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Establishment of a saliva donor selection for *in vitro* biofilm growth

Seleção de doadores de saliva para crescimento de biofilme *in vitro*

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Resumo

Introdução: O emprego de biofilmes polimicrobianos, utilizando a saliva como inóculo, é um modelo promissor para o estudo de biofilmes cariogênicos in vitro. Entretanto, ainda não existe uma padronização para seleção de doadores de saliva. **Objetivo:** O objetivo deste estudo foi estabelecer uma metodologia para seleção de doadores de saliva utilizando fatores salivares microbianos e características *in vitro* do biofilme. **Material e método:** Para doação de saliva foram selecionados vinte voluntários. Os voluntários permaneceram 24 horas sem escovar os dentes e ficaram em jejum por 2 horas antes da coleta da saliva. Foram avaliados os seguintes parâmetros: viabilidade das bactérias anaeróbias totais e *mutans* streptococci; concentração inibitória mínima (CIM) e concentração bactericida mínima (CBM) da clorexidina; capacidade de formação de biofilme por meio da biomassa; e a suscetibilidade dos biofilmes à clorexidina. **Resultado:** A viabilidade bacteriana da saliva, a capacidade de formação de biofilme e a suscetibilidade do biofilme à clorexidina foram apresentadas como média e intervalo de confiança (95%). A diferença entre a viabilidade do biofilme (*mutans* streptococci e bactérias totais) após tratamento com NaCl 0,9% e diacetato de clorexidina 0,2% foi comparada pelo teste t de Student com nível de significância estabelecido em 5%. A viabilidade total de bactérias anaeróbias (mediana) foi de 7,28 log 1+UFC/mL (unidades formadoras de colônia/mL). A viabilidade dos *mutans* streptococci na saliva apresentou mediana de 5,47 log 1+UFC/mL. Para capacidade de formação de biofilme a mediana da biomassa foi de 0,1172 A_{570}. **Conclusão:** O tratamento com clorexidina reduziu significativamente os *mutans* streptococci e a viabilidade total das bactérias. A metodologia para seleção do doador de saliva foi estabelecida com sucesso.

Descritores: Biofilme; biomassa; clorexidina; viabilidade microbiana; doador de saliva.

Abstract

Introduction: The utilization of polymicrobial biofilms, with saliva as an inoculum, represents a promising model for in vitro studies on cariogenic biofilms. However, there is still no standardization for selecting saliva donors. **Objective:** The aim of this study is to establish a methodology for the selection of saliva donors using microbial salivary factors and *in vitro* biofilm characteristics. **Material and method:** For saliva donation, twenty volunteers were selected. Volunteers remained 24 h without brushing their teeth and fasted for 2 h before saliva collection. The following parameters were evaluated: total anaerobic bacteria and *mutans* streptococci viability; minimum inhibitory concentration (MIC) and minimum bactericide concentration (MBC) of chlorhexidine; biofilm forming capacity by biomass assessment; and the susceptibility of biofilms to chlorhexidine. **Result:** Saliva bacterial viability, biofilm forming capacity and biofilm susceptibility to chlorhexidine were presented as mean and confidence interval (95%). The difference between biofilm (*mutans* streptococci and Total bacteria) viability after treatment with NaCl 0.9% and 0.2% chlorhexidine diacetate was compared using the Student t-test with a significance level established at 5%. Total anaerobic bacteria viability (median) was 7.28 log 1+CFU/mL (colony forming units/ mL). *Mutans* streptococci viability in the saliva showed a median of 5.47 log 1+CFU/mL. Biofilm

forming capacity showed that biomass had a median of 0.1172 A_{570}. **Conclusion:** Treatment with chlorhexidine significantly reduced *mutans* streptococci and total bacteria viability. The methodology for the selection of the saliva donor was successfully established.

Descriptors: Biofilm; biomass; chlorhexidine; microbial viability; saliva donor.

INTRODUCTION

Oral biofilms are communities of microbial cells that are incorporated into a matrix of organic polymers develop interlinked metabolic activities[1]. In the past decades, many efforts have been focused on developing *in vitro* biofilm models that allow a better understanding of the pathogenesis of oral biofilms, caries-like lesions development and the development of antimicrobial or antibiofilm substances[2-4]. In this context, most studies have focused on monotypic biofilms, particularly of *Streptococcus mutans*. Nevertheless, this model does not reproduce the ecological diversity and bacterial interactions present in naturally formed dental biofilm. As oral biofilm consists of more than 700 species[5,6], it is important to consider the physiological interactions between these species and not only the effects of a single species[7]. Moreover, studies have shown that the association of *S. mutans* with other microbial species enhance the cariogenicity of biofilm[8-10]. Thus, the limitations of using monotypic biofilms in caries-related studies are clear.

Given these limitations, a polymicrobial ("microcosm") biofilm model[11-13] emerges as a promising model for the study many features of cariogenic biofilms, such as: i) growth and development; ii) microbial succession; iii) microbial resistance to antibiotics; iv) biofilm response to environmental factors and v) screening of antiplaque agents. In this model, biofilms are developed from the saliva of volunteers and show similar complexity and heterogeneity, besides having similar potential of producing caries-like lesions[12]. Thus, it is possible to evaluate the ecology, pathology and performance of oral biofilms using a more realistic *in vitro* model[12,14].

Since the development of the polymicrobial ("microcosm") biofilm model, the ideal conditions to collect the inoculum regarding caries activity of the donor (caries-free or caries-active), origin of the sample (saliva, dental plaque or carious lesion) and the number of saliva donor have been explored. It is well established that, regardless of these conditions, the resulted *in vitro* polymicrobial biofilm will be cariogenic[13,15,16]. While the use of more than one volunteer may result in a greater microbial composition variability, may provide more microbial interactions[15], facilitates volunteer recruitment and requires less financial and human resources, pooling saliva from different volunteers may result in an instable and unrepresentative biofilm[17]. In addition, the presence of outliers[15] and the variation on microbial composition between individuals[17] can lead to the formation of a deficient biofilm. Observations from our research group have shown that, using the same growth conditions, some saliva might not develop adequate *in vitro* biofilms and some concerns have been raised about different susceptibility of biofilms to chlorhexidine (data not shown), which is an important milestone in new drug development.

Thus, it is of outmost importance that selection of the saliva donor is based on objective criteria. To date, there is no donor selection methodology in the literature that allows a standardization of the microbial viability, ability to form biofilms and susceptibility to chlorhexidine. In order to increase biofilm growth reproducibility, to ensure the development of cariogenic biofilms and to provide a more precise evaluation of biofilms susceptibility, we developed a methodology for the selection of the saliva donor based on confidence interval estimation, which can be applied in different laboratories to ensure to reproducibility of the studies. The methodology for the selection of saliva donors was established by evaluating salivary (bacterial viability in fresh saliva, ability to form biofilm and chlorhexidine MIC and MBC values) and biofilm parameters (susceptibility to chlorhexidine).

MATERIAL AND METHOD

Growing Conditions

The culture medium proposed by McBain et al.[17] was used for the culture of polymicrobial inoculum. The culture medium contained mucin (type II, porcine, gastric) (2.5 g/L), bacteriological peptone (2.0 g/L), tryptone (2.0 g/L), yeast extract (1.0 g/L), NaCl, (0.35g/L), KCl (0.2 g/L), $CaCl_2$ (0.2 g/L), cysteine hydrochloride (0.1 g/L), hemin (0.001 g/L), and vitamin K1 (0.0002 g/L), at pH 7.0, supplemented with 0.5% sucrose.

Saliva Collection and Processing

The use of saliva was approved by the Dentistry School of Araraquara Ethical Committee on Human Research (CAAE: 77697817.6.0000.5416). Informed consent was obtained from all volunteers in this study. Twenty healthy donors, aged between 20 and 40 years and with previous caries experience were selected. This research did not include pregnant and breastfeeding women, patients with oral prostheses and orthodontic appliances, total edentulous individuals, individuals with chronic periodontal disease or individuals who have received periodontal treatment in the last six months, individuals under head and neck radiotherapy with low salivary flow, smokers or those who made chronic use of alcohol, individuals with systemic diseases who were in drug therapy with drugs that could interfere with oral health conditions, such as opioids, anti-histamines, anti-depressives, anti-epileptics, anxiolytics and anticholinergic medications[18].

Donor volunteers refrained from antibiotics, antifungals and mouthwashes use in the previous six months and from anti-inflammatories and/or immunosuppressants in the last three months[18,19]. Individual saliva donor chewed parafilm before collection. The saliva was collected during the morning, after the volunteers abstained from toothbrushing for 24 h and without having breakfast for 2 h before collection. Furthermore, they were not allowed to consume alcoholic drinks in the last 2 h before saliva collection. The saliva samples were collected in the laboratory by responsible researcher and one volunteer at a time. After collection each saliva sample was processed immediately, filtered (0.22 µm polyethersulfone - PES) to remove debris and kept refrigerated. Next, saliva was diluted in glycerol/BHI (Brain heart infusion - final concentration of 30%), aliquots were placed in 2 mL tubes and stored at -80 ºC[20]. Exterkate et al.[20] demonstrated that there were no statistically significant differences in biofilm formed when fresh or frozen saliva was used.

Evaluation of Microbial Concentration in Saliva

In order to evaluate the microbial concentration in saliva, an aliquot of saliva was diluted in 0.9% NaCl to determine total anaerobic bacteria viability on Wilkins-Chalgren agar[21] and *mutans* streptococci viability on mitis salivarius agar supplemented with 15% sucrose and 0.2 IU/mL bacitracin (MSBS)[22]. Wilkins-Chalgren and MSBS agar plates were incubated in anaerobic conditions (5-10% CO_2; <1% O_2; Anaerobac - Probac do Brasil Produtos Bacteriológicos Ltda, Santa Cecília, SP, Brazil) at 37 °C for 48 h. Afterwards, the CFU numbers were counted and expressed in log (1 + CFU/mL).

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Chlorhexidine

The MIC and MBC were determined using the microdilution broth method based on the Clinical and Laboratory Standards Institute (CLSI, 2012), except that the Müeler-Hinton broth was replaced by McBain broth, since CLSI does not consider the growth of microorganisms from saliva. Two-fold serial dilutions were obtained in McBain broth in order to test concentrations between 0.0006 and 0.6 mg/mL (w/v) of chlorhexidine diacetate. As negative control, only McBain broth

was used. 20 µL of saliva from each volunteer were added to the wells containing 100 µL of broth. The plates were incubated in anaerobiosis at 37 ºC for 24 h. Growth inhibition (MIC) was evaluated after 24 h of incubation with the use of a spectrophotometer (λ = 620 nm)[23]. Thereafter, the wells were subcultured in Wilkins-Chalgren to determine MBC. MIC was determined as the lowest concentration to inhibit growth and MBC was determined as the lowest concentration to totally inhibit microbial growth. The experiments were performed in duplicate.

Ability to Form Biofilms

The ability to form biofilms was evaluated by analyzing the biomass formed. Glass coverslips (ø 13 mm; n=3/volunteer; sterilized at 121 °C for 15 min and later dried in an oven) were immersed in the wells of 24-well plates, containing 1.8 mL of broth and 0.4 mL of collected saliva, using an active adherence model[20], using an apparatus developed by Albuquerque et al.[24]. The glass coverslips were placed vertically in order to favor the formation of a biofilm only with cells capable of adhering to the specimens. This avoids deposition of microorganisms by the force of gravity, and their later adherence to the coverslips.

After 24 h of biofilm growth, the apparatus was removed from the culture medium, washed in NaCl 0.9% for 10 min and the biofilms were fixed with methanol for 15 min in a 24-well plate. After drying at room temperature, the glass coverslips were immersed in violet crystal solution. After 5 min, the glass coverslips containing the biofilms were washed with 0.9% NaCl in a 24-well plate. After drying again at room temperature, the glass coverslips were immersed in 33% acetic acid to remove the violet crystal. The contents of the wells were transferred in triplicate (200 µL each) to 96-well plates and the absorbance was read at 570 nm[25].

Evaluation of Polymicrobial Biofilm Susceptibility to Chlorhexidine

In order to evaluate the susceptibility to chlorhexidine, polymicrobial biofilms also were also grown using an active adherence model, as described before. After 24 h of growth, the culture medium was refreshed (2.2 mL) and the biofilms were cultivated for an additional 24 h. Next, the biofilms were washed in 0.9% NaCl to remove the non-adherent cells. Biofilms were immersed in 2.5 mL of 0.2% chlorhexidine diacetate or with NaCl 0,9% (control group for 2 min)[25]. After the treatments, the biofilms were washed in 0.9% NaCl.

Biofilms were dispersed in 2 mL of 0.9% saline solution using ultrasound bath for 10 s (Cristófoli ultrasound tank, Campo Mourão - PR, Brazil, 42 kHz). The dispersed biofilms were plated on Wilkins agar and MSBS agar to assess bacterial viability of total anaerobic bacteria and *mutans* streptococci, respectively, as previously described. The number of CFU was obtained and the results were expressed in log (1 + CFU/mL).

Data Analysis

The data were analyzed with GraphPad Prism version 3.02 (GraphPad Software Inc., San Diego, CA, USA). After checking normality and homoscedasticity, data from bacterial viability on saliva, biofilm forming capacity and biofilm susceptibility to chlorhexidine were presented as mean and confidence interval (95%). The difference between biofilm viability after treatment with NaCl 0.9% and 0.2% chlorhexidine diacetate was compared using the student t-test with a significance level established at 5%. The values of MIC and MBC were expressed as median, minimum and maximum.

RESULT

The variables bacterial viability in fresh saliva, biofilm forming capacity and MIC and MBC values of chlorhexidine and susceptibility to chlorhexidine for each volunteer (n=20) were shown in the dot plot (Figure 1).

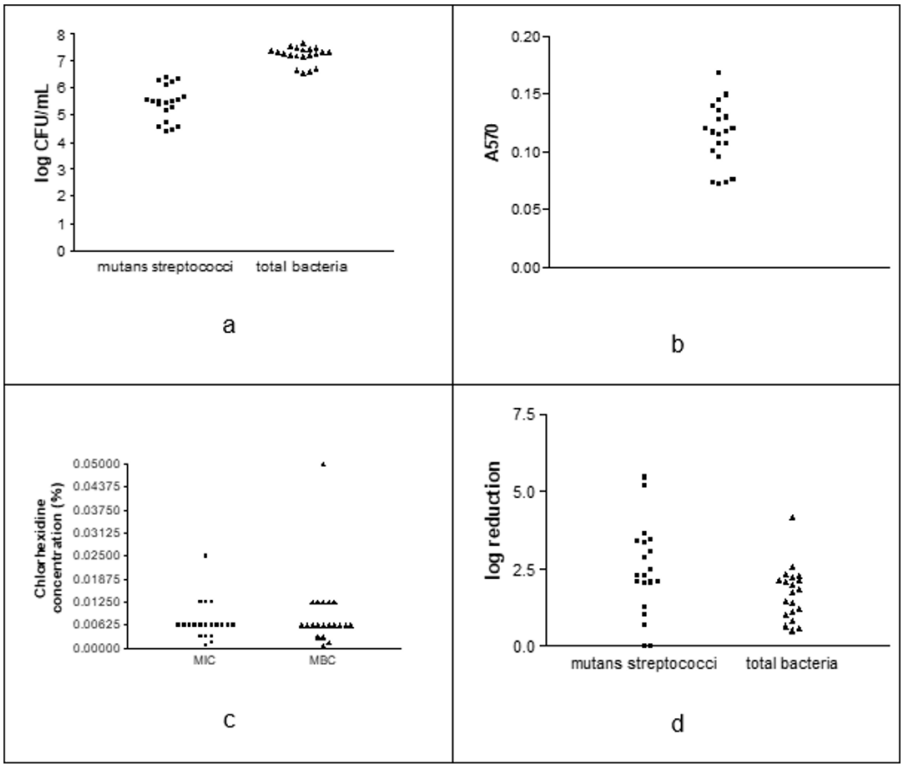

Figure 1. Distribution of individual volunteer data. a - Bacterial viability in fresh saliva (log CFU/mL); b- Biofilm forming capacity (A_{570}); c- Minimum inhibitory (MIC) and minimum bactericidal concentration (MBC) of saliva to chlorhexidine; d- Biofilm susceptibility to chlorhexidine. Each point represents one participant (n=20).

Mutans streptococci viability in saliva showed a median of 5.47 log 1+CFU/mL (min-max: 4.37 - 6.36). Total anaerobic bacteria in saliva were 7,28 log 1+CFU/mL (median) (min-max: 6.54 - 7.67). Biofilm forming capacity test showed that the biomass had a median of 0.1172 A_{570} (min-max: 0.07189 - 0.1686). The median for the MIC and MBC values of chlorhexidine was 0.00625% (min-max: 0.000781 - 0.0250) and 0.00625% (min-max: 0.000781 - 0.0500), respectively.

Table 1 shows the mean and confidence interval (95% CI) of the analyzed parameters (n= 20 participants). The mean concentration of total bacteria was 7.20 log CFU/mL (CI95 7.06 - 7.34) and of *mutans* streptococci was 5.40 log CFU/mL (95% CI 5.12 - 5.68). The mean biomass formed was 0.11 (95% CI 0.10 - 0.13). The mean reduction in microbial viability after chlorhexidine treatment was 1.71 log CFU/mL (95% CI 1.34 -2.08) for total bacteria and 2.43 log CFU/mL (95% CI 1.81 - 3.05) for *mutans* streptococci. For the data in Table 1, the smallest confidence intervals were for the variables bacterial viability in fresh saliva and biomass. Larger values were found for biofilm susceptibility to chlorhexidine.

Table 1. Mean and 95% confidence interval (95% CI) of the parameters analyzed (n= 20 participants)

Analysis	Variable	Mean	95% CI
Bacterial viability in fresh saliva (log (1+CFU/mL)	*mutans* streptococci	5.40	5.12 – 5.68
	Total bacteria	7.20	7.06 – 7.34
Ability to form biofilm (A_{570})	Biomass	0.11	0.10 – 0.13
Biofilm susceptibility to chlorhexidine (log reduction)	*mutans* streptococci	2.43	1.81 – 3.05
	Total bacteria	1.71	1.34 –2.08

Treatment with chlorhexidine significantly reduced *mutans* streptococci and total bacteria viability (Table 2). Treatment with chlorhexidine resulted in a log reduction of 2.81 Log 1+CFU/mL for *mutans* streptococci and 1.96 Log 1+CFU/mL for total bacteria.

Table 2. Biofilm viability after treatment with NaCl 0.9% (control) or 0.2% chlorhexidine diacetate (Log 1+CFU/mL)

	mutans streptococci		Total bacteria	
	control	treated	control	treated
Median	4.88[a]	2.07[b]	6.46[a]	4.50[b]
Percentile (25/75)	4.27/5.41	0.36/3.23	6.24/6.60	4.24/5.24

Medians followed by distinct letters indicate statistically significant difference within the same microbial group (t test; p=0.0001 for *mutans* streptococci and p < 0.0001 for total bacteria).

DISCUSSION

Although studies in the literature have evaluated the cariogenicity of biofilms from different conditions (caries-active or caries-free donors, saliva, dental plaque or dentine), there is a lack of a methodological study that systematizes saliva donor selection beyond the general requirements (good general health, normal salivary flow and not having used antibiotics). The present study established a methodology for the selection of saliva donors by evaluating salivary and biofilm parameters based on confidence interval estimation. The need for the development of this study arose from the following concerns: i) the occurrence of variability and heterogeneity of salivary composition from each individual, reflecting the variation in biofilm microbial composition[17,26,27]; ii) differences in therapeutic response to antimicrobial substances among volunteers[26]; iii) variations in biofilm forming capacity from saliva[24,28,29] and iv) the absence of methodology involving these parameters for volunteer selection. These concerns are based on the fact that most studies involving polymicrobial biofilm use only one volunteer to test substances with antimicrobial/antibiofilm properties.

Despite the increased cariogenicity found when saliva from more than one donor is pooled Viana et al.[15], the use of only one volunteer is useful to reduce costs and inter-subject variability. Moreover, there are evidence that the use of many subjects results in an unstable and unrepresentative biofilm[17]. Thus, it is desirable that parameters are established to produce reproducible research. By evaluating the 95% confidence interval, we can assure that, for the same population and under the same conditions, 95% of the saliva donors evaluated will have the salivary and biofilm parameters within the values found in the present study[30]. Moreover, narrow confidence intervals increase the certainty of the means estimated and the means are more representative of the source population[31].

The evaluated parameters of the 20 volunteers showed variability in their ability to form biofilm, susceptibility to chlorhexidine and bacterial viability in fresh saliva (total bacteria and *mutans* streptococci) (Figure 1). These results agree with a systematic review which showed considerable differences between the saliva for cariogenic biofilm formation and donor profiles among the studies. The authors also suggest a standardization for *in vitro* biofilm models[13]. *S. mutans* concentration in saliva also agrees with other studies[32,33]. This concentration can be considered high when compared to bacteria total amount (7.2 log 1+CFU/mL), but it can be explained by the fact that the volunteers had previous experience with dental caries[32]. Although caries experience may not be related to lesion development *in vitro*[15,34], the adoption of this inclusion criterion is recommended by several authors[2,14,19].

The highest 95% CI was found for biofilm susceptibility to chlorhexidine and the lowest variation was found for biofilm forming capacity (Table 1). These variations among individuals can be explained by the high bacterial diversity found in the oral cavity[3,35,36]. Kistler et al.[37] reported similar biofilm composition when the same saliva donor is used. On the other hand, a variation in biofilm composition was observed when different volunteers were used. This variation in salivary composition in different volunteers was also found in our study. Additionally, our study also provides a methodology of obtaining reference values for donor selection that can be used in future studies.

An interesting finding is that 50% of the volunteers presented the same MIC and MBC values (Figure 1). The determination of these values allows the identification of the antimicrobial substance concentration necessary to inhibit bacterial growth. As 50% of the volunteers presented the same MIC and MBC values, we can assume that the same chlorhexidine concentration was able to inhibit the microbial growth in the saliva of different donors included in this study. Therefore, this methodology suggests that the saliva selected in future studies presents MIC and MBC values to chlorhexidine similar to those found in this study. Also, the observation of MIC/MBC values is important because it avoids the use of saliva with lower susceptibility to chlorhexidine, which could overestimate the antimicrobial potential of the substance in tests. It is important to note that literature regarding chlorhexidine MIC and MBC values in saliva is scarce.

Considering the 95% CI, Table 1 can be used as reference values for volunteer's selection in future studies. However, more important than the values obtained, is the established of the methodology for saliva donor selection, since this methodology can be easily implemented in any laboratory routine. Data validation with confidence interval calculation is also important. To the best of our knowledge, there is no proposal in the literature for volunteer's selection methodology such as it is suggested in this work.

Furthermore, the findings of this study confirm that the model can form chlorhexidine-susceptible biofilms from the proposed methodology (Table 2), which agrees with the findings in the literature[16,24,38]. Therefore, the proposed model enables to select a volunteer that will provide chlorhexidine biofilm susceptible biofilms.

Pregnant and breastfeeding women, patients with oral prostheses and orthodontic appliances, total edentulous individuals, individuals under head and neck radiotherapy, smokers or those who made chronic use of alcohol, individuals who use some medications were not included in this study because they might present changes in salivary flow, which might result in changes in saliva microbial composition. These changes could impair our study results. The main causes of low salivary flow are related to alter autonomic secretion such as alcoholism, smoking and some drugs such as opioids, antihistamines, antidepressants, anti-epileptics, anxiolytics, and anticholinergic. Furthermore, patients in head and neck cancer treatment doing radiotherapy may present alterations in the secretory gland function, decreasing the salivary flow in these patients[18].

A recent systematic review showed that changes in microbial concentration, mainly in the *S. mutans* counts, oral pH and phosphate concentration decreases in the pregnant women. These changes can be extended to breastfeeding period[39]. Patients with orthodontic appliances also showed changes in microbial concentration, especially *S. mutans* and *lactobacilli*[40]. Patients using prosthesis or edentulous patients showed lower salivary flow and alterations in oral microbial concentration[41]. For these reasons these volunteers groups were not included in the study.

With the standards established in this study, future works using human saliva from donors do not need to evaluate and use several volunteers. The main idea of our study was to select only one volunteer and analyze whether his/her saliva is within the parameters found in this study. This increases confidence that this saliva donor selection methodology can be used to generate consistent results.

In short, this study showed the successful establishment of a methodology for volunteer selection in a way that guarantees biofilm formation capacity, and saliva and biofilm susceptibility to antimicrobial substances by means of simple, easy to perform and low-cost tests. These parameters, combined with the confidence interval calculation, are useful in the saliva donor selection and constitute an important step towards the development of satisfactory and reproducible biofilms. It is important to stress that values must be established for each population under study. However, once established, the values obtained can be used as reference for future studies.

CONCLUSION

It is concluded that, from the results presented in this study, there was a successful methodology establishment for saliva donor selection. It is recommended that this system be used in the volunteer's selection for cariogenic biofilms growth in future studies.

AUTHORS' CONTRIBUTIONS

Thalita Mendes: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization.
Luciana Solera Sales: Conceptualization, Methodology, Formal analysis, Data Curation Writing - Original Draft, Writing - Review & Editing, Visualization.
Marcelle Danelon: Conceptualization, Methodology, Formal analysis, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization.
Fernanda Lourenção Brighenti: Conceptualization, Methodology, Formal analysis, Resources, Data Curation, Writing - Original Draft, Writing - Review & Editing, Supervision, Project administration, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

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ETHICAL STATEMENT

The use of saliva was approved by the Dentistry School of Araraquara Ethical Committee on Human Research (CAAE: 77697817.6.0000.5416).

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CONFLICTS OF INTERESTS

The authors declare no conflicts of interest.

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