ADHESION OF *STREPTOCOCCUS MUTANS* TO SALIVARY PROTEINS IN CARIES-FREE AND CARIES-SUSCEPTIBLE INDIVIDUALS

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ABSTRACT

Adhesion of microorganisms to dental surfaces is the initial step in the formation of dental bacterial plaque. Streptococcus mutans (S. mutans) is considered the main causal agent of one of the most common diseases in humans: dental caries. Adherence of these bacteria results from the interaction of adhesins that form part of their structure with salivary components, specifically those that compose the acquired pellicle. The complexity of this interaction has been the subject of studies in past years, to the extent of identifying certain salivary components related to adhesion to enamel surfaces, such as proline-rich proteins (PRSs), Staherins, Histatins, Cystatins, etc. One of the objectives of this study was to determine the adhesion capacity of S. mutans to synthetic hydroxyapatite incubated with saliva samples of caries-active and caries-inactive individuals. For the purpose of these assays, both the whole saliva samples and the salivary protein extracts were used. They were obtained by separating the proteins contained in the simple SDS-PAGE, in three ranges of molecular weight, selected in accordance with the electrophoresis profile that was usually found. The results indicated that the adhesion of this microorganism was greater in caries-inactive patients, when tested with whole saliva and proteins in the 120-159 kDa molecular weight range. This suggests that adhesion, per se, does not have a definite effect on the mechanisms that cause the disease in some individuals. However, these are interesting findings that may contribute to the design of strategies to control the adhesion of S. mutans to the tooth's surface.

Key Words: *Streptococcus mutans, hydroxyapatite, bacterial adhesion, dental caries, salivary proteins.*

ADHESIÓN DE *STREPTOCOCCUS MUTANS* A PROTEÍNAS SALIVALES EN PERSONAS CON Y SIN EXPERIENCIA DE CARIES

RESUMEN

La adhesión de los microorganismos a las superficies dentales, es el paso inicial en la formación de la placa dentobacteriana, Streptococcus mutans (S. mutans) es uno de los encontrados en ésta y está asociado como el principal agente causal de una de las enfermedades más comunes en los humanos, la caries dental. La adherencia de esta bacteria se da por la interacción de adhesinas que la constituyen con los componentes salivales, específicamente con los que están formando parte de la película adquirida. La complejidad de esta interacción ha sido motivo de estudio durante los últimos años, hasta el punto de identificar algunos componentes salivales relacionados con la unión a las superficies del esmalte, tales como Proteínas ricas en prolina (PRPs), Estaterinas, Histatinas, Cistatinas, etc. En el presente trabajo se buscó determinar la capacidad de adhesión de S. mutans a hidroxilapatita sintética incubada con muestras de saliva de personas con y sin experiencia de caries. Para estos ensayos se utilizó tanto la muestra de saliva completa como extractos de proteínas salivales, obtenidos por medio de la separación de las proteínas contenidas en la muestra por SDS-PAGE, en tres rangos de peso molecular seleccionados de acuerdo con el perfil electroforético que fue comúnmente encontrado. Los resultados indican que la adhesión de este microorganismo es mayor en las personas sin experiencia de caries cuando se ensayó con saliva completa y con las proteínas separadas en el rango de peso molecular de 120-159 kDa. Sugiriendo que la adhesión por sí sola no tiene un efecto determinante en los mecanismos que producen la enfermedad en algunas personas. Sin embargo estos hallazgos son interesantes ya que pueden contribuir en el diseño de estrategias para intervenir en la adhesión de S. mutans sobre las superficies dentales.

Palabras Clave: *Streptococcus mutans, hidroxilapatita, adhesión bacteriana, caries dental, proteínas salivales.*

INTRODUCTION

Dental caries is one of the most common oral problems worldwide; in countries like Colombia, the experience of caries among adults is close to 87% (1). The participation of salivary components in the initiation and progress of caries has been recognized practically since the disease was first described. Today it is clearly accepted that the development of caries depends on the interaction of saliva protective and noxious factors that are part of the acquired pellicle and the plaque, in addition to the balance between the microbial cariogenic and non-cariogenic populations within the plaque and the physicochemical characteristics of the enamel, den-

tine and cement that make hydroxyapatite more or less vulnerable to acid attack (2).

The acquired pellicle is a biologic layer resulting from the selective adsorption of salivary components on the dental enamel surface (3), mostly (98%) made up of salivary proteins, which act as substrate for fixation of cariogenic bacteria such as *Streptococcus mutans* (*S. mutans*). Interactions between these bacteria and the proteins in the acquired pellicle are part of a very complex process that has been the subject matter of studies in recent years. Various studies of salivary proteins have identified some of them as responsible for the initial binding of *S. mutans* to the tooth's surface. Nevertheless, the potential differences between the salivary components of individuals with natural resistance to dental caries and individuals who are susceptible to caries have not yet been clearly established. Hydroxyapatite (HA), has been used for many years as an enamel model for studying salivary proteins, as its surface is similar to that of the tooth (3, 4, 5, 6).

The purpose of this research was to determine whether proteins present in saliva participate in the adhesion of *S. mutans* to synthetic hydroxyapatite. The results show the salivary proteins that participate in the adhesion of *S. mutans* to HA and the differences between the two study groups when testing whole saliva and salivary proteins separated in the 120-159 kDa molecular weight range.

MATERIALS AND METHODS

The working protocol for this research was approved by the Ethics and Research Committee of the Dentistry School of Pontificia, Universidad Javeriana. Salivary samples were obtained via spontaneous salivation from a total of 20 individuals (18-30 years old), who voluntarily agreed to participate in the study. Subjects were distributed in two groups based on an oral exam and the DMTS index (D: decayed, M: missing (due to caries), F: filled, S: surfaces). The control group consisted of 10 individuals without caries (caries-free individuals) and the experimental group comprised the same number of individuals but with caries (with restored lesions and multiple lesions in enamel and dentin). Patients with permanent dentition, without periodontal disease and no fluoride and sealants therapy participated in the study. Except for the presence of caries, all individuals were clinically healthy. Subjects were then instructed to hold saliva in their mouths for a period of time, and spit into an ice-chilled test tube. Between 5 and 10 ml of saliva were obtained from each individual in glass vials, which were placed in an ice-chilled container while the sample gathering process was completed. The saliva was clarified by centrifugation at 15000xg for 15 minutes; the precipitate was discarded and the supernatant containing the fraction of interest was added with a protease inhibiting solution at pH 7.5: Tris (0.1 M), Na_2EDTA (2%), n-propanol (10%) and fNMT (2mM) for each milliliter of sample (7) and stored at -20ºC until use.

Extraction of salivary proteins

To obtain extracts of salivary proteins, three ranges of molecular weights were selected according to the electrophoresis pattern observed in the samples (Fig. 1) as follows: range 1 between 60-69 kDa, range 2 between 90-99 kDa and range 3 for proteins found in the molecular weight range of 120-159 kDa. The saliva sample of each individual was subjected to electrophoresis in denaturant conditions (dodecylsulphate of sodium-electrophoresis in polyacrylamide gel SDS-PAGE), in a PROTEAN II (Biorad) vertical electrophoresis chamber. For this purpose, gels were produced at a 10% concentration in polyacrylamide; gel size was 20x20cm. The whole saliva sample and the molecular weight

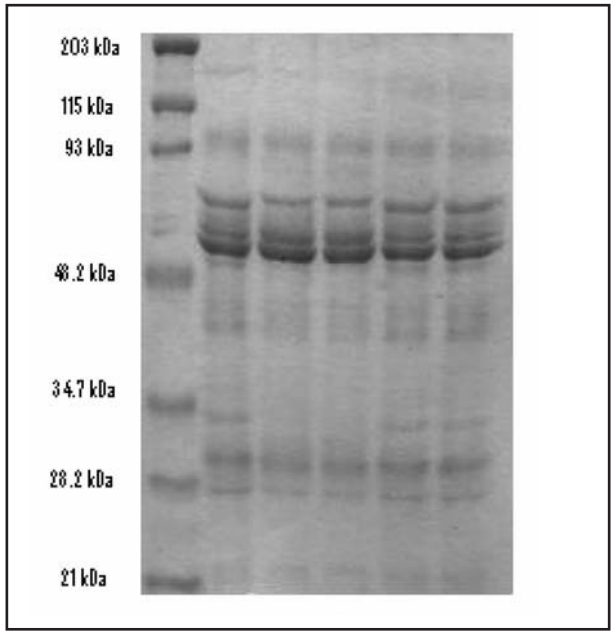

Fig 1: *Representative Electrophoresis pattern observed in the samples of whole saliva stained with Coomassie blue R250.*

marker (Biorad, broad range) were placed on each of the gels in a tris-glycine buffer at 150mV for 6 hours. Based on the distance of the electrophoresis run of marker proteins and employing linear regression calculations, the gels containing glasses were marked in fractions corresponding to each range of molecular weight; one of the glasses was removed and the gel piece containing that fraction was cut. The gel fraction obtained was divided in small pieces and left permanently stirring in sterile water to allow for elution of gel proteins for l5 minutes. It was centrifuged at 3500xg for 15 minutes, the supernatant was collected, and water was added once again to the precipitate (gel pieces). The procedure was repeated three times, and each time, after centrifugation, the supernatant contained in the proteins was collected. The collected supernatants were chilled at –70ºC for 6 hours and then lyophilized for storage at 4ºC until use in the acquired pellicle formation steps. To verify the elution of salivary proteins separated in the gel, the gel pieces were dyed with a *Coomassie* blue (0.25% p/v) solution and then discolored to prove the absence of proteins.

Culture and marking of S. mutans

S. mutans from the reference strain of ATCC (Ref. 31989) were placed in a soybean tripticase medium and incubated for 24 hours in a microaerophilic atmosphere. They were then placed in 6 ml of Todd Hewitt broth containing [^{3}H] tritiated thymidine in a proportion of 3µCi/ml and incubated for a further 24 hours. After this period the marked cells were collected and the total amount of cells was determined by spectrophotometry. The cells obtained were aliquoted in a $1x10^7$ cell/ml concentration to carry out the assays of adhesion to synthetic hydroxyapatite.

In vitro formation of acquired pellicle (PA) and binding of marked S. mutans

5 mg of commercial synthetic hydroxyapatite (Calcitec®) were weighed in microtubes; each one was added with 1 ml of pH 7.5 balancing buffer and left in permanent stirring for l6 hours. The HA balanced by centrifugation at 3500xg for 5 minutes was separated, and the buffer was removed with a Pasteur pipette. The precipitate (HA) was added with 1 ml of whole saliva or 1 ml of salivary protein extract, separated in three molecular weight ranges (range 1, range 2 and range 3), forming an S-HA saliva-hydroxyapatite suspension. This suspension was left under permanent stirring for two hours, at room temperature. It was centrifuged at 10000xg for 15 minutes, the supernatant was discarded and then the sediment was washed with NaCl (0.1M) to eliminate proteins that had not been adsorbed. The S-HA sediment was added with a bovine serum albumin (BSA) solution at 0.5% for one hour in permanent stirring, to block the HA points where the salivary proteins did not bind. After centrifuging, the supernatant was removed and the precipitate was added with the solution that contained the marked *S. mutans* cells in a $1x10^7$ cell/ml concentration. The system was incubated for 6 hours under permanent stirring, at ambient temperature, after which a microtube was placed on a vial containing 5 ml of flashing liquid. It was stirred vigorously and allowed to stand for 18 hours to reach photonic stability. It was then taken to the flashing meter, to take the readings of radioactivity emitted per sample. Radioactivity was determined with a Beckman 4000 liquid scintillation counter; the efficiency of the equipment was estimated in 96-97%. Blanks were run simultaneously, omitting the addition of saliva or the corresponding fractions. The radioactivity emitted in each case and expressed as disintegrations per minute (dpm) was used as the adhesion value of *S. mutans* to hydroxyapatite previously incubated with whole saliva and extracts from salivary proteins. The assays were performed according to a randomized design, with each determination at least in triplicate.

Statistical Analysis

The statistical analysis of the data corresponding to the replication of the same experiment was carried out using *Student's t* test. The results obtained were evaluated statistically using Windows, Excel 5; multiple comparisons were made using analysis of variance (ANOVA) to determine the various effects considered; the analysis included the significance *t* test. The results were expressed as mean ± standard deviation of the mean (MSD), with a variation coefficient of approximately 20%; statistical significance was set at $p < 0.05$.

RESULTS

The results of this research reveal significant differences ($p < 0.05$) in adhesion of *S. mutans* to HA

TABLE I. Adhesion of *Streptococcus mutans* to salivary proteins in caries-free and caries-susceptible individuals with whole saliva samples. The results (dpm/107cells/ml) are expressed as mean ± S.D. (n=3 for individual).

	Whole Saliva	
Individual	**Caries-free**	**Caries-susceptible**
1	152.97 ± 24.50	113.92 ± 31.15
2	127.22 ± 27.05	169.84 ± 15.85*
3	128.75 ± 7.97	237.47 ± 16.00*
4	155.06 ± 47.62	108.01 ± 25.56
5	191.02 ± 7.03	99.14 ± 18.10
6	152.98 ± 21.01	96.19 ± 10.04
7	147.80 ± 30.96	118.36 ± 28.88
8	163.75 ± 65.35	93.97 ± 4.64
9	156.61 ± 30.90	133.87 ± 22.32
10	161.60 ± 56.21	128.69 ± 39.90
Total	**153.78 ± 35.12**	**111.52 ± 24.02**

* Excluded of ANOVA.

between the two study groups. The average value of adhesion in the caries-inactive group was 35.12 dpm/10^7cells/ml while in the caries-active group it was 24.02 dpm/10^7cells/ml, 27% higher in incubations with whole saliva from individuals in the caries-inactive group. The adhesion with whole saliva samples is shown in Table I. The comparative values of adhesion of *S. mutans* to the proteins of whole saliva in the two study groups are shown in Figure 2.

Adhesion with salivary protein extracts is shown in Tables II, III and IV for ranges 1, 2 and 3 respectively. There were no statistically significant differences ($p < 0.05$) in adhesion in ranges 1 and 2; in range 3 there were statistically significant differences ($p < 0.05$) between the study groups, with greater adhesion in the caries-free group. Figure 3 shows the comparative values of adhesion of *S. mutans* expressed as dpm/10^7cells/ml and % for proteins

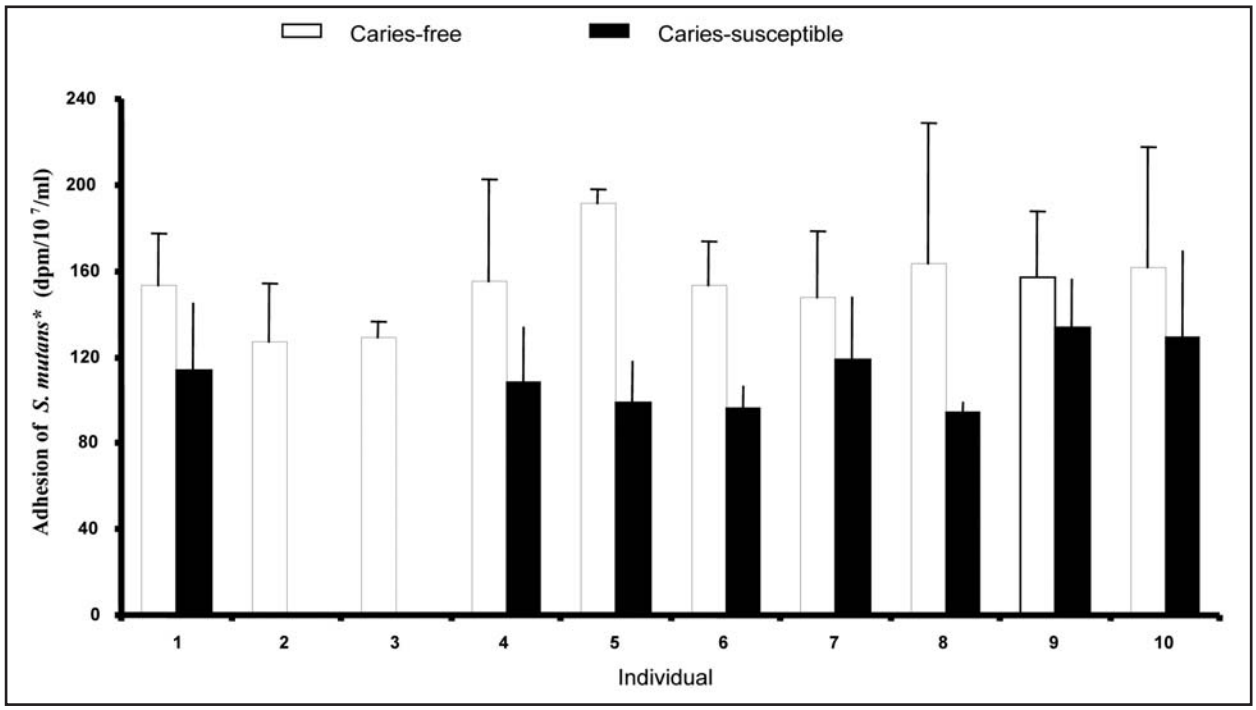

Fig 2: *Values of adhesion of* Streptococcus mutans *to the proteins of whole saliva in the two study groups, expressed in dpm/10^7 cells/ml.*

TABLE II. Adhesion of *Streptococcus mutans* to salivary proteins in caries-free and caries-susceptible individuals with salivary proteins extracts for range 1. The results (dpm/107cells/ml) are expressed as mean ± S.D. (n=3 for individual).

	Range 1	
Individual	**Caries-free**	**Caries-susceptible**
1	47.66 ± 10.63	64.15 ± 3.18
2	59.65 ± 2.25	97.65 ± 22.04
3	67.40 ± 2.25	82.05 ± 14.65
4	64.15 ± 48.12*	77.22 ± 8.41
5	29.68 ± 5.65	61.89 ± 15.71
6	91.00 ± 8.04	59.65 ± 3.18
7	33.81 ± 11.12*	43.91 ± 4.50*
8	63.36 ± 26.21	30.43 ± 3.17*
9	89.50 ± 11.44	90.99 ± 15.77
10	75.33 ± 17.47	62.64 ± 12.38
Total	**69.86 ± 21.32**	**73.81 ± 16.98**

* Excluded of ANOVA.

TABLE III. Adhesion of *Streptococcus mutans* to salivary proteins in caries-free and caries-susceptible individuals with salivary proteins extracts for range 2. The results (dpm/107cells/ml) are expressed as mean ± S.D. (n=3 for individual).

	Range 2	
Individual	**Caries-free**	**Caries-susceptible**
1	50.66 ± 15.90	50.64 ± 25.51
2	67.50 ± 14.28	66.37 ± 16.94
3	96.57 ± 7.88	82.04 ± 17.48
4	60.47 ± 2.02	70.84 ± 25.28
5	45.04 ± 14.30	95.46 ± 0.00
6	68.57 ± 34.87	64.12 ± 17.95
7	67.50 ± 11.09	33.80 ± 4.78
8	76.45 ± 20.57	70.86 ± 13.62
9	73.12 ± 6.72	92.12 ± 4.73*
10	75.34 ± 15.84	86.54 ± 0.00
Total	**8.36 ± 18.11**	**67.42 ± 21.50**

* Excluded of ANOVA.

TABLE IV. Adhesión of *Streptococcus mutans* to salivary proteins in caries-free and caries-susceptible individuals with salivary proteins extracts for range 3. The results (dpm/107cells/ml) are expressed as mean ± S.D. (n=3 for individual).

	Range 3	
Individual	**Caries-free**	**Caries-susceptible**
1	70.86 ± 13.65	52.91 ± 0.00
2	71.61 ± 18.27	69.75 ± 11.10
3	88.77 ± 0.00	200.91± 89.54*
4	97.68 ± 0.00	49.54 ± 4.77
5	32.67 ± 0.00	46.17 ± 0.00
6	97.68 ± 0.00	56.65 ± 14.62
7	64.14 ± 0.00	43.17 ± 6.87
8	69.72 ± 26.94	73.12 ± 5.93
9	71.61 ± 14.58	91.00 ± 9.46*
10	99.92 ± 0.00	70.88 ± 0.00
Total	**73.70 ± 19.10**	**57.96 ± 13.76**

* Excluded of ANOVA.

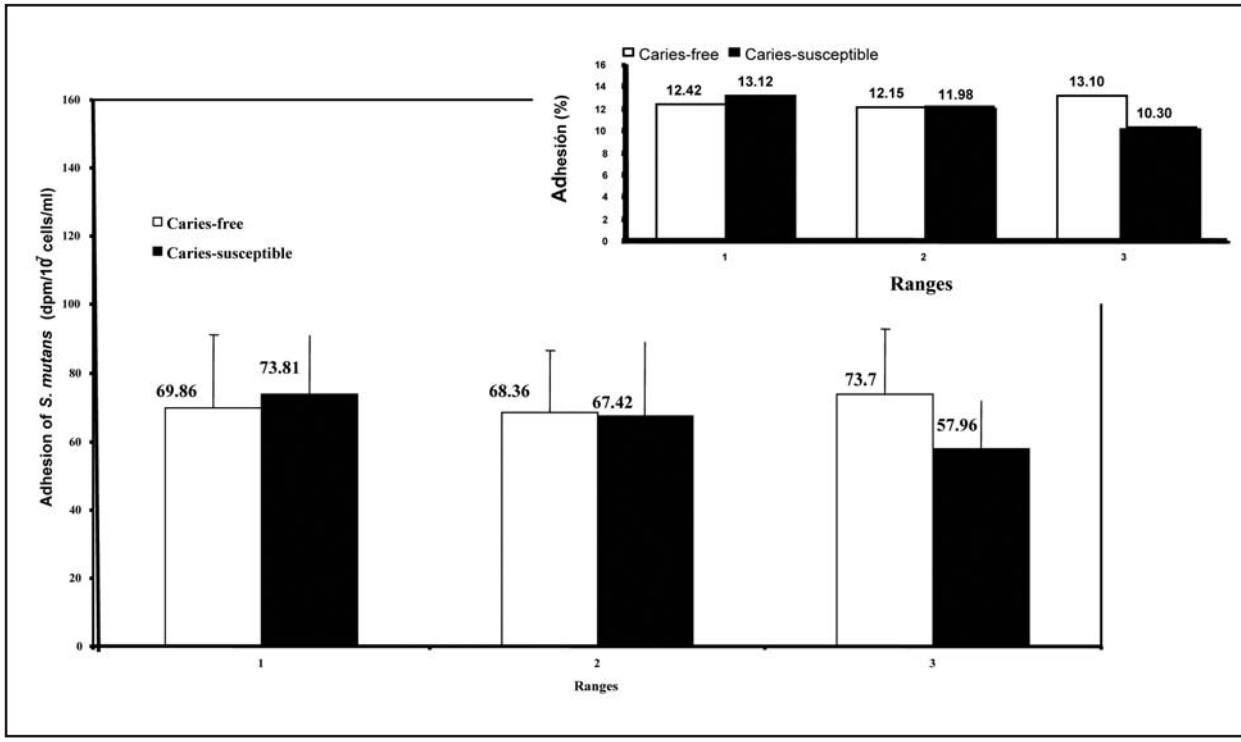

Fig 3: *Values of adhesion of* Streptococcus mutans *expressed in dpm/10^{7}cells/ml and in % for proteins separated in the three molecular weight ranges and the two study groups.*

separated in the three molecular weight ranges and for the two study groups.

These results show that it was impossible to detect proteins that would favor the adhesion of *S. mutans* in the ranges of 63-69 and 90-99 kDa. However, in the range of 120-159 kDa one or more proteins which favored the adhesion of *S. mutans* by 21% were found in caries-free individuals.

DISCUSSION

For many years now, hydroxyapatite (HA) has been used as an enamel model for studying salivary proteins (3). For the purposes of this study, HA was incubated with saliva samples and with salivary protein extracts in the study groups (caries-free and caries-susceptible individuals) and was then incubated with marked *S. mutans* cells. The radioactivity emitted per sample was determined and used to express adhesion. Adhesion was greater when experiments were carried out with whole saliva and with protein extracts in the molecular weight range of 120-159 kDa among caries-free individuals. These data would suggest the presence of proteins that favor the adhesion of *S. mutans* or that mediate certain biological phenomena.

The shape adopted by a protein would determine adhesion capacity of salivary components to *S. mutans*. The structure and orientation acquired by salivary proteins when they come into contact with hydroxyapatite influence their capacity to favor or impair adhesion. Statherin, for example, is one of the salivary proteins with the greatest affinity for mineralized surfaces in the oral cavity, which allows for adhesion of *S. mutans* (5). It has been proven that when in solution, it has virtually no folding, but when bound to an enamel mineral, it uses the bonding energy to establish a stable folding (8), which confirms that shape changes can influence the adhesion mechanisms.

On the other hand, the presence of heterotypical and homotypical complexes, regularly formed by salivary proteins and peptides (9), can modulate several interaction processes that contribute to the establishment of the acquired pellicle on the dental surface (5) and, therefore, govern the interaction between acquired pellicle and microorganisms. Within the various functions of salivary proteins, antagonistic characteristics may be found. Some act as receptors that provide ligands for bacterial fixation, but at the same time exert control on microorganisms, thus

preventing their establishment by aggregation. Once aggregates are formed, their elimination is easier through cleaning and deglution. Elimination of microorganisms adhered to dental surfaces may also occur by the action of proteins with antimicrobial properties, such as immunoglobulin A, Lysozyme, Lactoferrin and peroxidase. An increase in the concentration of these proteins caused by environmental conditions might be favored in individuals who are resistant to dental caries.

Most of the studies aimed at understanding the adhesion mechanisms used by microorganisms in the oral cavity have focused on the identification of the salivary components that are involved in these interactions and the part of these macromolecules that participate. Only some of them have correlated their findings with the presence or absence of dental caries in studied individuals. An example is a study where caries susceptibility was correlated with salivary components, that showed that there was a greater number of unknown peptides in saliva with high proteolytic activity and a reduced amount of proline-rich proteins (PRPs), histatins and statherins, in patients that were more caries-active. These findings suggested an association between these factors and caries susceptibility (5).

The interaction of salivary components when forming an acquired pellicle with a cariogenic bacterium such as *S. mutans* is the result of multiple factors that associate to establish a complex system, in equilibrium. Based on the assumption that such an equilibrium leads to a normal condition in humans: «being caries-resistant», any change that destabilizes this system can result in a significant alteration of the individuals, for instance in terms of their caries susceptibility. Two of the mechanisms present in the oral cavity that are part of this equilibrium and are directly related to adhesion are the content of salivary proteins, which is an inherent property of the host, and the various adhesion mechanisms inherent to the disease causing agent. In the former case, a group of over 40 salivary proteins that interact with hydroxyapatite have been reported (3); some of them show oligosaccharide chains, which suggests that these sugars are important in the binding to hydroxyapatite and thus in the binding with the enamel acquired pellicle (3, 10). Other studies have established that strong bindings are influenced, first, by sequences with phosphoserines, followed by glutamic and aspartic acid (11). Nevertheless, lysozyme, a protein lacking these amino acids has also been found to adhere to hydroxyapatite (12). In the latter case, it is well known that the adherence of *S. mutans* to dental surfaces involves, in its greatest expression, cellular surface protein antigen (PAc) (13) and that strains that are deficient in this protein adhere poorly to salivary pellicles, on an experimental basis (10, 14). On the other hand, *S. mutans* can also adhere by binding with glucans. This process is presumably influenced by glucan binding proteins (*Gbps*), associated with the wall of this microorganism and glucosyltransferase, which play an important role in adhesion of the microorganisms, as they synthesize and bind glucans (15). *S. mutans* secrete at least three of them: GbpA (molecular weight of 59 kDa), has more affinity with soluble than with insoluble glucans (16); GbpB (molecular weight of 41.3 kDa), whose most evident expression is in dental bacterial plaque and conceivably plays an important role in its formation process (17, 18) and GbpC (molecular weight of 63.5 kDa), associated with the aggregation of *S. mutans* dependent on dextrans (19).

CONCLUSION

Results indicate that adhesion of this microorganism is greater in caries-free individuals when whole saliva was tested, with separate proteins in the range of 120-159 kDa molecular weight. These are interesting finding, that can contribute to the design of strategies to influence the adhesion of S. mutans to the tooth's surface.

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