Influence of artificial saliva in biofilm formation of *Candida albicans in vitro*

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Abstract: Due to the increase in life expectancy, new treatments have emerged which, although palliative, provide individuals with a better quality of life. Artificial saliva is a solution that contains substances that moisten a dry mouth, thus mimicking the role of saliva in lubricating the oral cavity and controlling the existing normal oral microbiota. This study aimed to assess the influence of commercially available artificial saliva on biofilm formation by *Candida albicans*. Artificial saliva I consists of carboxymethylcellulose, while artificial saliva II is composed of glucose oxidase, lactoferrin, lysozyme and lactoperoxidase. A control group used sterile distilled water. Microorganisms from the oral cavity were transferred to Sabouraud Dextrose Agar and incubated at 37 °C for 24 hours. Colonies of *Candida albicans* were suspended in a sterile solution of NaCl 0.9%, and standardisation of the suspension to 10^6 cells/mL was achieved. The acrylic discs, immersed in artificial saliva and sterile distilled water, were placed in a 24-well plate containing 2 mL of Sabouraud Dextrose Broth plus 5% sucrose and 0.1 mL aliquot of the *Candida albicans* suspension. The plates were incubated at 37 °C for 5 days, the discs were washed in 2 mL of 0.9% NaCl and placed into a tube containing 10 mL of 0.9% NaCl. After decimal dilutions, aliquots of 0.1 mL were seeded on Sabouraud Dextrose Agar and incubated at 37 °C for 48 hours. Counts were reported as CFU/mL (Log10). A statistically significant reduction of 29.89% (1.45 CFU/mL) of *Candida albicans* was observed in saliva I when compared to saliva II ($p = 0.002$, considering $p \leq 0.05$).

Descriptors: Saliva, Artificial; *Candida albicans*; Biofilm.

Introduction

Humans produce and secrete between 1200 to 1500 mL of saliva per day. Approximately 90% of this volume is produced by the salivary glands, and the remaining 10% are produced by numerous minor salivary glands present in the submucosa of the oral and oropharyngeal cavity.[1]

Saliva is essential for protection of the mucosa as well as structures of the oral cavity and adjacent gastrointestinal epithelium.[1] In the mouth, saliva participates in the activities of chewing, speaking and swallowing, as well as taste sensitivity, lubrication of tissues, mucosal protection against penetration of various substances, regulation of pH and the formation of an oral biofilm. Further, saliva helps control oral microbiota, protects teeth against harmful substances and injuries and participates in the remineralisation process.[2]

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Hyposalivation, which is characterised by decreased salivary flow, and xerostomia, the subjective feeling of a dry mouth, may or may not be associated.[3,4]

Factors related to hyposalivation and xerostomia include aging, the presence of glandular or systemic diseases and the use of certain drugs (e.g., anticholinergics, anxiolytics, antihypertensives, diuretics, antihistamines, anti-reflux). In addition, it may be a side effect of head and neck radiotherapy. Xerostomia and hyposalivation can diminish quality of life by resulting in discomfort and pain when chewing, swallowing and talking. The decrease in salivary flow increases the risk of tooth decay and other oral fungal and/or bacterial infections, facilitating the emergence of opportunistic pathogens such as *Candida albicans*.[2,5]

Control and/or management of hyposalivation are usually done with the use of commercially available artificial saliva. Because it is typically not composed of any medications, it may be administered by the patient whenever they feel that their mouth is dry.[3] The composition of artificial saliva includes substances that soothe a dry mouth by lubricating the oral cavity.[6-9]

The species of the genus *Candida* are constituents of the oral microbiota of humans and are commensal microorganisms.[10] *Candida albicans* is considered the most common fungal pathogen in humans and is responsible for most superficial or systemic fungal infections.[11,12] *Candida* spp. can be easily isolated from the oral cavity in approximately 60% of healthy adults.[13,14] *Candida albicans* primarily colonises the tongue, flowing into the oral mucosa, tooth surfaces, the biofilm and saliva. Its pathogenic action is favoured by local or systemic changes. Of the local factors associated with risk for *Candida* infection of the oral cavity, poor oral hygiene, use of ill-fitting dentures and decreased salivary flow are the most common. The systemic factors include nutritional deficiencies, malignant tumours, use of broad-spectrum antibiotics, smoking, age, diabetes, disorders of the salivary glands and deficiencies in the immune response.[13,15]

In immunocompromised individuals, *Candida* spp. can cause fatal fungaemias.[16] Of the virulence factors associated with *Candida* spp., the most important include the ability to adhere to surfaces by producing filamentous growth and releasing hydrolytic enzymes capable of inducing damage to host cells.[10] In addition, *Candida* species are able to form biofilms,[10,17] and this feature has been associated with an increased expression of virulence factors,[10,18] as well as a reduced susceptibility to antimicrobial agents.[10]

The development of effective salivary substitutes requires an understanding of the physiological and biological properties of human saliva, which depend primarily on the role of salivary proteins and glycoproteins.[19-22] The appropriate action of salivary substitutes requires the interaction between viscosity and film formation. The oral cavity provides a favourable environment for the presence of both substances in the saliva substitutes and human saliva.[22] Of the salivary proteins, the mucins are the proteins primarily responsible for lubrication and film formation in human saliva.[21,23]

The objective of this study was to evaluate biofilm formation of *Candida albicans* in an acrylic resin disc after incubation in two artificial saliva samples of distinctly different chemical composition.

Methodology

Acrylic resin disc

A colourless acrylic resin disc was created that measured 5 mm in diameter and was 2 mm thick, yielding a total of 30 units. The disc was sterilised by gamma rays (cobalt-60) with a dose of 20 kGy for 6 hours (Embrarad, São Paulo, Brazil).

Microorganism and preparation of standardised suspension

We used a standardised suspension of a standard strain of *Candida albicans* (ATCC 18804) from the Laboratory of Microbiology, School of Dentistry of São José dos Campos - UNESP. The microorganisms were transferred to Sabouraud Dextrose Agar (Difco, Detroit, USA) and incubated in a bacteriological incubator at 37 °C for 24 hours. Colonies of *Candida albicans* were suspended in a sterile solution of NaCl 0.9%, and a standardisation of the suspension to 10^6 cells/mL was obtained in the spectrophotome-

ter (B582, Micronal, São Paulo, Brazil) with a wavelength of 530 nm and an optical density of 0.284.

Artificial saliva

We used two artificial salivas, artificial saliva I (AS I) - Salivan® (Apsen, São Paulo, Brazil), which is composed of carboxymethylcellulose, and artificial saliva II (AS II), Biotène® (GlaxoSmithKline, Moon Township, USA) consisting of glucose oxidase, lactoferrin, lysozyme and lactoperoxidase. For the control groups, we used sterile distilled water. The acrylic resin discs were immersed for 60 minutes at 37 °C in the artificial salivas tested and in the sterile distilled water, distributed in 10 units in each group. They were then washed in sterile water.

Biofilm formation of *Candida albicans*

The acrylic resin discs previously incubated in the saliva were inserted, with the aid of sterile forceps, in the first row of 24-well plates (Costar Corning, New York, USA) containing 2 mL of Sabouraud Dextrose broth (HiMed, Mumbai, India) plus 5% sucrose (Synth, Diadema, São Paulo). Thereafter, each well of the plate received a 0.1 mL aliquot of the suspension of *Candida albicans*. The plates were incubated at 37 °C for 5 days.

After incubation, the acrylic resin discs were transferred to the second row of wells containing 2 mL of sterile NaCl 0.9%, and the plates were shaken for five minutes on an orbital shaker (Solab, Piracicaba, Brazil). This procedure was performed twice with the goal of removing non-microbial cells that were adherent to the acrylic resin discs. After washing, each disc was placed in a Falcon tube containing 10 mL of sterile NaCl 0.9% and homogenised for 30 seconds in an ultrasonic homogeniser (HD 2200 Sonoplus, Bandelin Electronic, Berlin, Germany) with a power of 50 W.

The solution obtained was assumed to have a dilution factor of 10^{-1}. From this, decimal dilutions (10^{-2} and 10^{-3}) of the biofilm suspension of each acrylic resin disc were made. Aliquots of 0.1 mL were seeded in Petri dishes containing Sabouraud Dextrose Agar (Difco, Detroit, USA) and were incubated in a bacteriological incubator at 37 °C for 48 hours. Plates containing 30-300 colonies were counted for calculation of colony forming units per millilitre (CFU / mL), and the number obtained was transformed into base 10 logarithm (log10).

Statistical analysis

The results were statistically analysed using Tukey's test and Minitab software (Inc. PA, USA).

Result

The means and standard deviations obtained in the tests and the results of the statistical analysis of the CFU/mL (Log10) for biofilms isolated from the control, AS I and AS II groups are shown in Figure 1.

In the comparison between biofilms isolated from the AS I and control groups, the percentage reductions observed were 11.32% (0.43 CFU/mL) for the AS I group compared to the control group ($p = 0.508$). Comparing the biofilm from the AS II and control groups, there was a 26.48% (1.02 CFU/mL) percentage increase observed in the AS II group when compared to the control group, a statistically significant increase ($p = 0.035$).

When we compared the biofilms of the AS I and AS II groups, the percentage reduction in the AS I group when compared to the AS II group was 29.89% (1.45 CFU/mL), which was statistically significant ($p = 0.002$).

Discussion

Saliva is essential for human health. When secreted at a normal volume, it has many functions

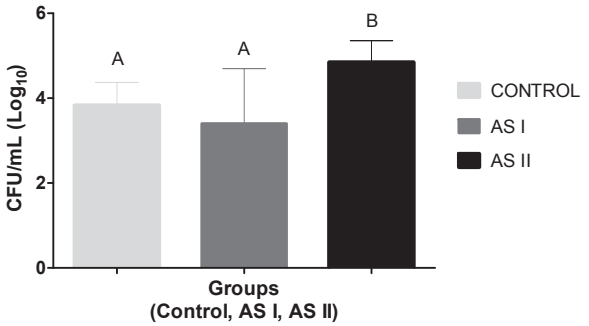

Figure 1 - Mean and standard deviation of the numbers of CFU/mL (log10) obtained in the tests for the control, AS I and AS II groups (AS: Artificial saliva; different letters indicate statistically significant difference between the experimental conditions; $p \leq 0.05$, ANOVA, Tukey's test).

and components, the primary being the lubrication of the oral cavity, as this is critical for protecting the oropharynx from infections. Much of its protective activity is mediated by its chemical makeup, which includes enzymes, minerals, proteins and substances that inhibit the growth and activity of various microorganisms.

The decrease in salivary volume within the oral cavity may cause discomfort and can cause the individual to be more susceptible to opportunistic infections. Many conditions can reduce salivary flow, e.g., the use of dental prosthetic devices, which are often made of acrylic resin and are easily colonised by microorganisms due to their surface roughness.[24] According to studies, *Candida albicans* is the primary organism identified in materials for making dentures, and the first phase of colonisation corresponds to adherence of the organism to a surface.[25]

Health professionals usually prescribe salivary substitutes or artificial saliva as treatment for hyposalivation. Ideally, these should have the same components as natural saliva; however, most of these products have only compounds that improve lubrication and moistening.[6] The high number of species of *Candida* in patients with xerostomia suggests that saliva substitutes participate in the control of oral microbiota. Since the 1990s, enzymes such as lysozyme, lactoferrin and lactoperoxidase have been proposed for use in patients with xerostomia with the goal of preventing related diseases.[26,27]

The present study compared the influence of two types of artificial saliva on biofilm formation by *Candida albicans* on acrylic resin discs. Based on the results, the artificial saliva products compared showed a statistically significant difference with respect to biofilm formation by *Candida albicans*. The AS II group had higher adherence of *Candida albicans* to the biofilm, although its formulation contains substances present in human saliva that would function to prevent diseases related to microbial growth. This result contrasts with that of Hahnel *et al*.[26] and Tenovuo *et al*.[27]

AS I resulted in a decreased development of *Candida albicans* biofilms. This solution is composed mainly of carboxymethylcellulose, a semisynthetic derivative of cellulose that is indigestible and is not absorbed systemically. It has a high sodium content (10.5% to 12.9%), which may interfere with the mechanisms of adhesion of oral microorganisms. The formation of a film by a cellulose-based product that is not consistently found in the oral cavity may also explain the lower adherence of *Candida albicans*, as this pathogen may not be expressing specific receptors for cellulose or derivatives. Thus, it is a drug that competes with the normal microbiota of the mouth. Carboxymethylcellulose is hypoallergenic, non-toxic, has high viscosity and does not interact with other medications. When tested *in vitro* in our study, it decreased the likelihood of *Candida albicans* biofilm formation; therefore, there may be a role for it in the prevention of oral candidiasis, and it may be a good option for patients who suffer with symptoms/signs of hyposalivation.

There have been studies that have studied the antimicrobial effect of commercially available artificial saliva that consists of lactoferrin, glucose oxidase, lactoperoxidase, lysozyme and immunoglobulins against *Streptococcus mutans, Lactobacillus acidophilus and Candida albicans*. These studies found that none of the evaluated compounds exerted anticandidal effects.[28]

Salivary substitutes are capable of forming films on the surfaces of teeth, tissue, mucous membranes and dental restorations, thus they are able to influence the initial adhesion of oral microorganisms.[26] In this *in vitro* study, we found that the initial adhesion of *Candida albicans* to the tested surface in the presence of artificial saliva varied depending on the components in the saliva. More specifically, AS II resulted in an increase in biofilm, while AS I decreased the amount of biofilm present.

Conclusion

The components in the formulation of artificial saliva II provided greater adherence of *Candida albicans* biofilms when compared to artificial saliva I, which contained carboxymethylcellulose.

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