

Stress as a Determinant of Saliva-Mediated Adherence and Coadherence of Oral and Nonoral Microorganisms

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Objective: The mucosal secretory proteins, such as the salivary proteins, play a key role in the acquisition and regulation of the mucosal microflora. Most notably, some microorganisms utilize the host's secretory proteins to adhere to the mucosa; a first step in colonization and infection. The secretory proteins also influence colonization by affecting the binding among microorganisms, a process denoted as coadherence. Previously we reported that acute stressors cause specific changes in saliva composition. The present study investigated to what extent these changes influence saliva-mediated microbial adherence and coadherence (ex vivo).

Methods: Thirty-two male undergraduates provided unstimulated saliva before and during a control condition and two stressors: A memory test and a surgery video presentation. We used saliva-coated microplates to test the adherence of bacteria for which the oral cavity is either a natural reservoir (eg, viridans streptococci) or a portal of entry (eg, *Helicobacter pylori*). We also tested the saliva-mediated co-adherence between *Streptococcus gordonii* and the yeast *Candida albicans*. Correlation analyses were performed to determine the relationships between changes in microbial adherence and the concentrations of potential salivary ligands, viz. cystatin S, the mucins MUC5B and MUC7, S-IgA, lactoferrin, α -amylase, and total salivary protein. **Results:** During the memory test, saliva-mediated adhesion of *Streptococcus sanguis*, *Streptococcus gordonii*, and *H. pylori* increased, whereas the coadherence of *C. albicans* with *S. gordonii* decreased. During the surgical video presentation the saliva-mediated adherence of *H. pylori*, *S. sanguis*, and *Streptococcus mitis* increased. These changes were independent of salivary flow rate, but correlated with specific changes in salivary protein composition. **Conclusion:** The results show that even moderate stressors, by altering the activity of the mucosal secretory glands, may affect microbial colonization processes such as adherence and coadherence. This study hereby presents a mechanism by which stress may affect the mucosal microflora and susceptibility to infectious disease.

Key words: laboratory stressor, oral health, psychoneuroimmunology, microbiology, MG1, MG2.

CHS = cleared human saliva; **DNA** = deoxyribonucleic acid; **ELISA** = enzyme-linked immunosorbent assay; **GEB** = Gibbons & Etherden buffer; **MANOVA** = multivariate analysis of variance; **OD** = optical density; **PBS** = phosphate-buffered saline; **S-IgA** = secretory immunoglobulin A.

INTRODUCTION

Among the most prevalent infectious diseases are the inflammatory periodontal diseases and dental caries (1, 2). The key role of bacteria in the etiology of both these oral diseases is well established. Although bacteria are essential, several host and environmental factors, including psychosocial stress, may increase susceptibility (3). The association between stress and heightened susceptibility to oral disease, periodontal disease in particular, has been demonstrated in both human and animal studies (4–9). In humans, this association has been found in studies that utilized case-control (10–14), longitudinal (11, 15–17), and epidemiological-correlational study designs (4, 18–21). This research literature further demonstrates that psychosocial stress is a risk factor independent of conventional risk factors (eg, social class, dental attendance, smoking, age, and dietary habits).

Saliva is essential for oral health: saliva contains many proteins, synthesized and secreted by the salivary glands, which play a critical role in the acquisition and maintenance of

the oral microflora (22–24). The secretion of these salivary proteins is under strong autonomic control (25) and salivary protein composition is modulated by psychosocial stress (25–30), thus providing a potential pathway linking stress to susceptibility to infectious oral diseases.

A useful, but complex, concept for understanding how saliva functions as a regulator of oral health is "amphifunctionality" (31). The term amphifunctionality is used to denote the observation that the salivary secretory proteins exhibit paradoxical properties. Some of these properties may inhibit microbial colonization (eg, by growth inhibition, killing, or prevention of adherence to host tissues), whereas other properties promote microbial colonization, thereby contributing to the commensal success of some species and the pathogenic potential of others. In particular, by absorbing to the oral tissues, the salivary proteins form an adhesive film or "pellicle." The pellicle promotes microbial adherence by functioning as a source of adhesion molecules that enable microbes to gain a stable foothold on the host (22–24). This adherence is a first and necessary step in infection and microbial colonization. The adherent microorganisms, in turn, also provide a source of microbial receptors that can bind other microorganisms, a process denoted as coadherence (22–24). As in adherence, in coadherence the salivary proteins play an important regulatory role. The role of the secretory proteins in adherence and coadherence is further illustrated in Figure 1. Each of these steps will determine the amount and the composition of the dental plaque (ie, the biofilm of microbes covering the oral tissues), which in turn is a critical determinant of oral health (22, 32).

We may add that the microbial colonization processes described here (ie, adherence and coadherence) are not unique to the oral cavity, but similarly take place on other mucosal surfaces (eg, nasorespiratory, gastrointestinal, and genitourinary).

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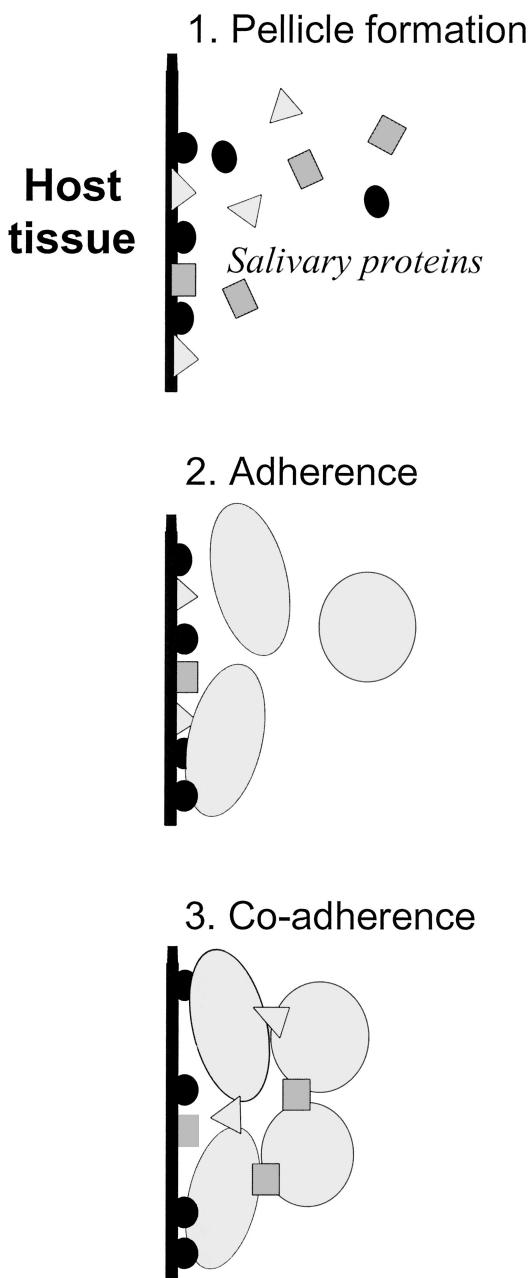


Fig. 1. The stepwise process of oral microbial colonization. 1) After secretion, a process almost exclusively governed by the autonomic nervous system, the salivary proteins form a pellicle on the host's tissues. Typical constituents of this pellicle are the mucins (eg, MUC7) and various other secretory proteins such as lactoferrin, S-IgA, and α -amylase. 2) Whereas this pellicle may form a barrier against colonization by some microorganisms, other microorganisms have the capacity to use this pellicle as a means to adhere to the tissues of the host. Examples of the latter are viridans streptococci such as *S. sanguis* and *S. gordonii*. 3) Adherence leads to the formation of an adhesive microbial layer that subsequently may promote colonization of other microorganisms through coadherence (ie, adherence among microbial species). The secretory proteins also play an important role in this coadherence by inhibiting some microbe-microbe interactions although promoting others. Inhibition of coadherence is caused when secretory proteins block microbial receptors, hereby preventing binding to other microorganisms. Promotion of coadherence may occur when two microbial species carry receptors for the same salivary protein; hereby these microbial receptors become "cross-linked" by their common salivary ligand.

nary), similarly mediated by the local secretions (eg, nasal or intestinal mucus, ocular fluid) (25, 33). Furthermore, these other mucosal secretions share similarities with saliva in both composition and functions (ie, host defense and tissue lubrication), and in the fact that their secretion is under autonomic nervous system control (25). Hence, saliva is considered a representative model system to study mucosal secretory immunity as well as the microbial colonization processes that are shaped by the secretory factors (25, 33, 34).

Previously we showed that laboratory stressors, which were selected for their potential to evoke distinct patterns of autonomic activation, alter both the concentration and secretion of salivary proteins that are potential ligands for microbes (26). The present study builds on these results by investigating the effects of these stressors on saliva-mediated adherence and coadherence (*ex vivo*). For this investigation we selected several strains of bacteria for which interactions with secretory proteins are well documented. Among these bacteria were three strains of viridans streptococci (ie, *Streptococcus sanguis*, *Streptococcus gordonii*, and *Streptococcus mitis*), which are a prominent group of bacteria in the dental plaque (32). These bacteria bind a broad spectrum of secretory proteins, such as MUC7, lactoferrin, and α -amylase, although the specific capacity to bind certain secretory proteins may vary from one species to another (24, 35, 36). Another bacterium tested was *Helicobacter pylori*, the causative agent of gastric and duodenal ulcers for which the oral cavity may act as a portal of entry. Previous studies of our group (28, 37, 38) have shown that this microorganism specifically binds to the secretory protein MUC5B. We further tested the coadhesion of the yeast *Candida albicans* to *S. gordonii*. Oral colonization by *C. albicans* involves coadhesion to oral streptococci such as *S. gordonii* (39), and this coadhesion is modulated by various salivary proteins (40, 41).

MATERIALS AND METHODS

Participants

Thirty-two male university undergraduates (mean age 23) volunteered to participate in this study. Participants gave written informed consent and received 40 Dutch guilders for their participation. None of the participants were using medication, and all reported to be in good health. In preparation of the study, participants were instructed to refrain from using alcohol or nonprescription drugs 24 hours before the experimental sessions. In addition, participants were instructed not to engage in physical exercise on the day of the experiment, and to abstain from smoking, drinking caffeinated beverages, and eating one hour before the experimental session.

Stress Tasks

All participants were subjected to two laboratory stressors and a control condition. The stressors were a time-pressured computerized memory test (42) and a video showing various surgical operations (26–28). In the control condition the participants were shown a didactic video presentation on birds. The conditions were administered in counterbalanced order, each experimental condition on a separate day, approximately one week apart. Previously, we showed that the stressors evoke clearly distinct patterns of cardiac autonomic activity (26, 27). The time-paced memory test evokes a typical "active coping" autonomic response pattern, characterized by a strong increase in cardiac sympathetic activation and a cardiac parasympathetic withdrawal, whereas the surgical video induces a so-called "passive coping" response,

characterized by enhanced cardiac parasympathetic activation and a moderate sympathetic coactivation. Analyses of self-report measures demonstrated that the two stressors were rated equally distressing. A detailed description of the cardiac autonomic and emotional responses to the stressors used for this study is presented elsewhere (26).

Procedures

Measurements were recorded between 1:30 PM and 4 PM. On arrival, the experimental procedure was explained, and participants rinsed their mouths with tap water. Subsequently, participants filled out questionnaires and were allowed to read self-selected magazines with neutral content for 30 minutes. While the subjects continued to read quietly, baseline saliva was collected. This baseline measurement was followed by one of three experimental tasks, which lasted 11 minutes. Saliva was collected again during the final part of each manipulation (stress sample) and also nine minutes after the end of the manipulation (recovery sample) when subjects again were engaged in quiet reading. For this study only baseline and stress samples were analyzed.

Saliva Collection

Saliva was collected by means of the "spitting-method," according to the directions given by Navazesh (43). This method is recommended for unstimulated whole saliva collection on the basis of a comparative study (44). The method of saliva collection was practiced before the start of the first experiment to familiarize the participants with the procedure. The collection trial started with the instruction to void the mouth of saliva by swallowing. Subsequently, saliva was allowed to accumulate in the floor of the mouth, without stimulation of saliva secretion by means of oro-facial movements. The participant spitted out into a preweighed, ice-chilled polypropylene test tube every 60 seconds. Saliva was collected for 4 minutes. After collection, saliva was homogenized by vigorous shaking with the use of a vortex mixer and clarified by centrifugation ($10,000 \times g$, 4 minutes), to eliminate buccal cells and oral microorganisms. The clear supernatant was divided into 500 μl aliquots and stored at -20°C until use.

Saliva Protein Assays

The salivary protein assays for this study are described in detail elsewhere (26). Briefly, amylase activity was determined by using the quantitative kinetic determination kit (no. 577) from Sigma Diagnostics (Dordrecht, The Netherlands) (29, 30). For the determination of cystatin S a sandwich ELISA was used, as described by Henskens et al. (45). Lactoferrin was quantified using a sandwich ELISA (46, 47). MUC5B and MUC7 were determined by ELISA, in which the antigen is directly coated to the microplate. Both the assays and antisera for the quantification of salivary MUC5B and MUC7 are described in detail elsewhere (48, 49). The monoclonal used for quantification of MUC5B specifically recognizes the terminal part of the carbohydrate moiety, sulfo-Lewis^a SO₃-3Galβ1-3GlcNAc. This structure is present on a subpopulation of MUC5B that is mainly secreted by the palatal and sublingual salivary glands (50). Total protein was determined using the bicinchoninic acid method (Pierce, Rockford, IL), as described by Bosch et al. (29, 30). Samples of the same participant were assessed in the same assay run. The intra-assay variability of each assay was <5%.

Microorganisms and Growth Conditions

H. pylori (ATCC 43504) (American Type Culture Collection, Manassas, VA) was grown under microaerophilic conditions on Dent agar plates (51) supplemented with 2,3,5-triphenyltetrazolium chloride (40 mg/ml), at 37°C for 4 days. With the use of a sterile cotton swab, bacteria were then collected and suspended in 100 mM sodium acetate buffer, pH 5.0. This suspension was washed and finally resuspended in the same buffer (supplemented with 0.5% Tween-20) to an OD_{700 nm} of 0.1.

S. gordonii HG222, *S. sanguis* ATCC 10556, and *S. mitis* ATCC 1479 (kind gifts from Dr. J.J. de Soet, Department of Oral Biology, ACTA, The Netherlands) were cultured on blood agar, containing hemin 1% (vol/vol), under anaerobic conditions for 48 hours at 37°C . Two colonies were picked up and suspended in Todd Hewitt broth, which was incubated anaerobically. Late exponential phase cultures were centrifuged (5000 $\times g$, 4°C , 10 minutes)

to pellet bacterial cells, washed two times in a buffer that simulates the ionic composition of saliva (GEB; 2 mM potassium phosphate, 50 mM KCl, 1 mM CaCl₂ and 0.1 mM MgCl₂, pH 6.0) (52), and resuspended into the same buffer to an OD_{700 nm} of 0.4.

Candida albicans (ATCC 10231) was grown on Sabouraud dextrose agar (Oxoid, Hampshire, UK) aerobically for 48 hours at 30°C . With the use of a sterile cotton swab, bacteria were collected and suspended in GEB (see above). This suspension was washed, and 10 μM of a cell permeable DNA-binding fluorescent probe (SYTO-13, Molecular Probes, Leiden, The Netherlands) was added and incubated for 30 minutes at room temperature in the dark. Subsequently, the suspension was washed again in GEB and resuspended in the same buffer to an OD_{700 nm} of 0.2.

Adherence Assays

Bacterial adherence to saliva coated microplates was quantified using a solid-phase fluorimetric assay with the use of a cell permeable DNA-binding stain (SYTO-13) for detection of bound bacteria. A detailed description of the development and biometric properties of this assay method can be found elsewhere (53). Optimal assay conditions (ie, highest maximal signal vs. lowest background) for each assay were determined by checkerboard titration, systematically varying saliva concentrations, OD of bacterial suspension, and stain concentrations. To study binding of *H. pylori*, CHS (starting dilution 1:200) was three-fold serially diluted, in triplicate, in coating buffer (0.1 M NaCO₃, pH 9.6) using a 96-well microtiter plate (high-affinity Greiner microlon, Fluotrac 600). For *S. sanguis*, CHS (starting dilution 1:160) was two-fold serially diluted, in duplicate, in coating buffer. For *S. mitis*, CHS was 1:1.5 serially diluted (in triplicate) in coating buffer, starting from a dilution of 1:48. For *S. gordonii* HG 222, CHS was 1:3 serially diluted (in duplicate) in coating buffer, starting from a 1:200 dilution.

After overnight incubation at 4°C , plates were washed four times in PBS with 0.1% Tween-20 added and aspirated using a plate washer (Mikrotek EL 403, Winooski, VT). Then 100 μl of a bacterial cell suspension containing 2.5 or 5 μM SYTO-13 was added to each well. Plates were incubated for 1 hour in the dark at 37°C , and were then washed four times with PBS-Tween-20 (0.1% vol/vol). Fluorescence was measured in a fluorescence microtiter plate reader (Fluostar Galaxy, BMG Laboratories, Offenburg, Germany) using 488 nm as excitation and 509 nm as emission wavelengths. Relative adherence was quantified by comparison to a pooled saliva sample of nine healthy males that did not participate in the present study. The level of adherence of this pooled sample was indexed on 100. Samples of the same participant were measured in one assay run to eliminate between-assay variability. The intra-assay variability (CV%) is 11.8% for *S. mitis*, 5.2% for *S. gordonii*, 4.9% for *S. sanguis*, and 6.0% for *H. pylori*.

Coadherence of *S. gordonii* and *C. albicans*

For the quantification of saliva-mediated coadherence of *C. albicans* to *S. gordonii*, approximately the same procedure was followed as described above. However, now microplates (high-affinity Greiner microlon, Fluotrac 600) were first incubated with a suspension of *S. gordonii* (OD_{700 nm} = 1.2, for 12 hours at 4°C). Plates were then aspirated and incubated at 80°C for 1 hour in order to fix the adherent bacteria onto the plates (54). Subsequently, saliva samples and a control sample were added in quadruplicate (starting dilution 1:20), two-fold serially diluted in GEB, and incubated for 2 hours at 37°C . After the samples were washed (four times with PBS containing 0.1% (vol/vol) Tween-20), 100 μl of the suspension containing stained *C. albicans* (see above) was added to each well, and incubated for 1 hour at 37°C in the dark. After a final washing, fluorescence was measured using 488 nm as excitation and 509 nm as emission wavelengths. Adherence was quantified by calibration against the dose-response curve of a pooled saliva sample. The level of adherence of this pooled sample was indexed on 100. Samples of the same participant were measured in one assay run.

Statistical Analyses

The effects of the stressors were examined by MANOVA for repeated measures. In this analysis, both the conditions and the two measurements within each condition are within-subjects factors. Two interactions were

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tested: 1) contrasting changes within the control condition with changes within the memory test, and 2) contrasting changes within the control condition with changes within the surgical video condition. The associations between changes in saliva (ie, flow rate and salivary protein concentrations) with changes in adherence were analyzed by computing Pearson's correlation coefficients. Both simple and partial correlations were computed. The partial correlation controlled for either changes in total salivary protein or changes in all other salivary protein measures (including total salivary protein), as described by Rudney et al. (55). Occasionally the data were incomplete due to insufficient saliva produced by the participant or due to the deletion of an extreme value (>2.5 SD from the mean). Data were analyzed with SPSS for Windows version 10.0.

RESULTS

Saliva-Mediated Adherence

The summary data of the saliva-mediated adherence of viridans streptococci and *H. pylori* are presented in Figures 2 and 3, respectively. As indicated in Figure 2, repeated-measures ANOVA (testing the time by condition interaction, see *Statistical Analyses*) showed that adherence of *S. sanguis* significantly increased during both the memory test ($F(1,29) = 32.08, p < .001$) and the surgical video ($F(1,30) = 11.35, p = .001$). The adherence of *S. gordonii* also increased during the memory test ($F(1,28) = 13.65, p < .001$), but remained unaffected during the surgical video ($F(1,30) = 0.76$ ns) (Figure 2). In contrast, the adherence of *S. mitis* was unchanged by the memory stressor ($F(1,27) = 0.10$ ns), but exhibited a small increase during the surgical video ($F(1,29) = 4.83, p < .05$) (Figure 2). Finally, saliva-mediated adherence of *H. pylori* increased by $\approx 50\%$ during the memory test ($F(1,29) = 4.67, p < .05$), but showed an increase of nearly 200% during the surgical video ($F(1,30) = 9.52, p < .005$) (Figure 3).

Coadherence of *S. gordonii* and *C. albicans*

Whereas saliva promoted the adherence of the viridans streptococci and *H. pylori* to saliva-coated plates, we found that the binding of *C. albicans* to microplates was unaffected by saliva (data not shown). However, we observed a strong coadherence between *C. albicans* and *S. gordonii*, and found that saliva attenuated the binding of these species. Likewise, the saliva-mediated coadherence decreased during stress. Figure 4 shows that changes in saliva composition during the memory test significantly inhibited the interaction between *S. gordonii* and *C. albicans* ($F(1,27) = 5.13, p < .05$). Compared with the control condition, no effect was seen for the surgical video ($F(1,28) = 1.22$ ns) (Figure 4).

Association Between Adherence and Secretory Measures

Tables 1 and 2 show the concordance between changes (using absolute differences) in salivary protein measures¹ and adherence measures, expressed as Pearson's correlation coefficients. Computing relative differences (%) or using rank-order correlations yielded comparable results. As the secretion

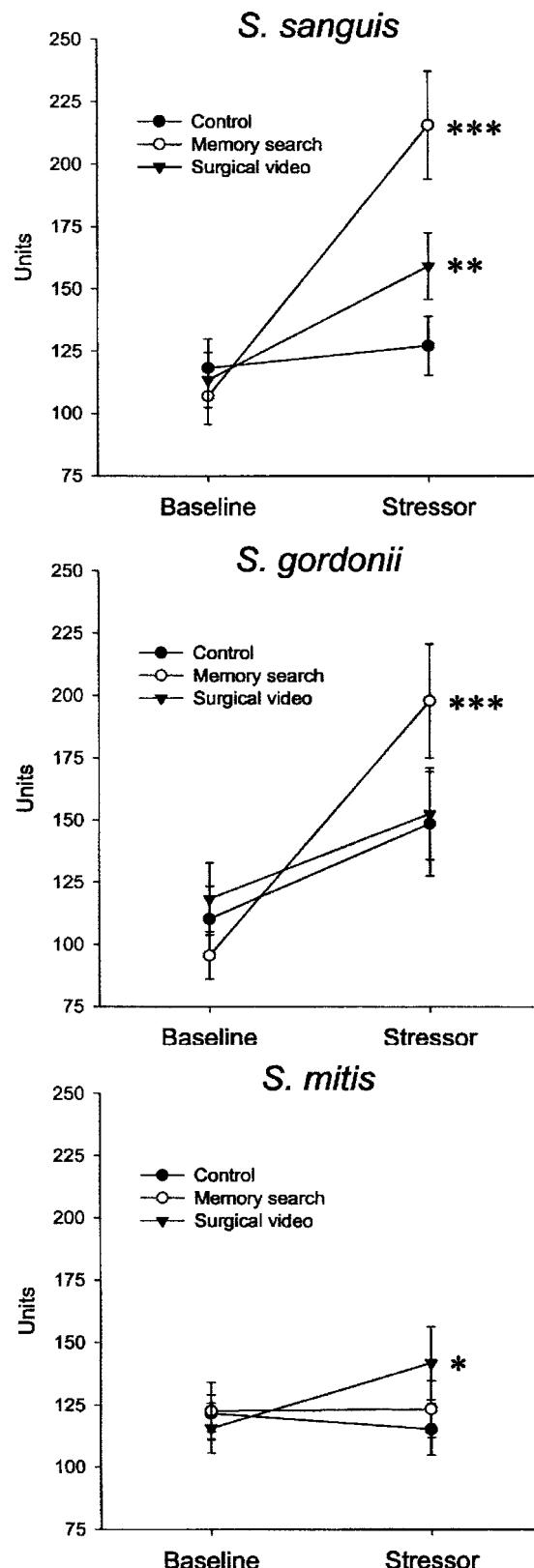


Fig. 2. Saliva-mediated adherence of three species of oral streptococci during the control condition, memory test, and surgical video. Dots indicate means, vertical bars indicate SEM. For statistical analyses, the changes within each stressor condition were contrasted with the changes within the control condition (ie, time by condition interaction). Time \times condition; * $p < .05$; ** $p < .01$; *** $p < .001$.

¹The data on the effects of stress on the secretion of these salivary proteins are described elsewhere (26).

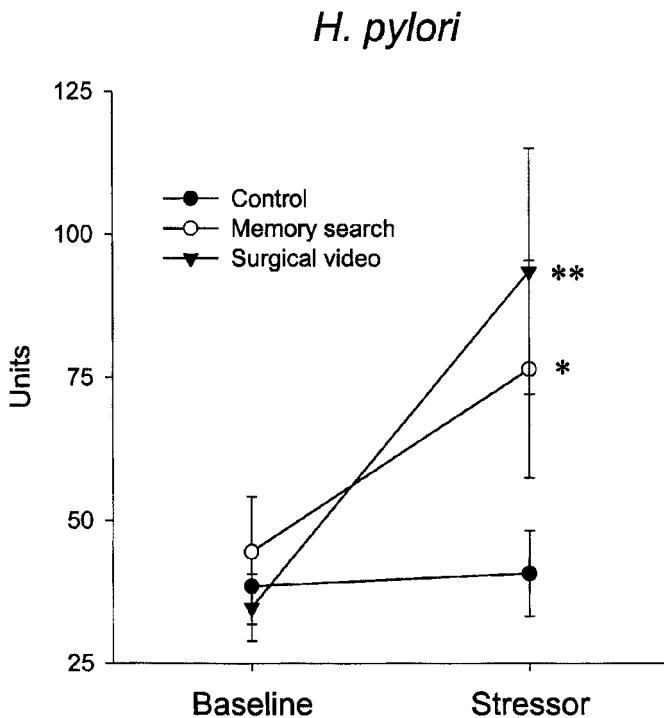


Fig. 3. Saliva-mediated adherence of *H. pylori* during the control condition, memory test, and surgical video. Dots indicate means, vertical bars indicate SEM. For statistical analyses, the changes within each stressor condition were contrasted with the changes within the control condition (ie, time by condition interaction). Time × condition; * $p < .05$; ** $p < .01$; *** $p < .001$.

of salivary proteins tends to be correlated (56), simple correlations might yield spurious results through associations with other proteins that are correlated with both the protein of interest and adherence scores. Therefore, partial correlations were computed as well, controlling for concurrent changes in either total salivary protein or all protein measures together in order to adjust for effects of common variation with other proteins. Thus estimating the unique contribution of each protein to individual changes in adherence scores (55).

Table 1 presents the associations between salivary and adherence measures during the memory task. The changes in adherence during this task appear unrelated to changes in salivary flow rate, but show a consistent relation with changes in protein concentration. For the specific proteins, both simple and partial correlations showed an association between adherence measures and MUC7. Other associations that stood out in these analyses were a positive correlation between both S-IgA and MUC5B and adherence of *H. pylori*, and a negative correlation between *S. mitis* binding and cystatin S concentrations.

Table 2 presents the associations between salivary and adherence measures during the video stressor. As with the memory stressor, changes in adherence were not significantly correlated with salivary flow rate but did, with the exception of *H. pylori* adherence, show a positive correlation with changes in total salivary protein. For *S. sanguis* and *S. mitis* the increase in adherence showed a positive correlation with

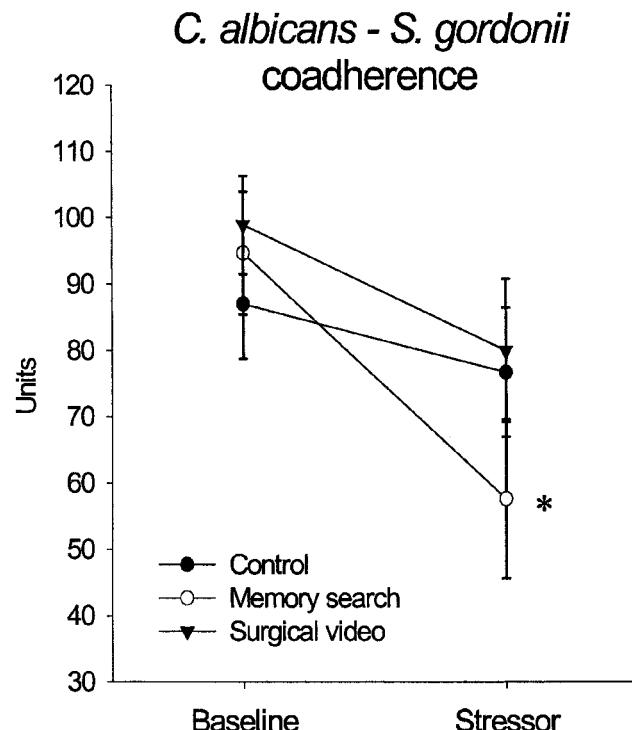


Fig. 4. Coadherence of the yeast *C. albicans* to saliva-coated layers of *S. gordonii* during the control condition, memory test, and surgical video. Dots indicate means, vertical bars indicate SEM. For statistical analyses, the changes within each stressor condition were contrasted with the changes within the control condition (ie, time by condition interaction). Time × condition; * $p < .05$; ** $p < .01$; *** $p < .001$.

changes in MUC7. Again a positive correlation was observed between MUC5B and increased adherence of *H. pylori*. A similar association was found for *S. mitis*. The adherence of *S. mitis* again showed a negative correlation with changes in cystatin S, whereas adherence of *S. sanguis* was positively correlated with cystatin S. *S. sanguis* adhesion was also positively correlated with changes in lactoferrin.

DISCUSSION

There is good evidence linking stress to susceptibility to infectious diseases. The most common route of acquisition of infectious agents is through adhesion and subsequent colonization of the mucosal surfaces (34). Little is known about how, or even if, stress affects the processes involved in this first step in infection. By using saliva as a paradigm for the mucosal secretory fluids (25, 33), we tested the hypothesis that stress may affect microbial colonization processes such as adhesion and coadhesion. The results showed that a salivapellicle formed by saliva that is secreted during acute stress, promotes the adherence of oral streptococci and *H. pylori*. These effects depended on the type of stressor that was used as well as on the type of bacterium that was assessed. In addition, we observed a decrease in the saliva-mediated coadherence between the bacterium *S. gordonii* and the yeast *C. albicans*. The effects of stress on adherence and coadherence were independent of changes in salivary flow rate, but corre-

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Table 1. Concordance Between Changes in Microbial Adherence and Changes in Salivary Proteins During the Memory Stressor.

	<i>S. sanguis</i>	<i>S. gordonii</i>	<i>S. mitis</i>	<i>H. pylori</i>	<i>C. albicans</i>
Saliva flow rate	.16	.07	.22	-.06	-.10
Total protein	.47	.30	.60	.34	-.41
MUC7					
Simple	.61	.45	.33	.33	-.31
Partial	.65 (.64)	.38 (.56)	.20 (25)	.34 (40)	-.27 (-.39)
MUC5B					
Simple	.08	-.06	.23	.44	.14
Partial	-.12 (-.11)	-.17 (-.19)	.04 (-.10)	.26 (.41)	.07 (.12)
Cystatin S					
Simple	.04	.27	-.17	.17	-.33
Partial	-.07 (-.16)	.22 (.29)	-.51 (-.54)	.06 (.17)	-.36 (-.43)
Lactoferrin					
Simple	.46	.42	.31	.42	.25
Partial	.23 (-.04)	.25 (.26)	.17 (.01)	.29 (.12)	.10 (.17)
S-IgA					
Simple	.26	.03	.16	.49	-.08
Partial	.04 (.11)	-.16 (-.19)	.24 (.15)	.45 (.56)	-.04 (.02)
Amylase					
Simple	.22	.16	.29	.16	-.38
Partial	-.09 (.22)	.01 (.00)	-.17 (-.08)	.03 (.34)	-.18 (-.25)

Presented are, respectively, simple Pearson's correlation coefficients, partial correlations controlling for changes in total protein, and (between parentheses) partial correlations controlling for all protein measures, including total protein. Significant results ($p < .05$) are printed in bold type.

Table 2. Concordance Between Changes in Microbial Adherence and Changes in Salivary Proteins During the Video Stressor.

	<i>S. sanguis</i>	<i>S. gordonii</i>	<i>S. mitis</i>	<i>H. pylori</i>	<i>C. albicans</i>
Saliva flow rate	-.17	-.08	.10	-.01	.02
Total protein	.38	.54	.53	.10	-.20
MUC7					
Simple	.61	.14	.40	.04	-.17
Partial	.57 (.66)	-.19 (-.22)	.33 (.32)	-.03 (-.15)	-.14 (-.09)
MUC5B					
Simple	.28	.20	.36	.60	-.28
Partial	.05 (.10)	-.13 (-.03)	.43 (.40)	.61 (.55)	-.10 (-.16)
Cystatin S					
Simple	.40	-.01	-.19	.00	-.20
Partial	.34 (.42)	-.18 (-.20)	-.37 (-.32)	.03 (-.17)	-.33 (-.17)
Lactoferrin					
Simple	.55	.45	.26	.25	.32
Partial	.51 (.46)	.12 (.18)	-.16 (-.05)	.23 (.38)	.07 (.13)
S-IgA					
Simple	.40	.27	.22	.13	-.24
Partial	-.22 (-.26)	-.25 (-.11)	-.19 (-.12)	.15 (.07)	.02 (-.12)
Amylase					
Simple	.01	.33	.21	.00	-.28
Partial	.15 (.16)	.36 (.23)	.02 (.28)	-.08 (-.12)	-.13 (-.20)

Presented are, respectively, Pearson's correlation coefficients, partial correlations controlling for changes in total protein, and (between parentheses) partial correlations controlling for all protein measures, including total protein. Significant results ($p < .05$) are printed in bold type.

lated with changes in salivary protein composition. These results present a novel mechanism that may explain the alterations in the mucosal microflora seen in humans and animals under various forms of psychological strain, such as depression (57), space flight simulations (58, 59), maternal separation (60), familial strains (61), animal fighting and relocation (62), and life events (63). Likewise, alterations in oral microbial colonization may form a pathway mediating the relation

between stress and oral diseases such as periodontal disease and dental caries (4–9).

The present study focused on processes that are relevant to oral microbial ecology and oral health (22, 24). Critical determinants of whether oral disease will develop are the amount of plaque covering the dental surfaces and the specific composition of that dental plaque (64). Our results indicate that both aspects may become affected during stress: First, there

was a general increase in the adherence of all species tested. An increase in bacterial adherence is conducive to the formation of dental plaque (22, 65). Second, the stressors exhibited differential effects on adherence, depending on the specific microbial species that was tested and on the type of colonization process (ie, adherence vs. coadherence) that was monitored. For example, within the group of viridans streptococci, the effects on adherence were in the following order: *S. sanguis*>*S. gordonii*>*S. mitis*. Such a selective advantage, or disadvantage, for the colonization of particular bacteria might contribute to a shift in the population of the established microflora (64), thereby potentially predisposing oral sites to disease.

Of course, critical to further discussion of our results is the question to what extent the results obtained ex vivo (or in vitro) can be translated into processes that occur in vivo. Studies in oral biology have demonstrated clinically relevant relationships between saliva-mediated bacterial adherence in vitro and mucosal colonization in vivo (22–24). For example, Carlen et al. (66) observed that bacteria that bind in greater numbers to a saliva pellicle in vitro are also found in larger proportions in dental plaque in vivo. Likewise, in vitro saliva-mediated adherence of *S. mutans*, a major etiological agent of dental caries, correlates with the rate of in vivo acquisition of this bacterium (67). Moreover, the saliva of caries-active subjects is more effective in promoting adherence than the saliva of caries-free subjects (68, 69). Similar relationships have been demonstrated outside the oral cavity. For example, *H. pylori*, which in vitro binds the gastric glycoprotein MUC5AC but not MUC6, colonizes the gastric mucosa (in vivo) in the proximity of MUC5AC-secreting cells but not in the proximity of MUC6-secreting cells (70). Thus, the ex vivo results do appear to generalize to the in vivo situation.

A notable feature of our results is that different stressors differentially affected microbial adherence. This is in line with our previous report demonstrating that the stressors used for this study exhibit very different patterns of protein release (as well as different patterns of autonomic activation) (26). For example, the “active coping” memory test induced a strong increase in MUC7, whereas not the slightest effect on MUC5B secretion could be detected. In contrast, the “passive coping” surgical video caused a nearly three-fold increase in MUC5B secretion, in addition to increasing MUC7 levels (26). It should be noted, however, that although these stressors had specific effects on the release of particular secretory proteins, the total amount salivary protein was increased to a comparable amount in both conditions. Thus, just as the stressors had distinct effects on protein composition, the adherent interactions that were shaped by secretory factors also differed.

We performed correlation analyses to obtain an indication of which salivary changes are involved in the observed changes in adherence and coadherence. Our results suggested that MUC7 is an important adherence factor for the oral streptococci tested in this study. This is consistent with studies of purified protein showing that MUC7, as a component of tooth pellicles, promotes the surface-binding of many oral

streptococci, including those tested here (35, 71–74). Also consistent with previous reports, is the positive correlation between mucin MUC5B and increased *H. pylori* adherence (28). Research by our group identified both the adhesion molecule on *H. pylori* (being the neutrophil activating protein NAP) and the ligand on MUC5B (being the carbohydrate moiety sulfo-Le^a) that are responsible for this interaction (37, 38). The correlation analyses further suggested that *S. mitis* could also adhere to MUC5B. In order to confirm this finding additional experiments with isolated protein are needed. Most oral bacteria, however, do not bind MUC5B (35, 75), and likewise we did not find an association between MUC5B concentrations and the ex vivo adherence of *S. sanguis* and *S. gordonii*, or *C. albicans* coadherence with *S. gordonii*. The association between adherence of *S. mitis* and cystatin S concentrations represented the only negative correlation, suggesting an inhibitory effect of cystatin S.

The finding that increases in S-IgA correlate with increased binding of *H. pylori* seems to contradict the notion that the function of this immunoglobulin is to inhibit microbial adhesion to the mucosal surfaces (62). However, such protective actions may largely depend on specific antibody-antigen interactions. Studies have shown that S-IgA is also capable of binding in a nonspecific manner to both Gram-positive and Gram-negative bacteria, and can subsequently promote the mucosal adherence of some species (76–78). The carbohydrate side chains on IgA are found to mediate nonspecific binding to *H. pylori* (79), which thus may explain the positive association presented in Table 2. For the other bacteria the associations with S-IgA were nonsignificant, which confirms the results of a large-scale ex vivo study that used a comparable methodology (55). We only assessed total S-IgA, and it is possible that a positive association would have appeared if bacterium-specific S-IgA had been measured (80, 81).

Two possible limitations of our study should be noted. First, although the observed associations between adherence and salivary mucin, lactoferrin, cystatin, amylase, and immunoglobulin concentrations are consistent with previous studies of purified proteins, those associations also may reflect the influence of adherence-promoting proteins not measured here. Spurious correlations are possible because the concentrations of many proteins are positively correlated (56). We addressed this issue by computing partial correlations to remove common relationships with other proteins. Nonetheless, the correlation between adherence and a particular protein may also reflect an association with another protein that was not measured here and that is released in parallel with that specific protein. A second potential limitation is that we only measured responses in males. The literature on gender differences in secretory responses to stress is limited and unequivocal (25), and deserves the attention of future studies.

Laboratory stressors are designed to model the physiological responses to the stressors encountered in everyday life. It is therefore relevant that we found that even relatively mild stressors can affect saliva composition to the extent that microbial adherence and coadherence is altered. This leads us to

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a strong hypothesis for future research; that real-life stressors of a protracted nature and larger impact may disregulate oral microbial ecology and oral health. We conclude that stress-induced changes in microbial adherence and coadherence may be a contributing factor in the relationship between stress and susceptibility to infectious disease.

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