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Award Number:  
W81XWH-05-2-0090

TITLE:  
Effect of Lactoferrin on Oral Biofilm Formation

PRINCIPAL INVESTIGATOR:  
Christine D. Wu, M.S., Ph.D.

CONTRACTING ORGANIZATION:  
University of Illinois at Chicago

Chicago, IL 60612

REPORT DATE:  
October 2009

TYPE OF REPORT:  
Final Addendum

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 01-10-2009		2. REPORT TYPE Final Addendum		3. DATES COVERED (From - To) 19 Sep 2007 - 18 Sep 2009	
4. TITLE AND SUBTITLE Effect of Lactoferrin on Oral Biofilm Formation				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-05-2-0090	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Christine D. Wu, M.S., Ph.D.				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Illinois at Chicago College of Dentistry  Chicago, IL 60612				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT 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. <i>Streptococcus gordonii</i> (Sg), <i>Streptococcus mutans</i> , <i>Fusobacterium nucleatum</i> (Fn) and <i>Porphyromonas gingivalis</i> (Pg) were used in this study. Glass disks of a two-track flow cell coated with flowing artificial saliva with and without Lf were used for studying bacterial attachment (3 h, 37C). The effects of $\beta$ -lactoglobulin, 2,2'-dipyridyl, an iron chelator, and FeCl <sub>3</sub> on attachment were also examined. <i>Results:</i> Lf inhibited the initial attachment of Sg (50.3%, $P < 0.05$ ) but not that of Fn and Pg. The attachment of biofilm containing Sg/Fn or Sg/Pg was significantly reduced by 48.7% and 62.1%, $P < 0.05$ ) in the presence of Lf. $\beta$ -Lactoglobulin did not affect the attachment of Sg. 2,2'-dipyridyl reduced attachment of Sg by 53.87%. No reduction in attachment was noted in Sg pretreated with Lf (100 $\mu$ g/ml) and FeCl <sub>3</sub> (20–200 $\mu$ M). <i>In conclusion</i> , Lf suppresses initial attachment of Sg and Sg coaggregates by iron sequestration, which may lead to subsequent inhibition of oral biofilm development.					
15. SUBJECT TERMS Oral Biofilms, Lactoferrin, Oral bacterial attachment					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT  UU	18. NUMBER OF PAGES  15	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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## A. INTRODUCTION

Lactoferrin, an iron-binding glycoprotein produced by neutrophils and glandular epithelial cells plays an important role in human innate defense against local mucosal infection. The bacteriostatic activity of lactoferrin is primarily due to its ability to withhold iron required for bacterial growth. An iron-independent bactericidal effect, mediated by binding of lactoferrin to cell surfaces leading to lysis has also been described.

Biofilms are medically important since few diseases are caused by microbes in their non-adherent and free-floating forms. In the oral cavity, microbial biofilms including dental plaque, are involved in the pathogenesis of caries, periodontitis, dental implant failures, denture stomatitis and oral yeast infections such as candidiasis. It is one of the most widely studied biofilm systems, yet many aspects regarding its formation, structure, and control remain to be elucidated. Although lactoferrin's interference with bacterial aggregation and biofilm development by *Pseudomonas aeruginosa* and *Streptococcus mutans* have been demonstrated, limited studies have been conducted on its effect on oral biofilms, especially multi-species biofilm. The hypothesis of the research project was that lactoferrin interfered with initial oral bacterial attachment to surfaces leading to subsequent biofilm formation. Our aim was to investigate the effect of lactoferrin on early stage of single and multi species oral biofilm development, and to investigate the mechanism involved in the inhibition.

## B. BODY

This is the final report submitted for the project titled "The Effect of Lactoferrin on Oral Biofilm" (Award #W81XWH-05-2-0090) funded by US Army Medical Research and Material Command (USAMRMC). The project research has been completed and the following represents a final report to fulfill the reporting requirements for the award.

### B.1. Previously Approved Statement of Work

**Title:** "Effect of Lactoferrin on Oral Biofilm Formation"

**Background:** Dental emergencies continued to threaten the readiness of deployed military forces. Majority of the dental emergencies are plaque-induced oral infections. The U.S. Army Dental and Trauma Research Detachment (USADTRD) is particularly interested in developing antimicrobial systems that could significantly reduce the incidence of dental emergencies among deployed forces by controlling plaque formation, allowing sustained operations in extreme environments while maintaining mission capabilities. The USADTRD has developed several candidate antiadhesive and antimicrobial peptides that could be used for controlling plaque-associated oral infections, including dental caries and periodontitis. One of these candidates is lactoferrin [LF].

Lactoferrin, a glycoprotein found abundantly in human external secretions, is a component of innate defense. LF is produced by exocrine glands and released by degranulating neutrophils at sites of infection and inflammation. The bacteriostatic activity exhibited by LF is primarily due to its ability to withhold iron, which is required for bacterial growth. An iron-independent

bactericidal effect, mediated by the ability of LF to bind to bacterial surfaces and cause bacterial lysis has also been described. Moreover, the impacts of lactoferrin on bacterial aggregation and biofilm development were examined. The aim of this study is to determine the effects of LF on oral biofilm formation using a flow cell model defined populations of oral pathogens.

In pursuit of this objective the USADTRD is interested in initiating Master Student Hire at the university of Illinois at Chicago (UIC) to conduct the study under the supervision of Drs. Kai P. Leung (USADTRD) and Christine Wu (UIC). The research performing site will be at Dr. Christine Wu's laboratory the University of Illinois at Chicago, College of Dentistry which has the set up to perform the study.

### **Scope:**

**USADTRD agrees to:** 1) provide UIC the necessary materials and supplies for doing the study; 2) Provide expertise on the bacteriostatic properties of lactoferrin; 3) Monitor the progress of the proposed study.

**Dr. Christine Wu (UIC) agrees to** provide her laboratory space and expertise in studying oral biofilm formation and its control in a flow cell model.

From time to time, USADTRD personnel may work in UIC laboratories and UIC personnel may work in USADTRD laboratories as necessary to accomplish the goals of these research collaborations.

### **B.3. Summary of work conducted over the research period (9/15/05 to 10/18/09)**

The project provided funding for supporting a Master degree student at University of Illinois at Chicago (UIC), College of Dentistry to perform the above mentioned research project. Sevim Yildiz Arslan (formerly Sevim Yildiz) was the student supported on this grant. She has successfully fulfilled the course and research requirements to obtain a Master's Degree in Oral Sciences. She defended her thesis and obtained her M.S. degree and the research generated through this effort has been detailed in her M.S. thesis entitled "The Effect of Lactoferrin on Oral Bacterial Adhesion." The research data was subsequently submitted to Oral Microbiology and Immunology journal for publication. Both the PI and Dr. Kai Leung at the USADTRD were listed as authors on the paper. Upon review, the reviewers of the Journal requested additional experiments to be performed before it can be considered for further review. We consulted with Dr. Leung and discussed and designed various microbiological and molecular biological experiments to fulfill the requirements of the reviewers. The revised manuscript was modified accordingly and resubmitted to the journal. It was accepted and published in 2009 in Oral Microbiology and Immunology ("The Effect of Lactoferrin on Oral Bacterial Attachment" by Arslan SY, Leung KP, and Wu CD. 24: 411-416, see attached).

We are grateful of the opportunity that the USADTRD has given us. It supported and helped mentored Sevim to excel in dental research. The grant greatly facilitated both knowledge and technology transfer and exchange between UIC and the USADTRD. Sevim is currently pursuing a PhD degree at the Microbiology and Immunology Department, UIC College of Medicine.

#### Brief summary of the research:

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. *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, 37C). 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  $\beta$ -lactoglobulin, 2,2'-dipyridyl (25–100 µg/ml), an iron chelator, and FeCl<sub>3</sub> on attachment were also examined. 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.  $\beta$ -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<sub>3</sub> (20–200 µM). In conclusion, Lf suppresses initial attachment of *S. gordonii* and *S. gordonii* coaggregates by iron sequestration, which may lead to subsequent inhibition of oral biofilm development.

#### **B.4. Published Manuscript and Presentations at Scientific Meetings**

##### Publication:

Arslan SY, Leung KP, Wu CD. 2009. The Effect of Lactoferrin on Oral Bacterial Attachment. *Oral Microbiology and Immunology*. 24: 411-416.

##### Presentations at Scientific Meetings:

Yildiz S, Leung KP, Wu CD. 2007. "Lactoferrin affects the initial stages of oral biofilm formation." Paper presented at 2007 UIC College of Dentistry Clinic and Research Day.

Coulburn J, Chin YW, Parekh H, Yildiz S, Beavers R, Kinghorn AD, Wu CD. 2007. "Oral Antimicrobial Compounds from *Hydrastis canadensis* L. Paper presented at 2007 UIC College of Dentistry Clinic and Research Day.

Yildiz S, Leung KP, Wu CD. 2007. "The Effect of Lactoferrin on Oral Bacterial Attachment." Paper presented at 2007 UIC Student Research Forum.

Yildiz S, Leung KP, and Wu CD. 2008 The Effect of Lactoferrin on Oral Bacterial Adhesion. Presented at the 2008 Annual Meeting of the American Association of Dental Research, Dallas, TX.

#### **B.5. Personnel Supported by the Award**

Sevim Yildiz was the graduate student supported by this award.

## C. KEY RESEARCH ACCOMPLISHMENTS

- The project supported the research of Sevim Arslan (formerly Sevim Yildiz) and provided her the opportunity to appreciate and learn how to perform dental research mentored by Dr. W and the mentors at the USADTRD including Dr. Leung. Sevim successfully obtained her M.S. degree in Oral Sciences.
- Sevim aspired to be a biomedical research scientist and is currently pursuing her PhD degree in microbiology and immunology at the UIC College of Medicine.
- The project facilitated both knowledge and technology transfer between UIC and the USADTRD scientists and strengthened the research collaboration relationship which benefited both institutions.
- Additional research collaboration relationships were built and nurtured which resulted in training of additional UIC or USADTRD scientists.
- One manuscript was published in a refereed journal on data obtained from this research project.
- Research was published in the thesis format which provided access to other scientists who are interested in the research topic.
- Several research presentations were made at local and national professional meetings.
- Sevim served as a great role model for other young scientists who want to pursue a career in biomedical research.
- The collaboration and co-publications enhanced the visibility and image of both UIC and USADTRD.

## D. REPORTABLE OUTCOMES

### Publication:

Arslan SY, Leung KP, Wu CD. 2009. The Effect of Lactoferrin on Oral Bacterial Attachment. *Oral Microbiology and Immunology*. 24: 411-416.

### Presentations at Scientific Meetings:

Yildiz S, Leung KP, Wu CD. 2007. "Lactoferrin affects the initial stages of oral biofilm formation." Paper presented at 2007 UIC College of Dentistry Clinic and Research Day.

Coulburn J, Chin YW, Parekh H, Yildiz S, Beavers R, Kinghorn AD, Wu CD. 2007. "Oral Antimicrobial Compounds from *Hydrastis canadensis* L. Paper presented at 2007 UIC College of Dentistry Clinic and Research Day.

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Yildiz S, Leung KP, and Wu CD. 2008 The Effect of Lactoferrin on Oral Bacterial Adhesion. Presented at the 2008 Annual Meeting of the American Association of Dental Research, Dallas, TX.

Student development: Under the mentorship of Dr. Christine Wu (UIC) and Dr. Kai Leung (USADTRD), Sevim successfully obtained her M.S. degree in Oral Sciences from UIC. She excelled in research and will pursue a career in biomedical research upon obtaining her PhD degree at the Microbiology and Immunology Department, UIC College of Medicine.

## **E. CONCLUSION**

Lactoferrin (Lf), an iron-binding salivary glycoprotein, plays an important role in human innate defense against local mucosal infection. Our data showed that Lf interfered with initial oral bacterial attachment to surfaces by iron sequestration and inhibited subsequent biofilm formation. This research demonstrated that Lf not only is important in the defense mechanism of a person but may also contribute to the antibacterial effects leading to the reduction of microbial biofilm and biofilm-mediated diseases.

Future studies investigating LF's effect on other oral pathogens, especially the opportunistic fungi especially *Candida albicans* that is associated frequently with oral mucosal infections.

## **F. REFERENCES**

For relevant references please see the bibliography section of the manuscript attached in the appendices section of this report.

## **G. APPENDICES**

Electronic files of a published manuscript (2009) and abstract presented at the annual meeting of the American Association of Dental Research (2008) are attached.

Arslan SY, Leung KP, Wu CD. 2009. The Effect of Lactoferrin on Oral Bacterial Attachment. *Oral Microbiology and Immunology*. 24: 411-416.

Yildiz S, Leung KP, and Wu CD. 2008 The Effect of Lactoferrin on Oral Bacterial Adhesion. Presented at the 2008 Annual Meeting of the American Association of Dental Research, Dallas, TX.

# The effect of lactoferrin on oral bacterial attachment

S. Y. Arslan<sup>1</sup>, K. P. Leung<sup>2</sup>, C. D. Wu<sup>1</sup>

<sup>1</sup>Department of Pediatric Dentistry, College of Dentistry, University of Illinois at Chicago, Chicago, IL, USA, <sup>2</sup>US Army Dental and Trauma Research Detachment, Great Lakes, IL, USA

Arslan SY, Leung KP, Wu CD. The effect of lactoferrin on oral bacterial attachment. *Oral Microbiol Immunol* 2009; 24: 411–416. © 2009 John Wiley & Sons A/S.

**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<sup>7</sup> 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<sub>3</sub> 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<sub>3</sub> (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

Christine D. Wu, Department of Pediatric Dentistry, College of Dentistry, MC850, University of Illinois at Chicago, 801 S. Paulina Street, Room 469J, Chicago, IL 60612-7212, USA  
Tel.: + 1 312 355 1990;  
fax: + 1 312 996 1981;  
e-mail: chriswu@uic.edu

Accepted for publication April 28, 2009

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<sub>2</sub>, 5% CO<sub>2</sub> and 85% N<sub>2</sub>; 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<sub>3</sub> was purchased from Fisher Scien-

tific (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<sub>2</sub>O) 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 Optonics 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 [ $5 \times 10^5$  colony-forming units (cfu)/ml for *S. gordonii* and *S. mutans*, and  $5 \times 10^6$  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<sub>3</sub> was examined. *S. gordonii* was pretreated with Apo-bLf (100 µg/ml), a combination

of Apo-bLf (100 µg/ml) and FeCl<sub>3</sub> (20–200 µM), and a combination of 2,2'-dipyridyl (25–100 µM) and FeCl<sub>3</sub> (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<sup>®</sup> 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

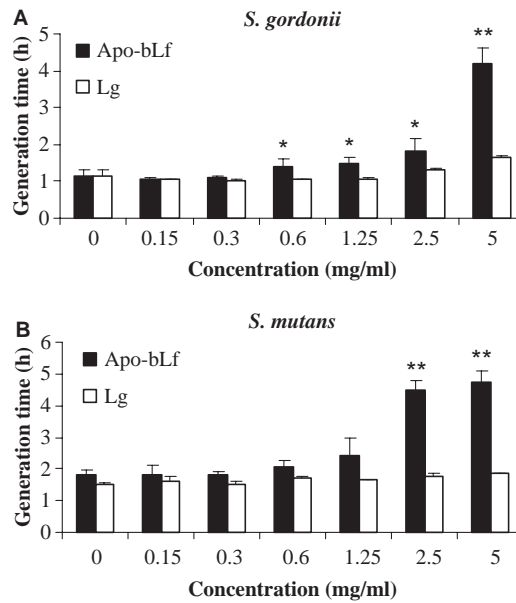


Fig. 1. Growth of *Streptococcus gordonii* (A) and *Streptococcus mutans* (B) in the presence of iron free lactoferrin (Apo-bLf) and  $\beta$ -lactoglobulin (Lg). Growth was represented by generation time of test bacteria. Three independent experiments were performed; \* $P < 0.05$ , \*\* $P < 0.01$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

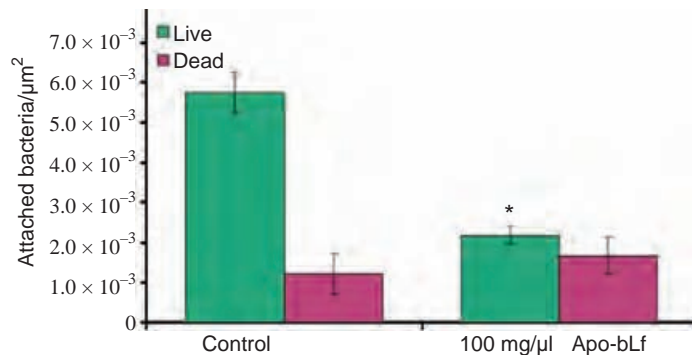


Fig. 2. Effect of lactoferrin (Lf) on *Streptococcus gordonii* adhesion. Attachment of *S. gordonii* to iron-free Lf (Apo-bLf; 100 µg/ml) pretreated glass disks was performed using a flow-cell model. Attached cells were stained with Live/Dead<sup>®</sup> to reveal the number of live (green) and dead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

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

Concentration (µg/ml)	Reduction (%)			
	Apo-bLf		$\beta$ -Lactoglobulin	
	<i>S. gordonii</i>	<i>S. mutans</i>	<i>S. gordonii</i>	<i>S. mutans</i>
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

\* $P < 0.05$ .

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

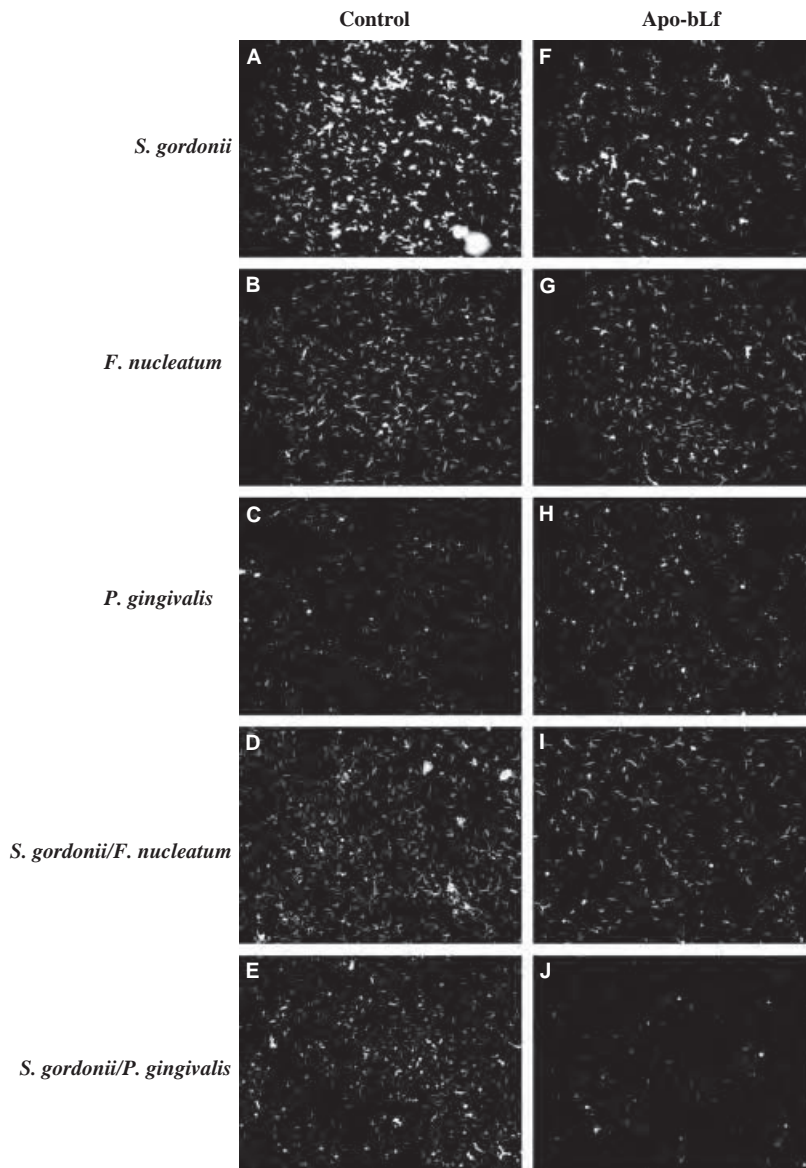


Fig. 3. Effect of lactoferrin (Lf) on oral bacterial attachment. Fluorescence microscopy images of selected oral pathogens on surfaces pretreated with artificial saliva either not containing iron-free Lf (Apo-bLf; A–E) or containing Apo-bLf (F–J). The attachment of *Streptococcus gordonii*, *S. gordonii*/*Fusobacterium 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  $\text{FeCl}_3$  (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/ $\text{FeCl}_3$  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  $\text{FeCl}_3$  (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).

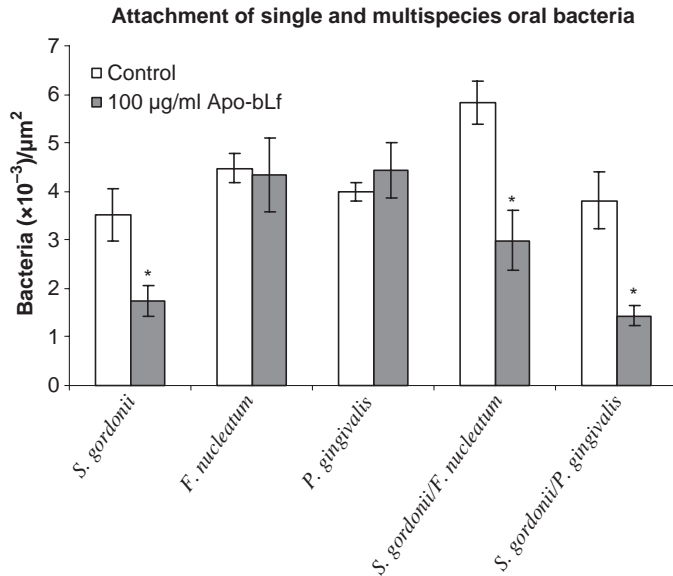


Fig. 4. Effect of lactoferrin (Lf) on oral bacterial attachment. Compared with the control, the attachment of *Streptococcus gordonii*, *S. gordonii/Fusobacterium nucleatum* and *S. gordonii/Porphyromonas gingivalis* to surfaces coated with iron-free Lf (Apo-bLf; 100 μg/ml) was reduced by 50.3%, 48.7% 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. \* $P < 0.05$ .

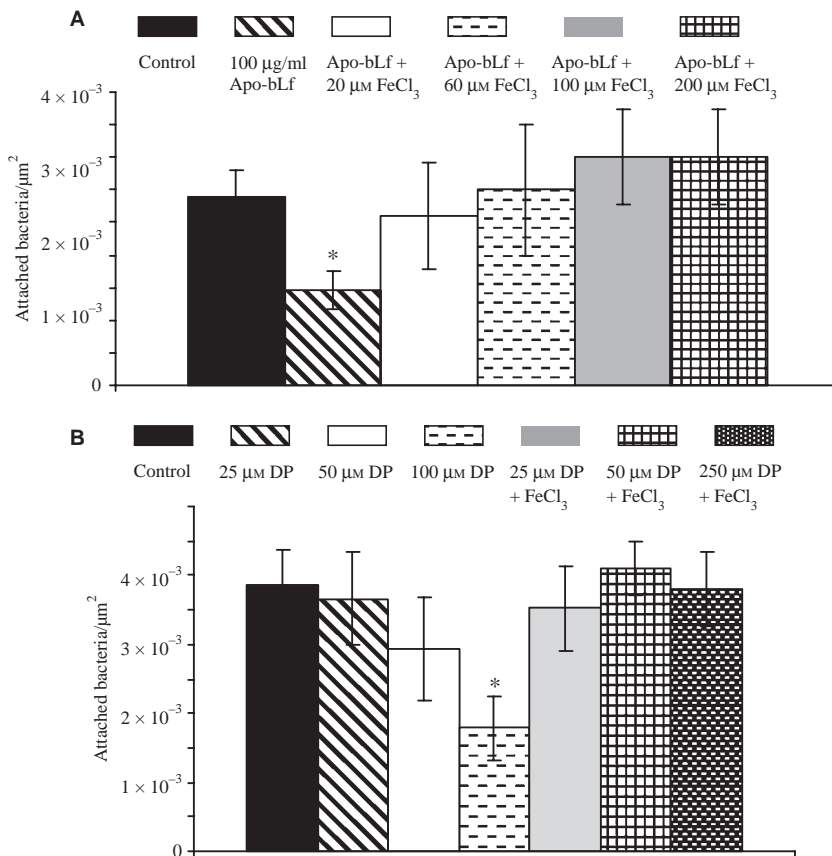


Fig. 5. Attachment of iron-free lactoferrin (Apo-bLf)/FeCl<sub>3</sub> (A) and 2,2'-dipyridyl/FeCl<sub>3</sub> (DP/FeCl<sub>3</sub>) (B) pretreated *Streptococcus gordonii* onto saliva-conditioned surfaces. No significant reduction in bacterial attachment was observed when the test bacterium was pretreated with Apo-bLf/FeCl<sub>3</sub>. 2,2'-dipyridyl (DP) (100 μM) reduced the attachment of *S. gordonii* by 53.87% whereas DP/FeCl<sub>3</sub> did not affect the attachment of the tested bacterium. In this study, the concentration of FeCl<sub>3</sub> 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<sub>3</sub> or 2,2'-dipyridyl/FeCl<sub>3</sub>. It is believed that the addition of FeCl<sub>3</sub> 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<sup>2</sup>–10<sup>7</sup> cfu/ml) of target bacteria. C<sub>T</sub> (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.

## Acknowledgments

This work was supported by the US Army Medical Research and Materiel Command. The views expressed in this article are those of the authors and do not reflect the official policy or position of the Department of the Army, the Department of Defense, or the US Government. The authors thank Dr Xu Xin and Habiba Sultana for their assistance in the qRT-PCR studies.

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## 0311 The Effect of Lactoferrin on Oral Bacterial Adhesion

**S. YILDIZ**, University of Illinois Chicago, Chicago, IL, K.P. LEUNG, US Army Dental and Trauma Research Detachment, Great Lakes, IL, and C.D. WU, Department of Pediatric Dentistry, University of Illinois at Chicago College of Dentistry, Chicago, IL

Lactoferrin (LF), an iron-binding glycoprotein, plays an important role in human innate defense against local mucosal infection. Although LF interference with bacterial aggregation and biofilm development has been demonstrated in *Pseudomonas aeruginosa*, limited studies have been conducted on its effect on oral biofilm formation. We hypothesized that LF interferes with initial oral bacterial attachment to surfaces by iron sequestration, thus inhibiting subsequent biofilm development. **Objective:** To investigate the effect of Lf on early stages of single and multi-species oral biofilm development. **Methods:** *Streptococcus gordonii* (Sg), *Streptococcus mutans* (Sm), *Fusobacterium nucleatum* (Fn) and *Porphyromonas gingivalis* (Pg) were used in this study. Glass coupons of a two-track flowcell perfused with artificial saliva (0.3ml/min) with and without LF (100µg/ml) were used for studying initial bacterial attachment (3hr, 37°C). Single or multi-species test bacteria (10<sup>7</sup>cfu/ml) were also added to glass slides precoated with LF or lactoglobulin (LG) (20-100µg/ml) for 30min at 37°C, gently rinsed, and stained with BacLight Live/Dead probe. Images of the attached bacteria were analyzed (Image-Pro Plus, version 5.1) and total attachment of test bacteria was determined. Bacterial attachment in the presence of an iron chelator, 2,2'-dipyridyl (25-100µg/ml) was also examined. **Results:** LF significantly reduced the initial attachment of Sg (50.3%), Sg/Fn (48.7%) and Sg/Pg (62.1%) compared to the control (p<0.05). However, the attachment of Fn and Pg was not affected by LF. In the presence of 100mM 2,2'-dipyridyl, attachment of Sg was reduced by 53.87%. LF did not inhibit Sg attachment in the presence of FeCl<sub>3</sub> (20-200µM). LG did not affect the attachment of Sg. **Conclusion:** Lactoferrin suppresses initial attachment of Sg and Sg coaggregates by iron sequestration. This may lead to subsequent inhibition of oral biofilm development (Supported by the US Army Dental and Trauma Research Detachment Great Lakes, IL).

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