary pellicle may also be able to colonize the oral cavity. The method employed in the present study allows quantitative measurement of the adhesion of radioactively labeled bacteria to saliva-coated surfaces. HA beads were chosen as a substrate that shares common surface characteristics with tooth enamel. Recently, Vesterlund et al. (33), by evaluating five different methods for assessing bacterial adhesion, have concluded that the use of radioactive labels offers the best reproducibility and sensitivity when poorly adherent bacteria (<1%) are being studied.

The primary aim of our *in vitro* study was to assess whether one of the main yoghurt starter microorganisms, namely *L. delbrueckii* subsp. *bulgaricus*, possesses adhesive properties allowing its putative prolonged establishment in the oral cavity. Yoghurt is one of the most common ways of delivering probiotics even though the probiotic effectiveness of yoghurt starter cultures has long been debated (14, 17, 35). By a recently reached consensus this species could be regarded as probiotic (10). However, in this respect the use of *L. delbrueckii* subsp. *bulgaricus* in the oral cavity has not been thoroughly investigated and the only clinical study has shown inconclusive results (24).

All the strains in our present study were found to adhere to sHA and polystyrene plates. However, when compared with the reference streptococcal strains the adhesion capacity of the studied lactobacilli was low. *S. sanguinis* is a distinguished front-line colonizer and anchoring species in the development of oral biofilms. *In vitro* studies have shown that the adhesion of

this microorganism to saliva-coated surfaces is mediated by both lectin-carbohydrate and non-lectin interactions (9, 13, 15). Among the lactobacilli we tested there was a single strain standing out as a strong binder, displaying similar adhesive properties comparable to the control streptococci. Yet, the commercially available probiotic *L. rhamnosus* GG adhered still better to the saliva-coated surfaces in all experiments when compared with most of the *L. delbrueckii* subsp. *bulgaricus* strains investigated. The *L. rhamnosus* GG species has already been ascertained as a putative probiotic in the oral cavity (1, 16).

The pattern of adherence to sHA and microtiter wells seemed similar, as shown by the high correlation coefficient. A relatively strong relationship has been observed in previous studies assessing the *in vitro* adhesion of potential probiotics to saliva-coated surfaces (12). Based on these results, it could be supposed that binding relies not solely on hydrophobic interactions, but also on specific adhesin–receptor reciprocal actions. The exact mechanisms of adhesion to salivary pellicle call for further investigations, however.

The assessment of cell surface hydrophobicity might be used as a test for studying adhesive properties of bacteria because this characteristic has been reported to objectively reflect microbial adhesion (6, 34). On the other hand, there are studies with results that contradict the former statement (20, 28). In the present experiment we observed a relatively strong correlation between the adhesion of strains to sHA and hydrophobicity, whereas no positive relationship was found for binding of bacteria to saliva-coated microtiter wells and cell surface hydrophobicity. Consequently, the correlation between hydrophobicity and adhesion remains debatable.

To be able to adhere to oral surfaces a probiotic candidate should be able to withstand the defense mechanisms in the oral cavity. In this perspective, we also evaluated the effect of lysozyme on the adhesive properties of lactobacilli. The lysozyme concentration used (0.05 mg/ml) was within the physiological limits for unstimulated whole saliva [0.01–0.20 mg/ml (32)]. Lysozyme possesses strong antimicrobial activity by breaking down bacterial cell walls and so releasing cell wall components. After 60 min of lysozyme pretreatment there was a significant increase in microbial binding to saliva-coated microtiter plates. The increase in adhesion was strain specific and observed for all strains. However, this observation is inconclusive and merits further investigations. Contrary to our results, Ouwehand et al. (21) have found that lysozyme pretreatment of *L. rhamnosus* GG leads to a significant reduction in its adhesion to immobilized intestinal mucus. The discrepancy between this observation and the results of our study might be the result of the different substrates used for assessing adhesion. Tellefson and Germaine (31) have found that lysozyme promoted the adherence of some oral streptococci (*S. sanguinis*) to sHA. The role of lysozyme pretreatment on probiotic properties has recently been addressed as a factor improving the immunostimulatory effect of probiotic species (2). The viability of strains after lysozyme pretreatment remains questionable. We observed a double reduction in the number of bacteria after lysozyme treatment compared to the PBS control (data not shown). Under the conditions of the present experiment it cannot be determined whether the increased adhesion was the result of the attachment of non-viable bacteria or of some cellular fractions of the microorganisms.

A commonly adopted principle of probiotics is that these bacteria should be able to survive the conditions from consumption to the transit to the specific target site. However, the definition of 'probiotics' may need to be reconsidered because the result of discoveries by Japanese scientists suggest that inactivated probiotic microorganisms or their cell structures may also have beneficial effects on human health (26). Consequently, even though lysozyme damages cell integrity, the increased adhesion observed in our series could predispose for probiotic activity, particularly if intracellular fractions have biological and beneficial health effects. This, however, remains to be investigated in further studies.

As a final part of the present study we evaluated the potential of lactobacilli to modulate the adhesion of *S. sanguinis*. The competitive inhibition for bacterial adhesion sites has been considered as a favorable mechanism of probiotic action (7). Despite the fact that lactobacilli adhered to various extents to the immobilized saliva they were not able to affect the adhesion of the target microorganism tested. It could therefore be concluded that the salivary receptors are different for dairy strains and *S. sanguinis* and that pretreatment with lactobacilli does not block streptococcal adhesion by steric hindrance. Similar results were observed for other probiotic species that also lacked the capacity to change the adhesive potential of several skin pathogens (19).

The present study was the first to evaluate the adhesive properties of *L. delbrueckii* subsp. *bulgaricus* strains to saliva-coated surfaces. Despite the limitations of the *in vitro* tests they are useful tools for screening and selecting bacteria for a particular probiotic use. Data obtained in our experimental study demonstrate that yoghurt starter cultures (*L. delbrueckii* subsp. *bulgaricus*) may also adhere to oral surfaces. However, well-designed human trials are necessary to draw further conclusions and for the eventual development of probiotic products targeted at the oral cavity.

References

1. Ahola AJ, Yli-Knuuttila H, Suomalainen T et al. Short-term consumption of probiotic-containing cheese and its effect on dental caries risk factors. Arch Oral Biol 2002: **47**: 799–804.
2. Bu HF, Wang X, Zhu YQ et al. Lysozyme-modified probiotic components protect rats against polymicrobial sepsis: role of macrophages and cathelicidin-related innate immunity. J Immunol 2006: **177**: 8767–8776.
3. Çaglar 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.
4. Çaglar E, Kuscu OO, Cildir SK, Kuvvetli SS, Sandalli N. A probiotic lozenge administered medical device and its effect on salivary mutans streptococci and lactobacilli. Int J Ped Dent 2008: **18**: 35–39.
5. Çaglar E, Sandalli N, Twetman S, Kavaloglu S, Ergeneli S, Selvi S. Effect of yogurt with *Bifidobacterium* DN-173 010 on salivary mutans streptococci and lactobacilli in young adults. Acta Odontol Scand 2005: **63**: 317–320.
6. Ellepola AN, Samaranayake LP. Investigative methods for studying the adhesion and cell surface hydrophobicity of *Candida* species: an overview. Microb Ecol Health Dis 2001: **13**: 46–54.
7. Fernandez MF, Boris S, Barbes C. Probiotic properties of human lactobacilli strains to be used in the gastrointestinal tract. J Appl Microbiol 2003: **94**: 449–455.
8. Gibbons RJ, Etherden I. Enzymatic modification of bacterial receptors on saliva-treated hydroxyapatite surfaces. Infect Immun 1982: **36**: 52–58.
9. Gong K, Mailloux L, Herzberg MC. Salivary film expresses a complex, macromolecular binding site for *Streptococcus sanguinis*. J Biol Chem 2000: **275**: 8970–8974.
10. Guarner F, Perdigon G, Corthier G, Salminen S, Koletzko B, Morelli L. Should yoghurt cultures be considered probiotic? Br J Nutr 2005: **93**: 783–786.
11. 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.
12. 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.
13. Hsu SD, Cisar A, Sandberg L, Kilian M. Adhesive properties of viridans group streptococcal species. Microb Ecol Health Dis 1994: **7**: 125–137.
14. Johns P, Pereira SL, Leonard AE et al. Cytoprotective agent in *Lactobacillus bulgaricus* extracts. Curr Microbiol 2007: **54**: 131–135.
15. Kolenbrander PE, London J. Adhere today, here tomorrow: oral bacterial adherence. J Bacteriol 1993: **175**: 3247–3252.
16. 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.
17. Nova E, Toro O, Varela P, Lopez-Vidriero I, Morande G, Marcos A. Effects of a nutritional intervention with yogurt on lymphocyte subsets and cytokine production capacity in anorexia nervosa patients. Eur J Nutr 2006: **45**: 225–233.
18. Ostengo MC, Nader-Macias E. Hydroxyapatite beads as an experimental model to study the adhesion of lactic acid bacteria from the oral cavity to hard tissues. Meth Molec Biol 2004: **268**: 447–452.
19. Ouwehand AC, Båtsman A, Salminen S. Probiotics for the skin: a new area of potential application? Lett Appl Microb 2003: **36**: 327–331.
20. Ouwehand AC, Kirjavainen PV, Grönlund M, Isolauri E, Salminen S. Adhesion of probiotic microorganisms to intestinal mucus. Int Dairy J 1999: **9**: 623–630.
21. Ouwehand AC, Tölkkö S, Salminen S. The effect of digestive enzymes on the adhesion of probiotic bacteria *in vitro*. J Food Sci 2001: **66**: 856–859.
22. Ouwehand AC, Tuomola EM, Tölkkö S, Salminen S. Assessment of adhesion properties of novel probiotic strains to human intestinal mucus. Int J Food Microbiol 2001: **64**: 119–126.
23. Pathirana RD, O'Brien-Simpson NM, Visvanathan K, Hamilton JA, Reynolds EC. Flow cytometric analysis of adherence of *Porphyromonas gingivalis* to oral epithelial cells. Infect Immun 2007: **75**: 2484–2492.
24. Petti S, Tarsitani G, D'Arca AS. A randomized clinical trial of the effect of yoghurt on the human salivary microflora. Arch Oral Biol 2001: **46**: 705–712.
25. Rosenberg M, Gutnick D, Rosenberg E. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol Lett 1980: **9**: 29–33.
26. Saito T. Selection of useful probiotic lactic acid bacteria from the *Lactobacillus acidophilus* group and their applications to functional foods. Animal Sci J 2004: **75**: 1–13.
27. Sakakibara J, Nagano K, Murakami Y et al. Loss of adherence ability to human gingival epithelial cells in S-layer protein-deficient mutants of *Tannerella forsythensis*. Microbiology 2007: **153**: 866–876.
28. Savage DC. Growth phase, cellular hydrophobicity, and adhesion *in vitro* of lactobacilli colonizing the keratinized gastric epithelium in the mouse. Appl Environ Microb 1992: **58**: 1992–1995.
29. Stamatova I, Kari K, Meurman JH. *In vitro* evaluation of antimicrobial activity of putative probiotic lactobacilli against oral pathogens. Int J Probiotic Prebiotic 2008: **2**: 225–232.
30. Stamatova I, Meurman JH, Kari K, Tervahartiala T, Sorsa T, Baltadjieva M. Safety issues of *Lactobacillus bulgaricus* with respect to human gelatinases *in vitro*. FEMS Immunol Med Microbiol 2007: **51**: 194–200.
31. Tellefson LM, Germaine G. Adherence of *Streptococcus sanguinis* to hydroxyapatite coated with lysozyme and lysozyme-supplemented saliva. Infect Immun 1986: **51**: 750–759.
32. Tenovuo J. Human saliva: clinical chemistry and microbiology, Vol. 2. Boca Raton: CRC Press Inc., 1989: 57–61.
33. Vesterlund S, Paltta J, Karp M, Ouwehand AC. Measurement of bacterial adhesion – *in vitro* evaluation of different methods. J Microbiol Meth 2005: **60**: 225–233.
34. Wadström T, Andersson K, Sydow M, Axelsson L, Lindgren S, Gullmar B. Surface properties of lactobacilli isolated from the small intestine of pigs. J Appl Bacter 1987: **62**: 513–520.
35. Wendakoon CN, Thomson AB, Ozimek L. Lack of therapeutic effect of a specially designed yogurt for the eradication of *Helicobacter pylori* infection. Digestion 2002: **65**: 16–20.