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The role of fructans on dental biofilm formation by *Streptococcus sobrinus*, *Streptococcus mutans*, *Streptococcus gordonii* and *Actinomyces viscosus*

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Abstract

Dental plaque biofilm plays a pivotal role in the progression of dental diseases. Polysaccharides are of great importance in the ecology of the dental biofilm. We studied the effect of fructans, glucans and a mixture of both fructans and glucans, synthesized in situ by immobilized fructosyltransferase or glucosyltransferase, on the adhesion of *Streptococcus sobrinus*, *Streptococcus mutans*, *Streptococcus gordonii* and *Actinomyces viscosus* to hydroxyapatite beads coated with human saliva (sHA). The adhesion of *A. viscosus* to sHA was found to be fructan-dependent. Adhesion of both *S. sobrinus* and *S. mutans* was found to be mediated mainly by glucans, while the adhesion of *S. gordonii* was found to be both glucan- and fructan-dependent. Treatment with fructanase prior to *A. viscosus* adhesion resulted in a significant reduction in adhesion to sHA, while adhesion of *S. sobrinus*, *S. mutans* and *S. gordonii* was slightly influenced by fructanase treatment. Treatment with fructanase after adhesion of *S. gordonii* to sHA resulted in a significant reduction in their adhesion to sHA. Our results show that fructans may play a role in the adhesion and colonization of several cariogenic bacteria to sHA, thus contributing to the formation of dental plaque biofilm.

Keywords: Adhesion; Fructan; Glucan; Fructanase; Dental biofilm

1. Introduction

Formation of dental plaque biofilm is a biological process associated with the attachment, detachment and proliferation of oral bacteria on the tooth surface. The dental biofilm is formed via adhesion of bacteria to an acellular pellicle coating the oral surface [1]. Several types of bacteria participate in the formation of the dental biofilm [2]. *Streptococcus gordonii* and *Actinomyces viscosus* are regarded as early colonizers of tooth surfaces, while mutans streptococci such as *Streptococcus sobrinus* and *Streptococcus mutans* are considered late colonizers of the dental biofilm.

S. gordonii can adhere to the salivary pellicle coating the teeth by specific interactions via sialic acid adhesion [3] or through binding sites to salivary α amylase [4]. *S. gordonii* produces glucosyltransferase (GTF), which synthesizes extracellular glucans from sucrose [5]. *A. viscosus* can adhere to teeth through interactions with salivary proline-rich proteins [6]. Both *S. gordonii* and *A. viscosus* produce fructosyltransferase (FTF), which synthesizes extracellular fructans from sucrose. These fructans function as short-term storage reservoirs in the plaque [7] and may also play a role in bacterial adhesion to the tooth surface. Mutans streptococci have been implicated as the principal etiological agent in dental caries formation, due to their prevalence in carious lesions [8]. Dietary sucrose is essential for the accumulation of this bacterium on teeth and for the initiation of carious lesions [9]. Both *S. sobrinus* and *S. mutans* synthesize extracellular glucans and fructans from sucrose which add to the pathogenicity of the dental plaque [7,8,10–12].

Studies on dental plaque have demonstrated the importance of fructans, in addition to glucans, in the pathogenicity of the dental biofilm. Munro et al. [13] have found that inactivation of the *ftf* gene, responsible for fructan production in *S. mutans*, resulted in reduced caries lesions. Furthermore, an increase in glucan production was found in *S. mutans* with inactivated *ftf* genes [14]. On the other hand, Wexler et al. [15] found that the caries process in the

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presence of a fructanase-deficient *S. mutans* in a rat caries model fed a high sucrose diet was similar in the number or severity of caries lesions to that caused by the wild-type. However, Burne et al. [16] demonstrated a cariogenic effect of fructans in a program-fed rat model on sulcal caries severity.

The purpose of the present study was to investigate the influence of in situ synthesized fructans and/or glucans by immobilized cell-free enzymes on the formation of a homogeneous mono-species dental biofilm composed of either *S. sobrinus*, *S. mutans*, *S. gordonii*, or *A. viscosus*.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains used in this study were *S. sobrinus* 6715 (laboratory strain), *S. mutans* ATCC 27351, *S. gordonii* ATCC 10558 and *A. viscosus* ATCC 43146. Radiolabeled bacteria were prepared as described previously [12,17]. Briefly, bacteria were grown to a late exponential phase in TY medium (1.4% tryptone and 0.8% yeast extract) with 5 μCi ml^{-1} of [^{3}H-methyl]thymidine (NEN-products, Boston, MA, USA) at 37°C under aerobic conditions supplemented with 5% CO_2. Following incubation, the bacterial suspension was washed four times by centrifugation and resuspended in buffered KCl pH 6.5 (50.0 mM KCl, 1.0 mM $CaCl_2{\cdot}2H_2O$, 0.65 mM KH_2PO_4, 0.35 mM K_2HPO_4 and 0.1 mM $MgCl_2{\cdot}6H_2O$).

2.2. GTF preparation

GTF was prepared as described previously [18]. Briefly, *S. sobrinus* 6715 was grown in 12 000–14 000 molecular mass cut-off dialysis tubing (Spectrum Industries Inc., Los Angeles, CA, USA) containing 150 ml semi-defined medium (1.67% casamino acids, 0.15% $(NH_4)_2SO_4$, 0.045% $MgSO_4{\cdot}7H_2O$, 0.96% KH_2PO_4 and 4.3% K_2HPO_4 in distilled water). The dialysis tubing was immersed in 3 l of enriched TY medium (1.4% tryptone, 0.8% yeast extract, 0.27% K_2HPO_4 and 0.05% $MgSO_4{\cdot}7H_2O$ in distilled water) supplemented with 0.67% D-sorbitol, 0.2% D-glucose and 0.09 mM $MnSO_4$. After 18 h incubation at 37°C, the bacteria were removed by centrifugation at $9000\times g$ for 20 min at 4°C and the supernatant fluid was supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) as a protease inhibitor and 0.02% sodium azide (NaN_3) as preservative. Next, the supernatant fluid was concentrated and washed with buffered KCl using ultrafiltration techniques with a 30-kDa cut-off membrane (YM 30, Amicon Inc., Danvers, MA, USA) in a stirred cell ultrafiltration device (Amicon model 8400) at 4°C. The GTF preparation used for the experiments polymerized 0.02 μmol glucose per minute, as determined using [^{14}C-glucose]sucrose, as previously described [12].

2.3. FTF preparation

FTF was prepared from *Streptococcus salivarius* ATCC 25975. Bacteria were grown in dialysis tubing and removed as described above. The supernatant fluid containing cell-free enzyme was filtered through a 100-kDa cut-off membrane (YM 100, Amicon) and concentrated using a 10-kDa cut-off membrane (YM 10, Amicon) in a stirred cell ultrafiltration device (Amicon) at 4°C. Next, the concentrate was loaded onto a DEAE-cellulose column (Bio-Rad laboratories, Hercules, CA, USA). The column was eluted with a gradient of 0–500 mM NaCl in 1.0 mM phosphate buffer pH. 6.5 at 4°C. The fractions were assayed for FTF activity using [^{3}H-fructose]sucrose and for fructanase activity as described below. The fractions containing FTF activity, but not fructanase activity, were combined and concentrated by ultrafiltration. The measured activity of the final FTF preparation was 0.024 μmol of fructose polymerized per minute.

2.4. Fructanase preparation

Fructanase was prepared from *S. mutans* V-1995, generated from the FTF hyperproducing strain V-403 by allelic exchange inactivation of the *gtf* genes [13]. After 24 h of incubation at 37°C in Brain Heart infusion (Difco Laboratories, Detroit, MI, USA) the bacteria were removed by centrifugation as described above. The supernatant fluid was supplemented with 1 mM PMSF and 0.02% NaN_3. The proteins in the supernatant fluid were precipitated step by step with ammonium sulfate at room temperature. The precipitates were collected after centrifugation at $9000\times g$ and dissolved in 10 mM phosphate buffer pH 6.5. Fructanase activity was detected at concentrations of ammonium sulfate between 2.0 and 2.7 M. These fractions were dialyzed against phosphate buffer and concentrated over a 10-kDa cut-off membrane (YM 10, Amicon) in a stirred cell ultrafiltration device (Amicon) at 4°C. The concentrate obtained after the ultrafiltrate was diluted to a final volume of 100 ml with phosphate buffer, pH 6.5, and loaded onto an anion exchange chromatography column of DEAE-cellulose (Bio-Rad). Fractions were eluted from the column with NaCl gradient (0–500 mM) in 1 mM phosphate buffer pH 6.5 and tested for fructanase activity by determining the amount of reducing sugar liberated by hydrolysis of 0.2% levan (used as substrate) after 24 h incubation at 37°C, using the Somogi method [19]. Fractions containing fructanase activity were eluted at 50 mM NaCl. The enzymatic activity of the above fructanase preparation was 0.005 μmol of liberated fructose per minute.

2.5. Saliva samples

Unstimulated clarified whole saliva samples were collected in the morning, 2 h before the beginning of each

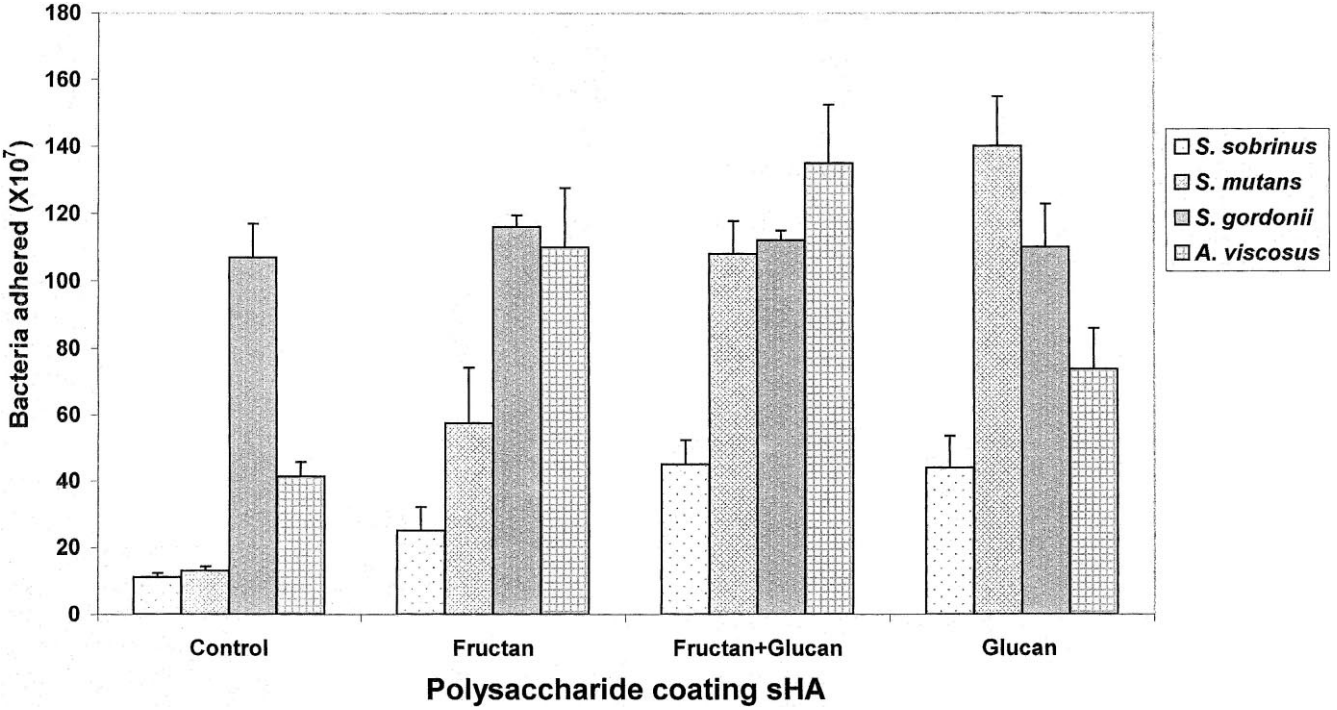

Fig. 1. Adhesion of *S. sobrinus* 6715, *S. mutans* ATCC 27351, *S. gordonii* ATCC 10558 and *A. viscosus* ATCC 43146 to sHA coated with different polysaccharides. Bacterial adhesion to sHA served as control. The number of bacteria adhered to the sHA surfaces was calculated from CFU ± S.D. of triplicate samples conducted in two different experiments.

experiment. One healthy female donor, aged 26, without active periodontal disease or active caries, was used as a single source for the salivary samples in order to minimize variation in the samples. The saliva samples were centrifuged for 20 min at 1500 × *g* (Universal 16, Hettich, Germany) at room temperature. The clear supernatant fluid was collected and used the same day for the experiments as described below. The above salivary samples did not contain significant amounts of bacteria. The activity of salivary enzymes, originating from the oral bacteria, was found to be non-significant under the experimental conditions.

2.6. Preparation of fructan- and/or glucan-coated pellicles

Experimental pellicle was prepared as previously described [18,20]. In brief, 40 mg hydroxyapatite (HA) beads (Bio-Rad) (size 80 μm, surface area 40 m^2 g^{-1}) were equilibrated with two washes of buffered KCl. The HA beads were then coated with unstimulated clarified whole saliva (sHA) (prepared as described above) for 30 min at 37°C on a shaker. Following incubation, the beads were washed twice with buffered KCl to remove unbound salivary components. Next, sHA beads were exposed for 60 min at 37°C to FTF enzyme, to GTF enzyme, or to a mixture of both FTF and GTF enzymes. After removal of the unbound FTF or GTF by washes with buffered KCl (98% of the enzymes remained bound to sHA after the washes), the FTF- or GTF-coated sHA beads were incubated for 60 min at 37°C with a 4% sucrose solution, while rotating. Following in situ fructan formation (when exposed to FTF) or glucan formation (when exposed to GTF), or fructan and glucan formation (when exposed to both FTF and GTF) on the surface of the sHA, the beads were washed by buffered KCl to remove unbound and loosely bound polysaccharides (fructans or glucans).

2.7. Bacterial adherence to HA beads coated with fructan and/or glucan polysaccharides

The influence of polysaccharides on sHA with respect to the adhesion of *S. sobrinus*, *S. mutans*, S. *gordonii* and *A. viscosus* was measured by using pre-labeled radioactive bacteria [12,17]. Radioactive bacteria (3.25–3.3 × 10^9 cells), prepared as described before, were exposed to sHA without polysaccharide coating (control) or to sHA coated in situ with fructan (1.45 μmol), glucan (1.25 μmol) or a mixture of both fructan and glucan (1.32 μmol) for 120 min at 37°C with gentle rotation. At the end of the incubation period, the beads were washed four times with buffered KCl to remove non-adsorbed bacteria, and then rinsed with 4 ml ethanol into vials containing 10 ml scintillation fluid (Ecoscint A, National Diagnostics, Atlanta, GA, USA). The amount of radioactively labeled bacteria adsorbed onto the sHA beads coated with different polysaccharides was measured using a β-counter (Beta-matic, Kontron, Switzerland). The specific thymidine incorporation value (counts per minute/vital bacteria) was determined as described previously [21]. Data are expressed as number of vital bacteria, measured as colony forming

units (CFU). The number of bacteria adsorbed onto sHA without polysaccharide coating was used as a control.

2.8. Influence of fructan degradation on adsorption and deposition of bacteria to biofilm

The influence of fructans on bacterial adhesion was examined with respect to the degradation of fructans with fructanase before or after bacterial adhesion. Fructans were selectively removed from the fructan-coated experimental pellicle by enzymatic treatment with fructanase before bacterial adhesion. Briefly, fructan-coated HA beads were incubated with fructanase in buffered KCl for 120 min at 37°C, while rotating on a shaker, after which the HA beads were washed twice with buffered KCl. Following the fructanase treatment, the HA beads were incubated with the bacteria ($6.5–6.6\times10^9$ cells) and the amount of *S. sobrinus*, *S. mutans*, *S. gordonii* or *A. viscosus* adsorbed onto the pellicle was measured as described above.

In additional experiments, the effect of fructanase on deposition of the pre-adsorbed bacteria to fructan-coated sHA was examined. After adsorption of radioactively labeled bacteria to fructan-coated HA beads, the beads were washed with buffered KCl, and then exposed to fructanase in buffered KCl for 120 min at 37°C, while rotating on the same shaker. Following the fructanase treatment, the HA beads were washed with buffered KCl and then rinsed into vials containing scintillation fluid in order to measure the amount of remaining adsorbed bacteria. In the control samples (no treatment), the adherence of radioactively labeled bacteria to fructan-coated HA beads without fructanase treatment was tested in the same conditions of incubation and washing by buffered KCl as described above.

2.9. Statistical analysis

The different sets of experiments described above were conducted in triplicate. These results were analyzed statistically using the Wilcoxon/Kruskal–Wallis tests of JMP 2 statistical program for Macintosh. The degree of significance was determined at $P<0.05$.

3. Results

3.1. Contribution of fructan and glucan polysaccharides to bacterial adherence to sHA

Results of the adhesion of *S. sobrinus*, *S. mutans*, *S. gordonii* and *A. viscosus* to sHA surfaces coated with fructans, glucans or a mixture of the polysaccharides are presented in Fig. 1. Adherence of the tested bacteria to sHA surfaces coated with different polysaccharides was higher compared to their adherence to sHA without polysaccharide coating (control). However, different adhesion profiles were found for the various polysaccharide coatings. *S. sobrinus* adhered 45% less ($P<0.05$) to fructan-coated sHA than to the glucan-coated pellicle. The presence of

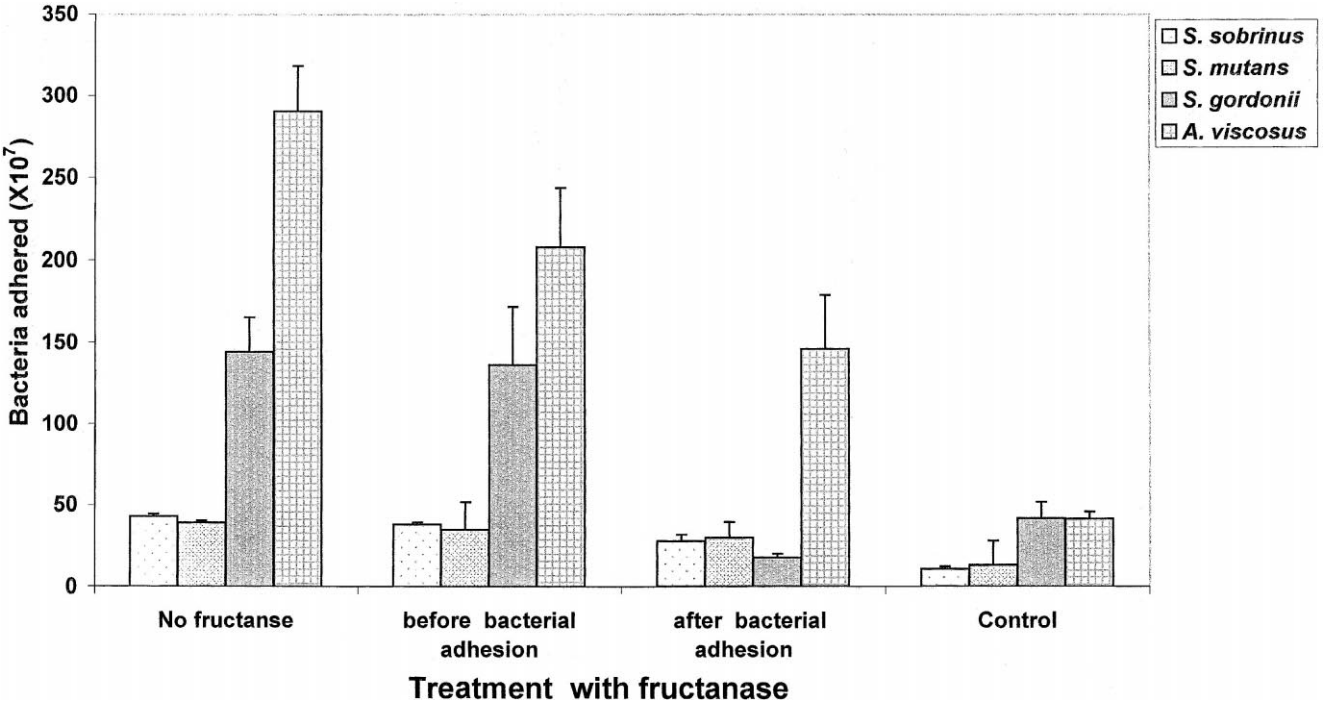

Fig. 2. Adhesion of *S. sobrinus* 6715, *S. mutans* ATCC 27351, *S. gordonii* ATCC 10558 and *A. viscosus* ATCC 43146 to fructans-coated sHA without treatment (no fructanase), and after fructanase treatment. Treatment with fructanase was tested before and after bacterial adherence. Bacterial adhesion to sHA served as control. The number of bacteria adhered to the sHA surfaces was calculated from CFU ± S.D. of triplicate samples conducted in two different experiments.

both fructan and glucan on coated sHA increased the adhesion of *S. sobrinus* to a level similar to the glucan-coated sHA. *S. mutans* adhered 60% less ($P < 0.05$) to fructan-coated sHA and 23% less ($P < 0.05$) to both fructan- and glucan-coated sHA compared to their adherence to the glucan-coated pellicles. Adhesion of *S. gordonii* to fructan-coated sHA was similar to its adhesion to the combined fructan- and glucan-coated sHA or to glucan-coated sHA, and was only slightly higher than control. Adherence of *A. viscosus* to the fructan-coated sHA was 33% higher ($P < 0.05$) compared to its adherence to the glucan-coated pellicle. The adherence of *A. viscosus* to the combined fructan- and glucan-coated sHA was 19% higher than to fructan-coated sHA ($P < 0.05$), and 46% higher than to glucan-coated sHA ($P < 0.05$).

3.2. The effect of fructanase treatment on bacterial adherence to HA beads coated with fructan polysaccharides

The results showing the effect of fructanase treatment on the adhesion of *S. sobrinus*, *S. mutans*, *S. gordonii* and *A. viscosus* to the fructan-coated experimental pellicle are presented in Fig. 2. Adherence of *S. sobrinus* to the fructan-coated pellicle was reduced by 36% ($P < 0.05$) when fructanase was added after bacterial adhesion, while treatment of the fructan-coated HA surfaces with fructanase prior to *S. sobrinus* adhesion had a minor influence on the adherence of bacteria to this surface compared to no treatment sHA. Adherence of *S. mutans* to the fructan-coated pellicle was reduced by 25% when the biofilm was exposed to fructanase after bacterial adhesion, while treatment of fructan-coated HA surfaces with fructanase prior to *S. mutans* adhesion has reduced the adherence by 12% compared to no fructanase treatment. Fructanase activity induced detachment of *S. gordonii* from sHA pre-coated with fructans by 88% ($P < 0.05$). Treatment with fructanase before *S. gordonii* adhesion reduced adherence of these bacteria to the fructan-coated sHA only by 5%. Adherence of *A. viscosus* to the fructan-coated pellicle was reduced by fructanase treatment by 50% ($P < 0.05$) as compared to the control. Treatment with fructanase prior to *A. viscosus* adhesion had reduced the adherence of this bacterium to the fructan-coated sHA by 29% ($P < 0.05$).

4. Discussion

The adhesion process of oral bacteria to the acquired pellicle is mediated by several mechanisms. One of the significant bacterial adhesion mechanisms associated with dental biofilm formation is polysaccharide-mediated adhesion [8]. Dental plaque is a complex biofilm [1,2] where various oral bacteria adhere to polysaccharide from different bacterial sources.

Previous studies have shown that different types of oral bacteria can adhere to sHA coated with glucans or fructans produced by GTF or FTF from various bacterial sources [12,17,22]. In our study, GTF from *S. sobrinus* and FTF from *S. salivarius* were used to study adhesion of various types of oral bacteria. GTFs from *S. sobrinus* synthesize predominantly H_2O-insoluble glucans [5,10] whereas FTF of *S. salivarius* synthesizes mostly levan-type fructans (β-2,6-linked) [5,24].

Both *S. sobrinus* and *S. mutans* adhered better to glucan-coated sHA than to fructan-coated sHA. Pellicle composed of both fructans and glucans supported adhesion of *S. sobrinus* at a level similar to pellicles composed of glucans only. The adherence of *S. mutans* to a pellicle composed of both types of polysaccharides was lower compared to a pellicle of glucans only. These findings are consistent with other studies that demonstrate a strong correlation between the presence of glucans and the adhesion of *S. sobrinus* and *S. mutans* [12]. Degradation of fructans with fructanase before *S. sobrinus* or *S. mutans* adhered to the pellicle had a minimal effect on adhesion, which supports the notion that *S. sobrinus* and *S. mutans* adhesion is mediated mainly by glucans. However, when glucans are not abundant, *S. sobrinus* and *S. mutans* can utilize fructans to facilitate adhesion to an sHA surface. In contrast to the adhesion mechanism of *S. sobrinus* and *S. mutans*, *A. viscosus* was found to have a higher affinity toward fructans than glucans. This adhesion process is fructan-dependent, as it was found that treatment with fructanase prior to the adhesion of *A. viscosus* resulted in a significant reduction in adhesion of this organism. In addition, *A. viscosus* may express dual lectin-binding sites, as it adhered to a mixture of fructans and glucans better than to either polysaccharide separately. Adhesion of *S. gordonii* to the biofilm was not dependent on the type of polysaccharide coating the sHA, since the adhesion profile of *S. gordonii* to fructans, glucans or a mixture of these polysaccharides was similar. However, detachment of *S. gordonii* from the biofilm did occur after treatment with fructanase.

It has been shown that expression of the genes encoding different fructanases is tightly regulated by environmental factors [25]. Fructanase used in our study was isolated from *S. mutans*. Fructanase of *S. mutans* degrades mainly levan-type fructans with a reduced hydrolytic activity on inulin-type fructans [7]. Our treatment with the levanase-type fructanase degraded 40% of the fructans adsorbed to the sHA (data not shown), and was sufficient to decrease adhesion of the oral tested bacteria. Treatment of fructan-coated pellicle with fructanase before bacterial adhesion had less of an impact on bacterial adhesion compared to treatment with fructanase after bacterial adhesion. These findings indicate that, in the absence of fructans, bacteria can adhere through alternative adhesion mechanisms, but when fructans are present, they may facilitate bacterial adhesion to form a dental biofilm. It may be assumed that pre-treatment with fructanase prior to bacterial adhe-

sion has exposed other binding sites that may have been blocked by steric hindrance when fructans were present in the pellicle. In addition, it cannot be ruled out that fructanase on the surface may serve as an additional binding site for bacteria.

The contribution of fructans to bacterial virulence stems mostly from the fact that they act as an extracellular nutrition reservoir for oral bacteria [7]. The data regarding their contribute to bacterial adhesion are less clear. Our results demonstrate another potential aspect of fructans in dental plaque. Bacteria that have the ability to synthesize fructans may also express a specific binding site for this type of polysaccharide. Such a lectin-binding site may facilitate an alternative avenue for colonization of tooth surfaces by oral bacteria. The existence of a fructan-binding protein similar to a glucan-binding protein remains to be explored.

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