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Antimicrobial Activity and Synergism of Lactoferrin and Lysozyme Against Cariogenic Microorganisms

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The present study evaluated the antimicrobial *in vitro* effects of the salivary proteins lactoferrin and lysozyme on microorganisms involved in the carious process, obtaining their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). *Streptococcus mutans* (ATCC 25175) and *Lactobacillus casei* (ATCC 7469) were submitted to broth macrodilution of lysozyme at 80 mg/mL and lactoferrin at 200 mg/mL. The tubes were read in a spectrophotometer after they had been incubated at 37 °C for 18 h, in a carbon dioxide chamber, in order to read the MIC. A new subculture was carried on agar plates to obtain the MBC. The agar diffusion method was also tested, using BHI agar with 100 µL of the standardized microbial inocula. Filter-paper disks soaked in 10 µL of the solutions lactoferrin (200 µg/mL) and lysozyme (80 µg/mL) were placed on the agar surface. Inhibition halos were not observed on the plates, showing the absence of the antimicrobial effects of these proteins in this method. The bactericidal and bacteriostatic effects of lysozyme on *L. casei* were 50.3 mg/mL and 43.1 mg/mL respectively. The bactericidal and bacteriostatic effects on *S. mutans* were 68.5 mg/mL and 58.7 mg/mL. Lactoferrin did not induce any inhibitory effects on any microorganism, even in the concentration of 200 mg/mL. There was not a synergic antimicrobial effect of proteins, when they were tested together, even in the concentration of 42.8 mg/mL of lysozyme and 114 mg/mL of lactoferrin (the highest values evaluated). *S. mutans* and *L. casei* were only inhibited by lysozyme, not affected by lactoferrin and by the synergic use of both proteins.

Key Words: lysozyme, lactoferrin, dental caries, *Streptococcus mutans, Lactobacillus casei.*

Introduction

There are many determining factors to dental caries, some of them related to saliva and bacterial colonization on the dental biofilm. Saliva protects oral tissues in many ways. A constant flow of saliva eliminates accumulations of microorganisms from the oral cavity but saliva also contains many innate or acquired (antibodies) defense mechanisms (1).

One of the unspecific immunological factors (innate) is antimicrobial proteins: lysozyme, lactoperoxidase system, lactoferrin, high weight molecular glycoproteins and other salivary components that can act as bacterial aglutinines (2-4). Innate human salivary defense proteins such as lysozyme, lactoferrin and peroxidase are known to exert a wide antimicrobial activity against a number of bacterial, viral and fungal pathogens *in vitro* (1).

The majority of these proteins can inhibit the metabolism, adherence or even the viability of the cariogenic microorganisms *in vitro.* Many unspecific immunological factors can interact with the antibodies (specific factors), resulting in mutual amplification of their respective activities (4,5).

Mutans streptococci and lactobacilli are microorganisms with known cariogenic capacities (6-8). The first is considered an etiological agent of carious lesions and is able to produce (acidogenic) and tolerate (aciduric) high quantities of lactic acid, and the second is considered particularly involved in the progression of carious lesions (8,9). Mutans streptococci and lactobacilli have been correlated positively with caries prevalence and caries increment and the existence of saliva-caries relationships have been reported (1,10-12).

The aim of this study was to evaluate, *in vitro,* the antimicrobial effects of the proteins lactoferrin and lysozyme on reference cultures of the microorganisms *Streptococcus mutans* and *Lactobacillus casei*, using the agar diffusion and broth macrodilution methods, in order to obtain their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), individually or synergistically.

Material and Methods

The experiments were performed at the Microbiology Laboratory of the North of Paraná University. Culture media Brain-Heart Infusion (BHI; Difco, Detroit, MI, USA) broth and agar were made. The tubes and the plates were

processed in autoclave, tested for their sterility and kept under refrigeration.

The solutions of lysozyme and lactoferrin (Sigma Chemical Co, Saint Louis, MO, USA) were obtained as powder weight on an analytical balance (Tecnal, Piracicaba, SP, Brazil) and diluted with ultra pure water in a volumetric balloon, and stored as aliquots in micro tubes and refrigerated at -20 °C.

Reference cultures of the microorganisms *S. mutans* (ATCC 25175) and *L. casei* (ATCC 7469) were used. These microorganisms, previously frozen, were reactivated in sterile BHI broth and were incubated at 37 °C in a carbon dioxide chamber (TE-399; Tecnal). After 48 h of culture growth, the Gram stain method was done and cultures were re-plated, in order to verify their purity.

The transference of the microorganisms to broth media was performed, and they had their absorbance index verified hourly in a spectrophotometer (Biomate 3; Thermo Scientific, Madison, WI, USA) in wavelengths of 540 nm. The absorbance numbers were utilized to create the graphics (Fig. 1). The log phase of bacterial growth or exponential growth period (about 6 h after transference) was the chosen moment for the antimicrobial test.

In the agar diffusion test, Petri dishes were filled with layer of Miller-Hinton agar and afterwards, they received the microbial inocula spread in BHI broth, standardized via a spectrophotometric reading.

Sterile filter-paper disks received 10 µL of the solutions lactoferrin or lysozyme, in respective concentrations of 200 µg/mL and 80 µg/mL. They were then placed over the agar surface in equidistant positions. Four plates were used: two of them contained the inoculum of *S. mutans.* One contained filter-paper disks saturated in the solution lysozyme, the other saturated in the solution lactoferrin. The two remaining plates contained *L. casei*. One was used to verify the inhibition by lactoferrin and the other by lysozyme. The plates remained in room temperature for 2 h to diffuse the antimicrobial substances through the agar, before the microbial growth could occur. Finally, the plates were incubated at 37 °C for 48 h in a carbon dioxide chamber. These procedures were executed in triplicate.

In the broth macrodilution, 16 tubes with 1 mL of BHI broth were used for each bacterial species, eight for each protein, numbered from 1 to 8. According to the calculations, the concentrations of the proteins in each tube were determined as shown in Table 1.

After preparation of the tubes, the standardized bacterial inocula of 5 x 10^5 CFU/mL (colony forming units *per* mL of medium), established via readings in a spectrophotometer, were introduced into each tube. The tubes were incubated at 37 °C for 18 h in a carbon dioxide chamber. After this period, the tubes had their absorbance numbers verified in the spectrophotometer to analyze the turbidity caused by bacterial growth and obtain the MIC.

In this sequence, 50 µL of the tube contents were transferred onto Petri dishes (15 x 60 mm) with BHI agar. After being spread on the plates, they were incubated at 37 °C for 18 h. Next, the plates were analyzed in order to verify the bactericidal activity. This experiment was also done in triplicate.

The susceptibility test of associated antimicrobial proteins followed the same protocol of the broth macrodilution method and was done only for the *S. mutans*. Inside tube number one, 4 mL of lactoferrin at 200 mg/mL and 2 mL of lysozyme at 150 mg/mL were added. From this tube, a serial dilution was made. The inoculum of *S. mutans* was then transferred to each tube of dilution. The final concentrations of lactoferrin and lysozyme are showed in Table 2.

Table 1. Concentrations of lysozyme and lactoferrin inside the tubes of standard dilution to test the susceptibility of microorganisms

Protein	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Tube 7	Tube 8
Lysozyme (mg/mL)	68.5	58.7	50.3	43.1	36.9	31.6	27.1	23.3
Lactoferrin (mg/mL)	171.4	146.9	125.9	107.9	92.5	79.3	67.9	58.2

Table 2. Concentrations of lysozyme and lactoferrin inside the tubes of standard dilution to test the synergism of proteins over *S. mutans*

Protein	Tube 1	Tube 2	Tube 3	Tube 4
Lactoferrin (mg/mL)	66.6	44.4	29.6	19.7
Lysozyme (mg/mL)	16.6	11.07	7.4	4.9

Results

The absorbance numbers recorded hourly in the spectrophotometer displayed the graphics of bacterial growth (Fig. 1). The start of the exponential growth of both bacteria occurred approximately in 6 h.

In the agar diffusion test, no zone of bacterial growth inhibition was observed, indicating absence of antimicrobial activity of both proteins in the tested concentrations.

In the broth macrodilution method, the MIC was obtained form the readings of turbidity of the culture medium with antimicrobial proteins at different concentrations. The values of absorbance and MIC are

shown in Tables 3 and 4, respectively.

The concentration of 43.1 mg/mL of lysozyme had a bacteriostatic effect over *L. casei*, but on *S. mutans* this effect was only obtained with 58.7 mg/mL.

Lactoferrin did not induce an inhibitory effect on any bacterium, even in concentrations of 200 mg/mL. There were no synergic antimicrobial effects of proteins when they were tested, until concentrations of 42.8 mg/mL of lysozyme and 114 mg/mL of lactoferrin were established.

The minimum bactericidal concentration (MBC) of lysozyme was 50.3 mg/mL against *L. casei* and 68.5 mg/mL against *S. mutans*.

Discussion

The overall clinical function of lysozyme and lactoferrin in the human mouth is not well documented, suggesting instead that they are important for controlling the microbial overgrowth, reducing the number of bacteria in the dental biofilm, decreasing the colonization of microorganisms and/or modifying the bacterial metabolism (13,14).

The antimicrobial activity of lactoferrin occurs via a high affinity linkage with iron, making this ion unavailable to many bacterial species. It also exerts antimicrobial activity using other mechanisms of action, such as, the direct interaction between lactoferrin and the components of bacterial cells (15). Because of this property, lactoferrin is used as a component for mouthwashes and dental creams (16).

Lysozyme is a prominent antimicrobial protein of human saliva. Lysozyme´s antibacterial properties are related to its muramidase activity, leading to the degradation of the murein-containing layer of the bacterial cell wall and eventually resulting in bacterial lysis. Lysozyme activity in the oral cavity has mainly been characterized against oral streptococci and may play a protective role in oral candidosis (17).

Table 3. Absorbance index of the tubes containing the antimicrobial proteins and the microorganism

Tubes	Lysozyme and *S. mutans*	Lysozyme and *L. casei*	Lactoferrin and *S. mutans*	Lactoferrin and *L. casei*
1	0.120	0.106	1.589	1.080
2	0.214	0.080	1.575	1.083
3	0.767	0.173	1.402	1.038
4	1.209	0.347	0.734	0.790
5	0.962	0.595	0.739	0.877
6	1.505	0.903	0.992	0.796
7	1.220	1.167	0.946	0.272
8	1.749	1.161	1.341	0.589

Felizardo et al. (18) found an association between concentrations of lactoferrin and the DMFT index (decayed, missed and filled teeth index) and a tendency of association between lysozyme concentrations and DMFT. This information corroborates the protective role of salivary proteins in the etiology of dental caries, in an indirect way. Proteins can inhibit the bacteria that can cause the dental disease.

The lactobacilli and mutans streptococci have a considerable cariogenic capacity (7,9,11). Therefore, these two microorganisms were selected for tests for the present work.

The use of different antimicrobial methodologies, without standardization, provides results that cannot be compared. In the present work, the broth macrodilution and agar diffusion methods were used. It was verified that in the agar diffusion test, there was no inhibitory halo formation, corroborating the findings of Koneman et al. (19), who saw the agar diffusion test with restrictions because the correlation between the inhibition zone sizes and the MIC results of macrodilution is not in agreement.

In the present study, the microorganisms utilized were only inhibited by the antimicrobial action of lysozyme, and were not affected by lactoferrin until the concentration of 200 mg/mL.

Arnold et al. (20) observed the bactericidal effect of lactoferrin on some strains of *S. mutans,* at the concentration of 83 µM, which correspond to 6.64 mg/mL. These concentrations are much lower than our results, which could be explained by the methodological differences. *L. casei* was not inhibited by lactoferrin in both studies, because this bacterial growth can be stimulated by this protein.

Lactoferrin does not only inhibit the growth of many pathogenical bacteria, but it also stimulates the growth of certain types of bacteria (21). According to Kim et al. (22), lactoferrin presents a prebiotic effect, promoting the growth of the probiotic bacteria *L. acidophilus* and *Bifidobacterium spp.* and they also suggest that there

Table 4. Concentration of the proteins that showed antimicrobial action (mg/mL)

Solutions	MIC	MBC
Lysozyme and *L. casei*	Tube 4: 43.1	Tube 3: 50.3
Lysozyme and *S. mutans*	Tube 2: 58.7	Tube 1: 68.5
Lactoferrin and *L. casei*	-	-
Lactoferrin and *S. mutans*	-	-
Lysozyme + lactoferrin and *L. casei*	-	-
Lysozyme + lactoferrin and *S. mutans*	-	-

are other lactoferrin-binding proteins, which promote the bacterial growth stimulation of lactoferrin. This could explain why lactoferrin did not inhibit *L. casei,* but it could not explain why *S. mutans* was not inhibited.

Lysozyme from different sources were tested by Iacono et al. (23), against some strains of *S. mutans,* and they found that human lysozyme was more effective than hen egg white Lysozyme, however, many authors, have used this, because the facilities of extractions and good correlations with human lysozyme. Iacono et al. (23) showed a total inhibition of different strains of *S. mutans* by egg lysozyme at a concentration of 5 mg/mL, a lower value than our concentrations. They also stated that different mechanisms may be responsible for the bacteriostatic, lytic and bactericidal properties of the enzyme and that lysozyme is a selective but effective antibacterial factor for oral microorganisms.

Little is known about the interaction among lysozyme, lactoferrin, lactoperoxidase and secretory IgA (antibody) *in vivo,* but it is reasonable to presume that it occurs. *In vitro* evidence suggests that interactive effects may vary with different concentrations of the proteins involved; and salivary concentrations of lysozyme, lactoferrin, lactoperoxidase and secretory IgA show considerable variations between individuals. It is therefore likely that patterns of interactions will also vary between individuals (24). Many antimicrobial proteins contain multiple functional domains, which makes that one protein may have more than one mechanism of antimicrobial activity (17).

In the present study, the association of two enzymes did not promote a better antimicrobial effect with the concentrations utilized. It is hypothesized that the antimicrobial effects of lactoferrin and other salivary proteins are associated in a synergistic way, as immunoglobulins and inorganic compounds, also justifying the lack of inhibitory effects of lactoferrin alone.

According to Felizardo et al. (18), who found correlations among the lactoferrin and lysozyme concentrations and the caries index, it can be hypothesized that there are more interactions among these and other salivary proteins, which can influence the individual susceptibility of dental caries through antimicrobial activity. Therefore, the proteins interactions should be further investigated.

In conclusion, *S. mutans* and *L. casei* were only inhibited by lysozyme and were not affected by lactoferrin and by the synergic use of lysozyme and lactoferrin.

Resumo

O presente estudo avaliou, *in vitro*, o efeito antimicrobiano das proteínas salivares lactoferrina e lisozima sobre micro-organismos envolvidos no processo carioso, obtendo suas concentrações inibitórias mínimas (CIM) e bactericidas mínimas (CBM). Cepas de *Streptococcus mutans* (ATCC 25175) e *Lactobacillus casei* (ATCC 7469) foram submetidas a macrodiluição em caldo das soluções de lisozima a 80 mg/mL e lactoferrina a 200 mg/mL. A leitura dos tubos foi realizada em espectrofotômetro, após a incubação a 37 °C por 18 h em estufa de CO_2, para verificação da CIM. Uma nova subcultura foi semeada em placas de ágar para a obtenção da CBM. O método de difusão em ágar foi também testado utilizando-se placas de Petri com ágar BHI com100 µL do inóculo microbiano padronizado. Discos de filtro de papel embebidos com 10 µL das soluções de lactoferrina (200 µg/mL) e lisozima (80 µg/mL) foram colocados sobre a superfície do ágar. Não foi observado halo de inibição nas placas, demonstrando ausência de efeito antimicrobiano das proteínas neste teste. Os efeitos bactericida e bacteriostático da lisozima sobre *L. casei* foram 50,3 mg/mL e 43,1 mg/mL respectivamente. Os efeitos bactericida e bacteriostático sobre *S. mutans* foram 68,5 mg/mL e 58,7 mg/mL. A lactoferrina não induziu nenhum efeito inibitório sobre nenhuma bactéria, mesmo na concentração de 200 mg/mL. Não houve efeito antimicrobiano sinérgico das proteínas, quando testadas conjuntamente, e mesmo até em concentrações de 42,8 mg/mL de lisozima e 114 mg/mL de lactoferrina (os maiores valores avaliados). *S. mutans* e *L. casei* foram inibidos somente pela lisozima, não sendo afetados pela lactoferrina e pelo uso sinérgico de ambas proteínas.

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