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The effect of lactoferrin on oral bacterial attachment

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Introduction: Lactoferrin (Lf), an iron-binding salivary glycoprotein, plays an important role in human innate defense against local mucosal infection. We hypothesized that Lf interferes with initial oral bacterial attachment to surfaces by iron sequestration, so inhibiting subsequent biofilm formation. The objective was to investigate the effect of Lf on the early stages of single-species and multi-species oral biofilm development.
Methods: *Streptococcus gordonii*, *Streptococcus mutans*, *Fusobacterium nucleatum* and *Porphyromonas gingivalis* were used in this study. Glass disks of a two-track flow cell coated with flowing artificial saliva (0.3 ml/min) with and without Lf (100 μg/ml) were used for studying bacterial attachment (3 h, 37°C). Attachment was also examined by incubating single or multiple species of test bacteria (10^7 colony-forming units/ml) with Lf-coated (20–100 μg/ml) and uncoated glass slides. The effects of β-lactoglobulin, 2,2′-dipyridyl (25–100 μg/ml), an iron chelator, and $FeCl_3$ on attachment were also examined.
Results: Lf inhibited the initial attachment of *S. gordonii* (50.3%, $P < 0.05$) but not that of *F. nucleatum* and *P. gingivalis*. However, the attachment of a dual-species biofilm containing *S. gordonii* (i.e. *S. gordonii*/*F. nucleatum* or *S. gordonii*/*P. gingivalis*) was significantly reduced (48.7% or 62.1%, respectively, $P < 0.05$) in the presence of Lf. β-Lactoglobulin did not affect the attachment of *S. gordonii*. In the presence of 100 μM 2,2′-dipyridyl, attachment of *S. gordonii* was reduced by 53.87%. No reduction in attachment was noted in *S. gordonii* pretreated with Lf (100 μg/ml) and $FeCl_3$ (20–200 μM).
Conclusion: Lf suppresses initial attachment of *S. gordonii* and *S. gordonii* coaggregates by iron sequestration. This may lead to subsequent inhibition of oral biofilm development.

Key words: iron; iron chelator; lactoferrin; oral bacterial attachment; oral biofilm

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Biofilms are communities of microorganisms attached to a surface (30). They may be comprised of a single or multiple microbial species and can form on various biotic and abiotic surfaces. Biofilms are associated with a variety of human infections and are more resistant to antibiotics and host immune responses (10). Microbial attachment and adhesion lead to subsequent colonization of biofilm on biotic and abiotic surfaces. Dental plaque, found commonly on tooth surfaces, is a microbial biofilm consisting of a diverse community of oral microorganisms (23). It plays a role in the etiology of oral diseases such as dental caries and gingivitis. Therefore, compounds capable of hindering initial bacterial attachment and adhesion will be an effective means to control dental plaque-related oral diseases.

Lactoferrin (Lf) is an iron-binding glycoprotein in saliva that is produced by neutrophils and glandular epithelial cells. It is a multifunctional bioactive molecule that functions in many important physiological pathways (25). The molecule possesses bacteriostatic, bactericidal, anti-inflammatory, fungicidal and antiviral properties (13, 19, 22, 24, 37). Hence, it is an important component of our innate immunity, specifically in the context of protecting mucosal surfaces from microbial infections (28).

Lf plays an essential role in iron delivery and in the regulation of iron homeostasis. It can be found in two forms: the iron free 'Apo-Lf' form; or the iron bound 'Fe-Lf' form. It is well established that Lf inhibits bacterial adhesion by iron sequestration. Apo-Lf can effectively inhibit the growth of many bacterial species reversibly (35). It was noted that additional iron could overcome its bacteriostatic effect in *Staphylococcus aureus* (1). Arnold et al. (3, 4) reported that the bactericidal activity

of human Lf is distinct from its iron-withholding activity. Lf has been found to bind non-specifically to bacteria or hosts (5). Singh et al. (28) reported that Lf prevented the formation of *Pseudomonas aeruginosa* biofilm *in vitro*. Both Apo-Lf and Fe-Lf have been shown to inhibit the adhesion of free and aggregated *Streptococcus mutans* cells to Lf-coated dental polymers (7) and hydroxyapatite (32).

In view of the earlier observations that Lf is able to inhibit surface adhesion of oral pathogens including *S. mutans* (32), *Aggregatibacter actinomycetemcomitans* (formerly *Actinobacillus actinomycetemcomitans*) and *Prevotella intermedia* (2), we hypothesized that Lf could interfere with the surface attachment of a selected group of early colonizers resulting in the inhibition of subsequent biofilm formation. The objectives of this study were to investigate the effect of Lf on the early stages of single-species and multi-species biofilm development of oral bacteria including *Streptococcus gordonii*, *S. mutans*, *Fusobacterium nucleatum* and *Porphyromonas gingivalis*. The role of iron in oral bacterial attachment was also examined.

Materials and methods

Bacterial strain, media and culture conditions

S. gordonii challis I, *S. mutans* Ingbritt, *F. nucleatum* ATCC 10953 and *P. gingivalis* ATCC 33277 were used in this study. All test bacteria were obtained from the culture library collection at the College of Dentistry, University of Illinois at Chicago. *S. gordonii* and *S. mutans* were grown in brain–heart infusion (BHI) broth (Becton, Dickinson and Company, Sparks, MD) and incubated at 37°C for 24 h. *P. gingivalis* was grown in trypticase soy broth–yeast extract supplemented with 0.05% cysteine hydrochloride, 0.02 μg/ml menadione, 5 μg/ml hemin and 0.02% potassium nitrate. *F. nucleatum* was grown in Schaedler broth (Oxoid Ltd., Basingstoke, UK). *F. nucleatum* and *P. gingivalis* were incubated at 37°C in an anaerobic growth chamber (10% H_2, 5% CO_2 and 85% N_2; Forma Scientific, Inc., Marietta, OH) for 48 h.

Chemicals

The iron-free form of bovine Lf (Apo-bLf) and iron-saturated bovine Lf (Fe-bLf), purchased from Life Diagnostic, Inc. (West Chester, PA), were used in this study. $FeCl_3$ was purchased from Fisher Scientific (Fair Lawn, NJ). An iron chelator, 2,2′-dipyridyl, was used to test the role of iron in inhibition of bacterial attachment (Sigma, St Louis, MO). Bovine β-lactoglobulin (Lg), an 18.4-kDa molecular weight milk protein, was purchased from (Sigma). In the attachment assays, artificial saliva (1 g lemco (refined meat extract of very light colour), 2 g yeast extract, 5 g peptone, 2.5 g mucin, 0.2 g potassium chloride, 0.2 g calcium chloride and 1.26 ml 40% urea solution in 1 litre distilled H_2O) was used to better standardize the experimental conditions and to minimize the effects of antimicrobial components that are present in the human saliva (12).

Microscopy and image analysis

Image analysis techniques were used to determine the initial attachment of oral bacteria to surfaces. After the attachment of oral bacteria to surfaces, the cells were observed with the use of fluorescent Live/Dead® BacLight™ stain (Molecular Probes, Invitrogen, Carlsbad, CA) and placed on the stage of a Leica DMRE microscope (Wetzlar, Germany) for quantitative analysis. The total numbers of live and dead cells were calculated from a minimum of seven images, captured at 20× magnification using a digital Optronics Magnafire camera (Meyer Instruments, Inc., Houston, TX) and processed using the image analysis software IMAGE-PRO PLUS, version 5.1 (Media Cybernetics Inc., Bethesda, MD) for the attached population.

Antibacterial activity and bacterial growth assay

Minimal inhibitory concentrations were determined using procedures described previously (12). Bacterial cultures in 0.05 M phosphate-buffered saline (PBS; pH 6.8) were used for the experiment. In a 96-well microtiter plate, each well contained test bacteria [5×10^5 colony-forming units (cfu)/ml for *S. gordonii* and *S. mutans*, and 5×10^6 cfu/ml for *F. nucleatum* and *P. gingivalis*], Apo-bLf (0–5000 μg/ml) and the respective growth medium. Lg (0–5000 μg/ml) was used as a control. Additional controls included inoculated growth medium without test compounds, and sample blanks containing uninoculated growth medium only. Plates were incubated at 37°C under appropriate atmospheric conditions and the optical readings were taken at 660 nm hourly up to 24 h for bacterial growth. The generation time was calculated for each test bacteria grown with and without Apo-bLf.

Attachment of single-species and multi-species oral bacteria to surfaces

For initial bacterial attachment, a flow-cell model modified from that of Leung et al. (20) was used. The two-track flow-cell system was purchased from BioSurface Technologies (Bozeman, MT). The flow-cell consisted of two compartments, each containing a polycarbonate flow chamber with two recesses to hold the glass disks (10 mm in diameter and 2 mm in thickness) upon which biofilms were formed. Glass disks provided appropriate background that allowed visualization of the initial attachment of oral bacteria using fluorescence microscopy. To form biofilms, glass disks in both chambers were precoated with flowing artificial saliva (0.3 ml/min) with and without Apo-bLf (100 μg/ml) for 30 min. *S. gordonii* (10^6 cfu/ml) was inoculated into the respective chambers and flow resumed for 3 h. At the end of 3 h, the flow-cell system was flushed with fresh artificial saliva at 0.3 ml/min for 15 min and the Live/Dead® stain was introduced into each chamber. After incubation for 15 min at room temperature, the images of attached bacteria on the disks were obtained and analysed using an *in situ* image process program as described previously.

In addition to using the flow-cell system, a slide model was also adopted to examine bacterial attachment. Four-well chamber slides (Nunc Lab-Tek, Rochester, NY) were used to conduct the attachment study for *S. gordonii* and *S. mutans* in the presence of Apo-bLf (20–100 μg/ml) or Lg (20–100 μg/ml). The attachment of *F. nucleatum* and *P. gingivalis* followed by multispecies bacterial attachment in the presence of Apo-bLf-coated (100 μg/ml) glass surfaces was also examined. The multispecies pairs included *S. gordonii*/*F. nucleatum* (1 : 1 ratio) and *S. gordonii*/*P. gingivalis* (1 : 1 ratio). Slides were preconditioned with Apo-bLf or Lg for 30 min at 37°C under appropriate atmospheric conditions. Test bacteria (10^7 cfu/ml) were added and incubated at 37°C for 15 min. The slides were washed three times with PBS, the attached bacteria were stained, and images were obtained and analysed as described previously.

The role of iron on bacterial attachment

To test the role of iron in association with bacterial attachment, the effect of an iron chelator, 2,2′-dipyridyl, or $FeCl_3$ was examined. *S. gordonii* was pretreated with Apo-bLf (100 μg/ml), a combination

of Apo-bLf (100 μg/ml) and $FeCl_3$ (20–200 μM), and a combination of 2,2′-dipyridyl (25–100 μM) and $FeCl_3$ (100 μM) at 37°C for 1 h. The treated cells were then added to saliva-preconditioned slides, and incubated at 37°C for 15 min. The slides were washed three times with PBS, and the attached bacteria were stained and images analysed as described above.

Statistical analysis

All experiments were independently repeated at least three times and the mean ± SD of attached bacteria were calculated. A minimum of seven images was analysed in each attachment experiment. The results were analysed for statistical significance ($P < 0.05$) using analysis of variance. A pairwise multiple comparison test, using Scheffe's post-hoc test ($P < 0.05$) was employed to evaluate the effectiveness of the experiments.

Results

The effect of lactoferrin on growth of oral bacteria

The effect of Apo-bLf (0–5 mg/ml) on the generation time of *S. gordonii* and *S. mutans* was tested. Apo-bLf at 0.6, 1.25, 2.5 and 5 mg/ml increased the generation time of *S. gordonii* by 22.3, 27.5, 58.1 and 263.5%, respectively ($P < 0.05$) compared with the control (Fig. 1A). No difference in growth was observed when *S. gordonii* was grown in the presence of Lg compared with the control. When *S. mutans* was grown in the presence of Apo-bLf at 2.5 and 5 mg/ml, the generation time was increased by 1.5-fold and 1.6-fold ($P < 0.01$), respectively compared with the control. However, no significant difference in the generation time of *S. mutans* was noted between the Lg treatment (5 mg/ml) and the control (Fig. 1B).

The effect of lactoferrin on oral bacterial attachment

Attachment of *S. gordonii* to Apo-bLf-pretreated glass disks (100 μg/ml) was examined using the flow-cell model. A 45% reduction in total bacterial attachment was noted when compared with the non-treated control (two sample *t*-test, $P < 0.05$). Live/Dead® staining showed that the attachment of live cells to Apo-bLf-pretreated disks was reduced by 62% compared with the control ($P < 0.05$) (Fig. 2). Similar inhibition of attachment by Lf was also observed in *S. gordonii* and *S. mutans* using the slide model. In this case, cells were added onto Apo-bLf or Lg preconditioned glass surfaces. As compared with the control, Apo-bLf (100 μg/ml) significantly reduced the attachment of *S. gordonii* and *S. mutans* by 51.1% and 52.9%, respectively ($P = 0.008$, $P = 0.004$). However, the attachment of *S. gordonii* and *S. mutans* was not affected in the presence of Lg (Table 1).

To study the effect of Lf on the attachment of multispecies bacteria to

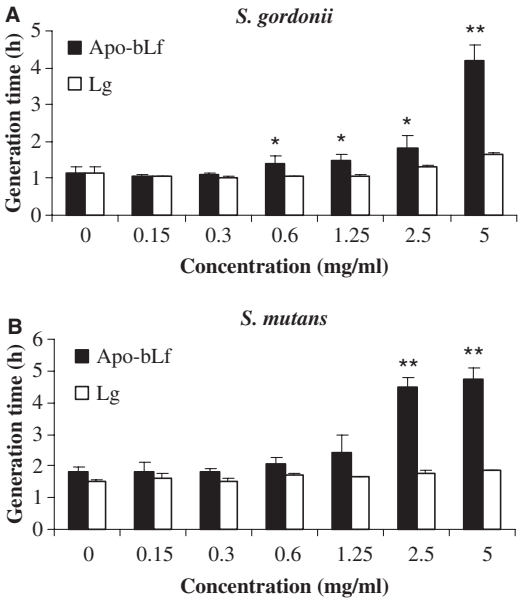

Fig. 1. Growth of *Streptococcus gordonii* (A) and *Streptococcus mutans* (B) in the presence of iron free lactoferrin (Apo-bLf) and β-lactoglobulin (Lg). Growth was represented by generation time of test bacteria. Three independent experiments were performed; $*P < 0.05$, $**P < 0.01$ vs. non-treated control.

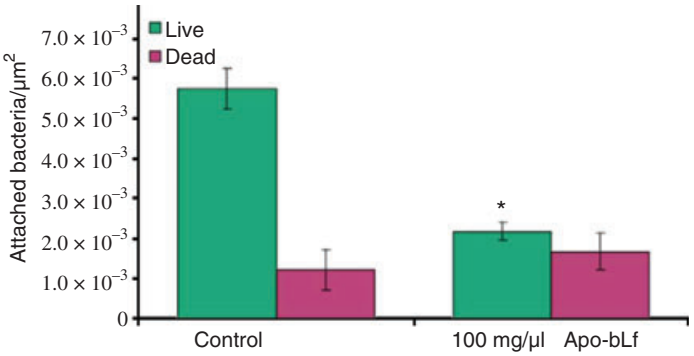

Fig. 2. Effect of lactoferrin (Lf) on *Streptococcus gordonii* adhesion. Attachment of *S. gordonii* to iron-free Lf (Apo-bLf; 100 μg/ml) pretreated glass disks was performed using a flow-cell model. Attached cells were stained with Live/Dead® to reveal the number of live (green) and dead (red) bacterial cells. Control surfaces were coated with artificial saliva only. $*P < 0.05$.

Table 1. Effect of iron-free lactoferrin (Apo-bLf) and β-lactoglobulin on the attachment of *Streptococcus gordonii* and *Streptococcus mutans*

Concentration (μg/ml)	Reduction (%)			
	Apo-bLf		β-Lactoglobulin	
	S. gordonii	*S. mutans*	*S. gordonii*	*S. mutans*
20	12.10 ± 1.08	14.00 ± 1.00	4.40 ± 0.70	24.00 ± 0.67
60	20.90 ± 0.55	38.40 ± 0.41	1.10 ± 0.70	15.00 ± 0.78
100	51.10 ± 0.42*	52.90 ± 0.43*	3.30 ± 0.50	8.70 ± 0.87

$*P < 0.05$.

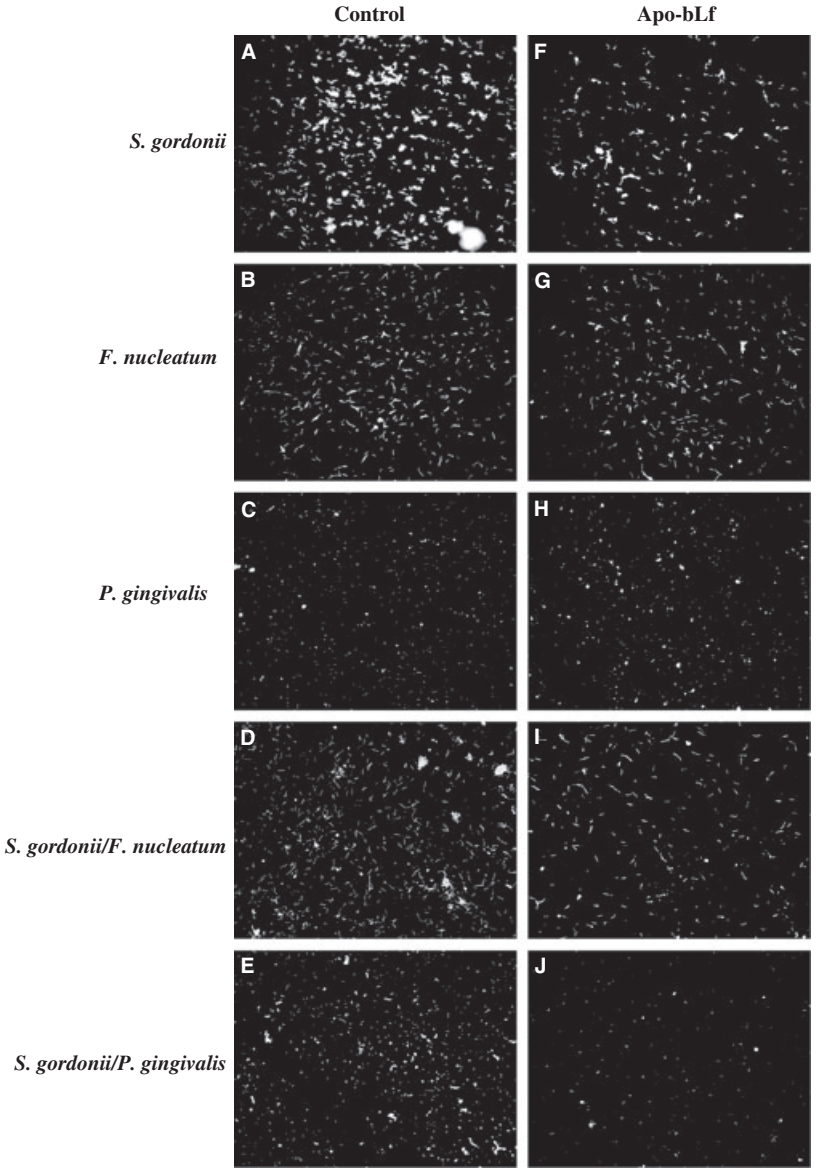

Fig. 3. Effect of lactoferrin (Lf) on oral bacterial attachment. Fluorescence microscopy images of selected oral pathogens on surfaces pretreated with artificial saliva either not containing iron-free Lf (Apo-bLf; A–E) or containing Apo-bLf (F–J). The attachment of *Streptococcus gordonii*, *S. gordonii*/*Fusobacterium nucleatum* and *S. gordonii*/*Porphyromonas gingivalis* to Apo-bLf-coated surfaces was reduced compared with the control. However, the attachment of individual species of *F. nucleatum* and *P. gingivalis* was not affected. Control surfaces were coated with artificial saliva only.

Apo-bLf-conditioned surfaces, *S. gordonii*, *F. nucleatum*, *P. gingivalis*, *S. gordonii*/*F. nucleatum* (1 : 1 ratio) or *S. gordonii*/*P. gingivalis* (1 : 1 ratio) were used as a static inoculum. As shown in Fig. 3(A–J) test bacteria attached to artificial saliva-coated surfaces were distributed uniformly. Attachment of *S. gordonii* to the Apo-bLf-coated surface was reduced by 50.3% compared with the control ($P < 0.05$) (Figs 3A, F and 4). Neither the attachment of *F. nucleatum* (Fig. 3B, G) and *P. gingivalis* (Fig. 3C, H) nor of the dual species *P. gingivalis*/*F. nucleatum* was inhibited by Apo-bLf. However, when *S. gordonii* was paired with *F. nucleatum* (Fig. 3D, I) or *P. gingivalis* (Fig. 3E, J, the attachment of these dual species pairs was reduced by 48.7% and 62.1%, respectively ($P < 0.05$) (Fig. 4).

The role of iron on oral bacterial attachment

To assess whether iron plays a role in the attachment of test bacteria to glass surfaces coated with artificial saliva, the attachment of *S. gordonii* pretreated with Apo-bLf (100 μg/ml) alone or Apo-bLf (100 μg/ml) in the presence of $FeCl_3$ (20–200 μM) was examined by using the slide model. The attachment of Apo-bLf-treated *S. gordonii* to surfaces was reduced by 50.3% (Fig. 5A). However, no significant reduction was noted in *S. gordonii* pretreated with Apo-bLf/$FeCl_3$ combinations (Fig. 5A). Treatment of *S. gordonii* with 100 μM 2,2′-dipyridyl resulted in a 53.87% reduction in their surface attachment ($P < 0.01$). No significant reduction in bacterial attachment was observed when test bacteria were pretreated with the combination of 2,2′-dipyridyl (25–100 μM) and $FeCl_3$ (100 μM) (FIG. 5B). In addition, no difference in the attachment of *S. gordonii* to surfaces was noted in the presence of Fe-bLf (100 μg/ml) (data not shown).

Discussion

The bacteriostatic and bactericidal activities of Lf have been tested against a wide variety of bacteria (9, 21, 34). Among these studies, Arnold et al. (3) demonstrated the reduction of the viability of *S. mutans* and *Escherichia coli* by 4.2 μM Apo-Lf. In comparison, our data showed that a much higher concentration of Apo-bLf at 7.3 μM (600 μg/ml) was needed to inhibit the growth of *S. gordonii* and *S. mutans*. This difference may be the result of different growth conditions and exposure time of cells to Lf in the respective growth media.

The initial attachment of *S. gordonii* and *S. mutans* to surfaces was reduced in the presence of subinhibitory levels of Apo-Lf. Our data indicated that this inhibition by Apo-Lf is specific because the extent of the attachment by these target bacteria was not affected by Lg. The results suggest that Lf could prevent biofilm formation by inhibiting the initial surface attachment of the target organisms. By contrast, Singh (29) reported that *P. aeruginosa* when exposed to subinhibitory concentrations of Lf, was able to attach and multiply on surfaces. However, iron-unsaturated Lf stimulated the twitching motility of the organism, prohibiting the cells from remaining attached to surfaces and forming microcolonies that would lead to biofilm development. The anti-biofilm activity observed was attributed to the ability of Lf to chelate free iron, an essential nutrient for organisms (29, 31, 36). Similar observations were found in, *S. aureus* and *S. mutans*, which show that iron is essential for biofilm formation (6, 7, 14, 29).

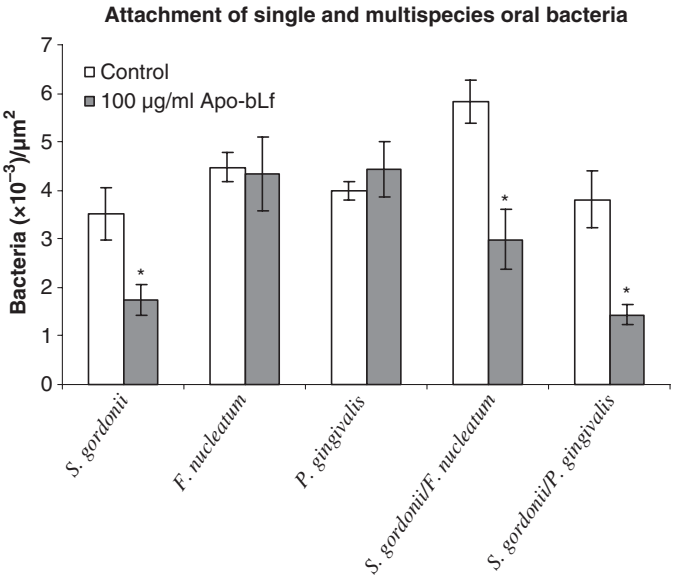

Fig. 4. Effect of lactoferrin (Lf) on oral bacterial attachment. Compared with the control, the attachment of *Streptococcus gordonii*, *S. gordonii*/*Fusobacterium nucleatum* and *S. gordonii*/*Porphyromonas gingivalis* to surfaces coated with iron-free Lf (Apo-bLf; 100 μg/ml) was reduced by 50.3%, 48.7% and 62.1%, respectively ($P < 0.05$). However, the attachment of individual species of *F. nucleatum* and *P. gingivalis* was not affected. Control surfaces were coated with artificial saliva only. *$P < 0.05$.

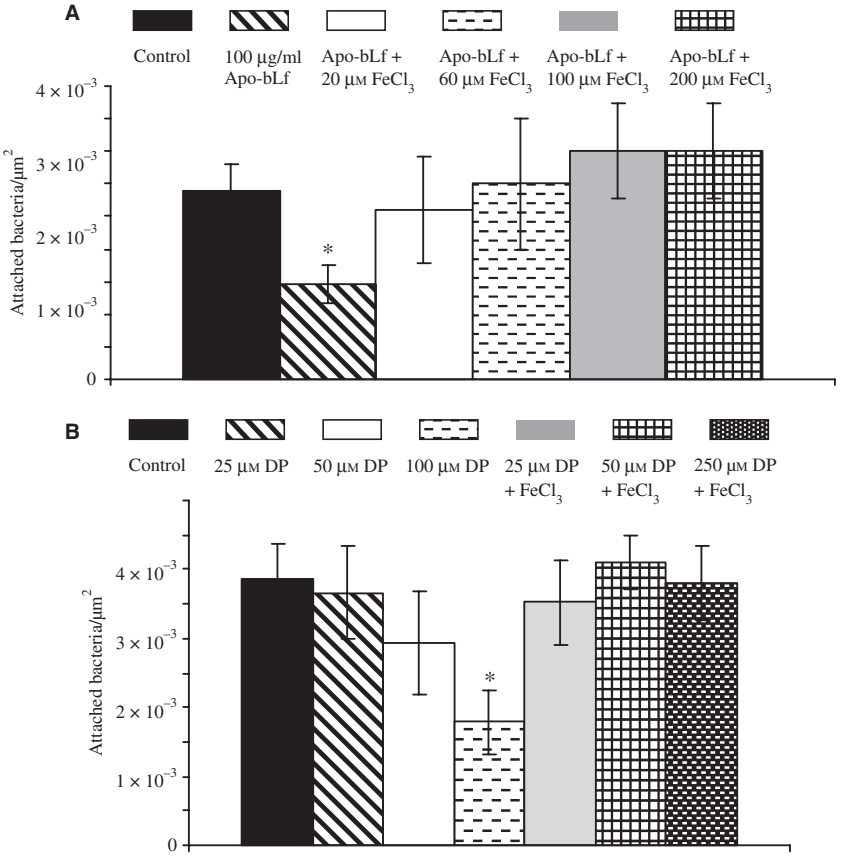

Fig. 5. Attachment of iron-free lactoferrin (Apo-bLf)/$FeCl_3$ (A) and 2,2′-dipyridyl/$FeCl_3$ (DP/$FeCl_3$) (B) pretreated *Streptococcus gordonii* onto saliva-conditioned surfaces. No significant reduction in bacterial attachment was observed when the test bacterium was pretreated with Apo-bLf/$FeCl_3$. 2,2′-dipyridyl (DP) (100 μM) reduced the attachment of *S. gordonii* by 53.87% whereas DP/$FeCl_3$ did not affect the attachment of the tested bacterium. In this study, the concentration of $FeCl_3$ was 100 μM. Control test bacteria were pretreated with artificial saliva only. *$P < 0.05$.

Rhodes et al. (27) showed that fewer cell aggregates formed when *A. actinomycetemcomitans* was grown in the presence of a synthetic iron chelator, 2,2′-dipyridyl. Our data are consistent with these previous studies because both Apo-bLf and 2-2′-dipyridyl (100 μM) reduced the attachment of *S. gordonii* to surfaces. No significant reduction in attachment was observed in *S. gordonii* pretreated with Apo-bLf/$FeCl_3$ or 2,2′-dipyridyl/$FeCl_3$. It is believed that the addition of $FeCl_3$ overcame the inhibitory effect of Apo-Lf. No inhibition in attachment was noted in the presence of Fe-bLf (data not shown). It is therefore likely that the inhibition of attachment observed was the result of iron sequestration by Apo-Lf. In the oral cavity, Lf could reduce the attachment of oral bacteria to surfaces, rendering them more susceptible to mechanical removal by host saliva. The removal could lead to the reduction of oral biofilms, specialized microbial communities for long-term survival on surfaces in the oral cavity.

Data obtained from the *in situ* image analysis in this study demonstrated that Apo-bLf specifically suppressed initial attachment of the single species *S. gordonii* but not *F. nucleatum* or *P. gingivalis*. However, when *F. nucleatum* or *P. gingivalis* was mixed with *S. gordonii* as coaggregating pairs, the attachment to Apo-bLf-coated surfaces was inhibited. Although microscopic and image analysis methods were used to evaluate the effect of Lf on dual species biofilms in this study, we have made additional attempts to confirm these results. Methods including gram staining and viable colony count determination were unsuccessful in differentiating *S. gordonii* from *P. gingivalis* or *F. nucleatum*. Therefore, quantitative real-time polymerase chain reaction (qRT-PCR) was performed to quantify the test bacterial species adherent to the slides after Lf treatment. The adherent *S. gordonii*/*P. gingivalis* cells after Lf treatment were recovered by sonication and their chromosomal DNA was extracted and purified using QIAamp DNA mini kit (Qiagen, Valencia, CA). Species-specific primer sets targeting 16S ribosomal DNA were used for qRT-PCR (SYBR green PCR Master Mix, Applied Biosystems, Foster City, CA). Quantitative standard curves were generated by qRT-PCR amplification of DNA from 10-fold serial dilutions of known cell concentrations (10^2–10^7 cfu/ml) of target bacteria. C_T (the cycle number at the threshold level of log-based fluorescence) values generated from control and treated groups were compared with these standard

curves and cell concentrations of *S. gordonii* and *P. gingivalis* were quantified. Data obtained showed that Lf preferentially inhibited the attachment of *S. gordonii* in the dual species mixture, i.e. 22.5% inhibition for *S. gordonii* while 7.3% inhibition for *P. gingivalis*, when compared with the non-treated *S. gordonii*/*P. gingivalis* pair (data not shown). Although 62.1% inhibition was noted in the *S. gordonii*/*P. gingivalis* mixture after exposure to Apo-bLf as shown in Fig. 4, this discrepancy could have been the result of the differences in methodologies for detecting adherent cells on glass surfaces after Apo-bLf treatment. The sonication method for recovering adherent cells might have resulted in some cell loss.

It is well documented that *S. gordonii* is a known initial colonizing bacterium on tooth surfaces and acts as an anchor for subsequent attachment of other bacterial species for the establishment of complex oral biofilms (15, 16, 18, 26, 33). The preferential inhibition of *S. gordonii* attachment when present in a mixed bacterial environment may represent a host defense mechanism to reduce colonization of microbial biofilm in the oral cavity. Because many periodontal pathogens require iron for growth (8, 11, 17), the iron sequestering ability of Lf may contribute to its clinical significance in oral disease prevention.

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