Special Issue (October)

INTERNATIONAL JOURNAL OF APPLIED PHARMACEUTICS

INNOVARE ACADEMIC SCIENCES Knowledge to Innovation

ISSN - 0975-7058

Research Article

ANALYSIS OF THE POTENTIAL OF *STREPTOCOCCUS SALIVARIUS* ISOLATED FROM THE SALIVA AND TONGUE DORSUM TO INHIBIT THE GROWTH OF *FUSOBACTERIUM NUCLEATUM*

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Received: 21 April 2017, Revised and Accepted: 18 August 2017

ABSTRACT

Objective: Analyzing the potential of *S. salivarius* isolated from the saliva and tongue dorsum of adults to inhibit the growth of *Fusobacterium nucleatum*.

Methods: Polymerase chain reaction, deferred antagonism test, and well-diffused agar test.

Results: Inhibition of the growth *F. nucleatum* by *S. salivarius* isolated from the tongue dorsum ($p>0.05$). No inhibition to the growth of *F. nucleatum* by *S. salivarius* isolated from the saliva. No inhibition to the growth of *F. nucleatum* by the protein produced by *S. salivarius*.

Conclusions: The growth of *F. nucleatum* was not inhibited by *S. salivarius* isolated from the saliva but by *S. salivarius* isolated from the dorsum of the tongue.

Keywords: Streptococcus salivarius, Fusobacterium nucleatum, Probiotic.

 DOI: http://dx.doi.org/10.22159/ijap.2017.v9s1.06

INTRODUCTION

For years, a general decrease in the effectiveness of antibiotics to cure infections has been occurring globally at an increased pace [1], leading to the development of other approaches, including a natural alternative, probiotic agents, to address this problem. Probiotics are defined by the World Health Organization as living microorganisms that can be beneficial to their host's health [2]. Various studies have shown that isolated oral probiotics can prevent caries and the formation of biofilm plaque and can treat pharyngitis and halitosis [2], which has led to their use as treatments and prevention methods in oral and dental health. Of existing oral bacteria species, the *Streptococcus* genus has proved to have beneficial effects on oral infections [3]. Probiotics work by inhibiting the adhesion of pathogens to the host's tissue, stimulating and modulating the immune system, and killing or inhibiting the growth of pathogens by producing products toxic to them, including bacteriocins, which are peptides or antimicrobial proteins produced by bacteria that inhibit the growth of or kill other bacteria without endangering themselves. *Streptococcus salivarius* produces Bacteriocin-like inhibitory substance, a bacterial product that has an inhibitory effect similar to that of a bacteriocin [3].

S. salivarius is an oral microbiotic organism abundant in healthy human beings that play a major role in maintaining a balanced oral ecosystem. In healthy human beings, *S. salivarius* is the main component in biofilm that forms on the buccal mucosa, tongue, dorsum epithelial, and pharynx mucosa. Some strains of *S. salivarius* on the tongue release bacteriocins that are toxic to oral flora bacteria and that change characteristics from commensal to pathogenic [4,5]. The literature shows *S. salivarius* as toxic to the oral streptococci involved in tooth decay, including *Streptococcus mutans, Streptococcus sobrinus,* and *Streptococcus pyogenes*, and to the pathogens involved in periodontitis [6]. *Fusobacterium nucleatum* is an oral organism that can be pathogenic to periodontal tissue, as it invades oral epithelial cells and facilitates the infiltration of non-invasive bacteria to form a biofilm on periodontal tissue [7]. Therefore, early elimination of the bacteria that cause periodontal disease can delay its development. The most common periodontal disease is periodontitis, an infection in the tooth-supporting structure caused by inflammation, which causes a progressive destruction in the periodontal ligament and alveolar bone structure due to the formation of plaque, calculus, and periodontal pockets. One of the major causes of periodontal disease is bacterial accumulation, which triggers the formation of microbial plaque [7].

Because of its relatively limited spectrum, protein produced by *S. salivarius* could be a natural alternative to antibiotics [3]. Even though the probiotic effect of *S. salivarius* has been known, to date no studies have addressed the potential of protein secreted by *S. salivarius* isolated from the dorsum of the tongue and from the saliva to inhibit the growth of *F. nucleatum.* This study aimed to analyze the potential of protein and other molecules secreted by *S. salivarius* isolated from the dorsum of the tongue and the saliva of healthy adults to inhibit the growth of *F. nucleatum.*

METHODS

Subject selection and preparation

Participants were selected by comparing the oral condition of each potential participant to the inclusion criteria. Ten participants who met these criteria were given an explanation of the study and were asked to sign an informed consent form agreeing to participate. Participants were asked not to consume any food for 3 hrs before sample collection and to brush their teeth before sample collection.

Sampling the saliva and the dorsum of the tongue

Samples were collected in the morning. Immediately before collection, participants were asked to rinse their mouths with water to eliminate food debris, and collection was delayed 10 minutes after rinsing to avoid dilution of saliva with the rinse water. Then, 10 ml of saliva was collected after stimulation with Parafilm M, in which participants were instructed to chew for 10 minutes before their saliva was collected into sterilized vials, which were sealed and refrigerated at 4°C until use. Before the collection of isolated samples of the dorsum of the tongue, each participant's tongue was isolated using a sterilized cotton roll. Sampling was done by moving a citobrush from the circumvallate papillae to the tip of the tongue. Each citobrush containing a sample

was placed into a vial with phosphate buffered saline (PBS) of pH 7.2 and refrigerated at 4°C until used.

Identifying *S. salivarius*

The samples of saliva and isolated dorsum of the tongue were cultured on mannitol salt agar (MSA) medium to isolate the *S. salivarius* colonies [8]. The medium containing the target bacteria was stored in anaerobic jars, subjected to mixed gas for 2 minutes, and then incubated at 37°C for 24 hrs. In addition, colony morphology was identified by observing the size and surface consistency of each colony for softness and smoothness (Fig. 1) [9]. Colonies identified by this observation were confirmed as *S. salivarius* using the polymerase chain reaction (PCR) technique, with *S. salivarius* ATCC 13419 serving as a positive control and SalAUS (5'-GTAGAAAATATTTACTACATACT) and SalADS (5'-GTTAAAGTATTCGTAAAACTGATG) serving as primers [10]. Colonies confirmed by PCR were then grown on Columbia blood agar, given mixed gas for 2 minutes, and incubated at 37°C for 24 hrs.

The results of this CPR amplification were analyzed using 1% agarose subjected to electrophoresis for 30 minutes at 100 V. *S. salivarius* colonies were identified by the appearance of a DNA band of 118 bp. Colonies identified as *S. salivarius* were provided with glycerol stock and stored at −80°C. Cultivation was also conducted on brain-heart infusion (BHI) agar medium and BHI liquid for 18 hrs.

Identifying and calculating *S. salivarius* protein concentrations

The results of the liquid-medium cultivation were centrifuged. The resulting pellets were separated from spent medium using cell lysate buffer and centrifuged. Both the pellets and the spent medium were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (150 V, 80 mA, 60 minutes) and colored using Coomassie blue. SDS-PAGE identified 4 participants with similar protein profiles based on the appearance of bands having sizes up to 70, 40, and 10 kDa (Fig. 2).

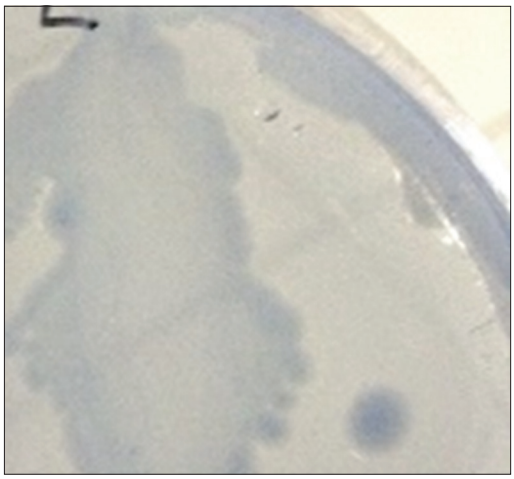

Fig. 1: Morphology of *Streptococcus salivarius* culture on Mitis Salivarius agar medium

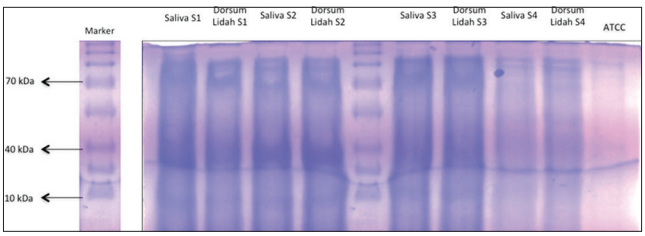

Fig. 2: Profile of protein *Streptococcus salivarius* based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis

The Bradford method was used to calculate concentrations of protein that had the same molecule mass [11], which identified that on the whole cell, the concentration of protein produced by *S. salivarius* from isolated saliva was 20,625 µg/ml; from isolated dorsum of the tongue, 20,313 µg/ml; and from ATCC 13419, 14,622 µg/ml. On the spent medium, the concentration of protein produced by *S. salivarius* from isolated saliva was 28,263 µg/ml; from the isolated dorsum of the tongue, 27,972 µg/ml; and from ATCC 13419, 18,127 µg/ml. Colonies of *S. salivarius* with similar profiles were placed in Eppendorf tubes filled with 30% glycerol and stored frozen at −80°C as stock.

Deferred antagonism testing to analyze inhibitory potential of *S. salivarius*

Clinical *S. salivarius* and *S. salivarius* ATCC 13419 (control) were diluted into 4 concentrations: 100%, 10%, 1%, and 0.1%, each of which was inoculated on a 1-cm band on BHI agar. The agar plate was placed inside an anaerobic jar, given mixed gas, and incubated at 37°C for 24 hrs. Likewise, *F. nucleatum* 10,593 was cultured on BHI agar medium, placed inside an anaerobic jar, given mixed gas, and incubated at 37°C for 24 hrs. After 24 hrs, bacteria visible on the agar's surface were wiped with a glass slide. Then, the agar's surface was sterilized with chloroform for 30 minutes by placing the agar plate upside down against a circle of filter paper soaked in chloroform, with the assumption that the bacteria on the agar's surface would die and those inside the agar would live. After 30 minutes, the plate was aired for 15 minutes to eliminate remaining chloroform. One colony of *F. nucleatum* was placed into an Eppendorf tube filled with soft agar and centrifuged to homogenize it. A cotton bud was used to spread the *F. nucleatum* mixed with soft agar perpendicular to *S. salivarius* that had been cleaned. Each procedure was performed twice. Then, the agar plate was placed inside an anaerobic jar, given mixed gas, and incubated at 37°C for 24 hrs preparatory to identifying the inhibitory zone of *F. nucleatum* growth.

Analyzing the inhibitory potential of *S. salivarius* protein

A 2-inoculating loops colony of *F. nucleatum* was placed into an Eppendorf tube filled with PBS and centrifuged until homogenated. Then, 100 ml of BHI agar was sterilized and cooled to 55°C. *F. nucleatum* was diluted with PBS and then inoculated into the liquid BHI agar. After being placed on an orbital shaker for 30 seconds, the BHI agar was poured into a Petri dish. Once the agar hardened, 4 wells were made in it, each 4 mm in diameter. Protein was placed into each well, and the dish was incubated at 37°C for 24 hrs, at which time, the distance between each well's edge and the edges of the bacteria colonies was observed and measured.

Data analysis

Data analysis was performed by comparing the number of participants who harbored *S. salivarius* colonies in both their isolated saliva and isolated dorsum of the tongue with those who harbored no colonies in either isolated source. This was done using Fisher's test (F-test). In addition, a one-way ANOVA *post hoc* test was conducted to compare the mean values of the inhibitory zones of *S. salivarius* and its secreted protein against the growth of *F. nucleatum* on each isolated source. Furthermore, the inhibitory potential of *S. salivarius* and its secreted protein on each concentration was analyzed using a paired t-test. Finally, a one-way ANOVA test was used to compare the mean value of the inhibitory zone of *S. salivarius* with the mean value of the inhibitory zone of its secreted protein.

RESULTS

Identification of *S. salivarius* was conducted by comparing the morphology of clinical *S. salivarius* with that of a control, *S. salivarius* ATCC 13419, which was grown on MSA medium. Of the 10 participants, 6 from each isolated sample had *S. salivarius* colonies with similar morphologies: Large, sticky, and mucoid. PCR test was conducted on these six participants to confirm that the colonies were *S. salivarius*, which was done by observing the fragment band of 118 bp. Fig. 3 shows the results of the PCR test (Table 1).

After *S. salivarius* was isolated from the isolated sources, the interaction of *S. salivarius* from four subjects who had protein profiles similar to

those of *F. nucleatum* 10,593 was tested using a deferred antagonism test. Analysis was conducted by comparing the means of the inhibitory zone of *F. nucleatum* growth of each concentration from each isolated source. The results of this test could be interpreted for only one of the four participants. Therefore, this stage of the study and the next one used a sample from only one participant. For both the isolated dorsum of the tongue and the control, the mean values of the zone of inhibition of *F. nucleatum* growth differed significantly from that of isolated saliva. The mean value of the zone of inhibition of isolated saliva could not be compared with that of the control because both had the same results (Fig. 4).

The value of the inhibitory zone of the isolated dorsum of the tongue was significantly larger than those for both isolated saliva and the control. However, the values of the inhibitory zone of the isolated saliva and the control did not differ significantly from each other. In fact, on each concentration of each isolate, the mean value of the inhibitory zone of *S. salivarius* differed slightly, but not significantly. The potential of protein secreted by *S. salivarius* to inhibit the growth of *F. nucleatum* was tested using the well-diffused agar method. Tables 2 and 3 show that for each isolated source, the mean value of the inhibitory zone around the well and at all concentration levels was 0 mm.

A dense colony of *F. nucleatum* grew inside the agar but did not reach the surface, so microscopic observations were made around the wells to confirm the presence of the colonies (Fig. 5). These observations confirmed that circles of colonies were spread throughout the agar, indicating that there was no inhibition of colony growth inside the agar.

DISCUSSION

This study was conducted to analyze the potential of *S. salivarius* and its secreted protein to inhibit the growth of *F. nucleatum* isolated from both the saliva and the dorsum of the tongue. The results showed that

Table 1: Identifying *S. salivarius* from clinical isolated sources

Isolated source	*S. salivarius*	
	+ (%)	− (%)
Saliva	6 (60)	4 (40)
Tongue	6 (60)	4 (40)

S. salivarius: Streptococcus salivarius

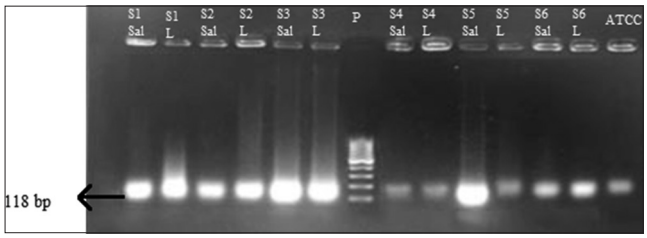

Fig. 3: Polymerase chain reaction test results for *Streptococcus salivarius* colonies, isolated saliva, and isolated dorsum of the tongue. Sal: Isolated saliva. L: Isolated dorsum of the tongue. ATCC: S. *salivarius* ATCC 13419

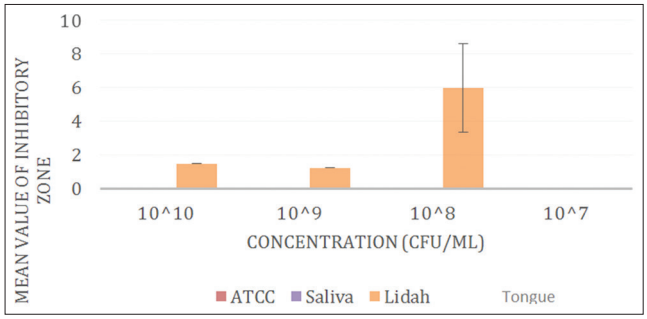

Fig. 4: Potential of *Streptococcus salivarius* based on isolate to inhibit the growth of *Fusobacterium nucleatum* as measured using the deferred antagonism test

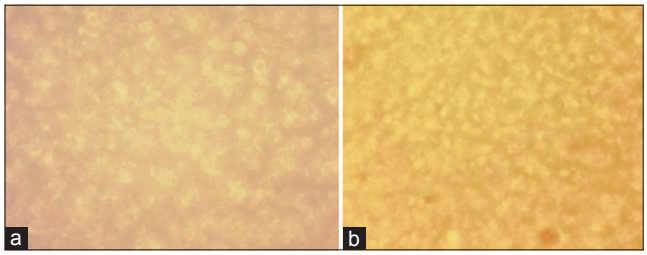

Fig. 5: (a and b) Microscopic images of *Fusobacterium nucleatum* colonies inside agar

Table 2: Mean value of inhibitory zone for each concentration on each isolated whole cell, as measured using the well-diffused agar method

Description	Results of culture based on protein concentration				
Concentration of protein *S. salivarius* ATCC 13419	Control	14.62 µg/ml	1.46 µg/ml	1.46×10^{-1} µg/ml	1.46×10^{-2} µg/ml
Mean value of inhibitory zone	0 mm	0 mm	0 mm	0 mm	0 mm
Concentration of clinical protein *S. salivarius* (saliva)	Control	20.62 µg/ml	2.06 µg/ml	2.06×10^{-1} µg/ml	2.06×10^{-2} µg/ml
Mean value of inhibitory zone	0 mm	0 mm	0 mm	0 mm	0 mm
Concentration of clinical protein *S. salivarius* (dorsum of the tongue)	Control	20.31 µg/ml	2.03 µg/ml	2.03×10^{-1} µg/ml	2.03×10^{-2} µg/ml
Mean value of inhibitory zone	0 mm	0 mm	0 mm	0 mm	0 mm

S. salivarius: Streptococcus salivarius

Table 3: Mean value of inhibitory zone for each concentration on each isolated spent medium, as measured using the well-diffused agar method

Description	Results of culture based on protein concentration				
Concentration of protein *S. salivarius* ATCC 13419	Control	18.12 µg/ml	1.81 µg/ml	1.81×10^{-1} µg/ml	1.81×10^{-2} µg/ml
Mean value of inhibitory zone	0 mm	0 mm	0 mm	0 mm	0 mm
Concentration of clinical protein *S. salivarius* (saliva)	Control	28.26 µg/ml	2.82 µg/ml	2.82×10^{-1} µg/ml	2.82×10^{-2} µg/ml
Mean value of inhibitory zone	0 mm	0 mm	0 mm	0 mm	0 mm
Concentration of clinical protein *S. salivarius* (dorsum of the tongue)	Control	27.97 µg/ml	2.79 µg/ml	2.79×10^{-1} µg/ml	2.79×10^{-2} µg/ml
Mean value of inhibitory zone	0 mm	0 mm	0 mm	0 mm	0 mm

S. salivarius: Streptococcus salivarius

of 10 participants, 6 had *S. salivarius* in both sample sources (saliva and dorsum of the tongue). The results aligned with those of a previous study, which found that *S. salivarius* was a pioneer colony in the oral cavity and remained there as predominant bacteria as long as the human host lived [12]. That study concluded that *S. salivarius* could be isolated from the human oral cavity, especially in samples of saliva and dorsum of the tongue. However, that study found minimal numbers of *S. salivarius* colonies. The diets of participants could be a factor in this, as diet can influence changes in microbiotic composition, depending on the basic capacity of each species to use the diet substrate [12]. *S. salivarius* needs organic components and nutrients such as sucrose for microbial growth [13]. Therefore, eliminating sucrose from the diet could drastically decrease the amount of *S. salivarius* in the saliva. Ogawa *et al*. found that *S. salivarius* needs a certain amount of energy to grow and develop colonies [12]. This energy comes from sucrose, which is degraded into fructose with the help of an extracellular enzyme called fructosyltranferase, which is secreted by *S. salivarius*.

Besides diet, factors that influence growth of *S. salivarius* in the human oral cavity include components of enzymes in the saliva, including lysozyme, lactoperoxidase, and amylase, all of which have antibacterial activity [13] that might decrease the growth of *S. salivarius* to the point that colonies are undetected when grown on MSA medium. The present study used multiple isolated sources and concentrations to analyze the potential of *S. salivarius* and its secreted protein to inhibit the growth of *F. nucleatum*. A one-way ANOVA test showed no significant difference between either the inhibitory zones of isolated dorsum of the tongue and saliva or between the inhibitory zones of any of the sources and the control (*S. salivarius* ATCC 13419). Likewise, a *post hoc* test of each concentration of each isolate and the control found no significant differences, indicating that the inhibitory potential of *S. salivarius,* whether from one of the isolated sources or from the control, was not dependent on the concentration of the bacteria.

To function properly, lantibiotic must bond with a lipid II precursor in the peptidoglycan. Gram-negative bacteria have three layers of cell membrane: The outer membrane, a thick layer of peptidoglycan in the periplasmic space, and an inner membrane. The outer membrane acts as a barrier to stop macromolecules, including bacteriocins, from entering [14]. Therefore, to reach the peptidoglycan, products of *S. salivarius* must penetrate the outer membrane of Gram-negative bacteria. An external agent called a permeabilizer can destroy the integrity of this outer membrane [15]. Lactic acid is a permeabilizer, and *S. salivarius,* as lactic acid bacteria, can produce lactic acid. Therefore, in the present study, *S. salivarius* was assumed to be able to produce lactic acid that would destroy the outer membrane of *F. nucleatum*, allowing lantibiotic to bond with the lipid II precursor on the peptidoglycan, create pores in the *F. nucleatum* membrane, inhibit the cell membrane's biosynthesis, and destroy the target bacteria.

The present study formed inhibitory zones differently from those in MacDonald's study, which found no inhibition against the growth of *F. nucleatum* [16]. However, that study found that although there was no inhibition against the growth of *F. nucleatum*, there was coaggregation between *S. salivarius* and *F. nucleatum*. This difference in results might be caused by the characteristics of salivaricin. MacDonald's study used *S. salivarius* K12 and M18, reas the present study used *S. salivarius* 13,491 (control) and a clinical strain [16]. One of the lantibiotics secreted by *S. salivarius* K12 and M18 is salivaricin B, which instead of forming pores on the cell's membranes, resists the transglycosylation that leads to inhibition of the cell wall's biosynthesis [17]. That was why in the study, salivaricin appeared only to reduce the thickness of the cell walls. In that study, after 24 hrs, the target cell had undergone only partial lysis.

In the present study, purification was not conducted to encourage a specific antibiotic, so it could not be determined which type of lantibiotic would be produced by any particular strain. Likewise, the present study did not conduct microscopic tests of the *F. nucleatum* after exposure to *S. salivarius*, so data on changes to the structure of the cell layers cannot be provided. However, given the differences in the results between McDonald's study and the present one, it can be assumed that the strain used by the latter produced a type of lantibiotic that produced pores and inhibited the growth of the target bacteria. Paired t-test conducted on the inhibition zones produced by the isolated dorsum of the tongue and by *S. salivarius* 13,419 (control) showed that the clinical *S. salivarius* strain had a larger inhibition zone than *S. salivarius* did, even though the difference was not statistically significant ($p>0.05$). However, the inhibition zone produced by isolated saliva could not be compared statistically with that produced by *S. salivarius* 13,419 because the results of both were the same, with neither forming an inhibition zone.

The results of the present study also were aligned with those of another previous study that identified three receptors on the surface of *S. salivarius* [18]. The first receptor mediates the host's adhesion and aggregation, including aggregation of saliva and adhesion to buccal epithelial cells. The second receptor functions in coaggregation with *Veillonella alcalescens* VI, and the third receptor functions in coaggregation with *Fusobacterium nucelatum* LF. That study found that the receptors on both clinical and wild-type strains functioned well *in vitro*, meaning that they could adhere to the buccal surface, the teeth, and the dorsum of the tongue, as well as coaggregate with *Veillonella* and *F. nucleatum*. That study found no adhesion to either epithelial cells or saliva and no coaggregation with *Veillonella*. To further analyze the microbial activity of *S. salivarius*, the present study conducted another test of the inhibition potential of its secreted protein, this time using the well-diffused agar method. The bacteria that produced the protein were grown on liquid medium BHI broth. After 24 hrs, protein produced by the bacteria on whole cells was harvested using the cell-lysate method, while protein on the spent medium was harvested using centrifugation.

The results of this test showed no potential of *S. salivarius* from either saliva or from the dorsum of the tongue to inhibit the growth of *F. nucleatum* in any concentration. These results supported the theory of Barbour *et al*. and Ross *et al*., which states that more bacteriocins would be produced by *S. salivarius* if the bacteria were grown on a solid medium [19]. In general, bacteriocins were not expressed and did not show inhibition activity when the bacteria were grown on liquid medium. However, liquid medium could be used if the method used to harvest the protein was the auto-induction method [19]. This theory was also supported by a study by Barbour and Philip, which found that regulating lantibiotic production involved peptides secreted as molecules that play a role in bacterial communication [8]. These peptides accumulate in an environment during bacterial growth, and when they reach a certain concentration, lantibiotic production is induced in high concentration. However, production of salivaricin from the *S. salivarius* strain did not guarantee that the bioactive molecule would be well expressed. Therefore, in the present study, the auto-induction method was not used, and the possibility that peptide-molecule concentration would not reach a point sufficient to induce lantibiotic production was assumed to be one of the causes of the unexpressed protein of *S. salivarius* when it was tested using a bacterial indicator.

Another factor that might influence the results of the present study is the possibility that the lantibiotic could not bond with the lipid II precursor on the peptidoglycan. In general, lantibiotic interferes with the integrity of the cytoplasmic membrane by forming pores and inhibiting the biosynthesis of cell walls [17,19]. As explained previously, lantibiotic can work on Gram-negative bacteria only if the bacteria's outer membrane is compromised [20], which can be accomplished only by lantibiotic that is relatively large (1800-4600 Da) [21]. To penetrate the outer membrane, a permeabilizer is needed, such as lactic acid produced by *S. salivarius*. However, in the present study, *F. nucleatum* was exposed only to protein produced by *S. salivarius*. Therefore, there was no fermentation of lactic acid to enable destruction of the outer membrane, so the lantibiotic could not act on the *F. nucleatum*. The present study assumed that without a permeabilizer, produced either by *S. salivarius* or another substance acting as one, lantibiotic could not influence the growth of *F. nucleatum* because it would not be capable

of penetrating the outer membrane to reach the peptidoglycan layer. Because of time limitations and constraints on the materials available, the present study did not use either the purification method or the auto-induction method to harvest a certain lantibiotic. Each class of lantibiotic has a different mechanism. This constraint on the study's method prevented further elaboration of differences in the mechanism of inhibition between *S. salivarius* and *F. nucleatum*. Therefore, studies should investigate lantibiotic protein that is produced using a clinical strain from both isolated saliva and isolated dorsum of the tongue.

CONCLUSION

In general, the present study concluded that *S. salivarius* had the potential to inhibit the growth of *in vitro F. Nucleatum.* However, this inhibitory potential differed, depending on whether the *S. salivarius* came from the participant's saliva or dorsum of the tongue. However, the source of the *S. salivarius* made no difference in the ability of the protein it produced to inhibit the growth of *F. nucleatum.* Suggestions for further studies include the following: (1) Purifying lantibiotic produced by *S. salivarius*; (2) acquiring a more specific type of lantibiotic; (3) analyzing *S. salivarius*'s inhibitory potential using samples isolated from both saliva and the dorsum of the tongue and using *S. salivarius* K12 and M18 as positive controls; (4) analyzing the inhibitory potential of protein produced by *S. salivarius* when combined with a permeabilizer; and (5) analyzing the antimicrobial activity of a clinical strain of *S. salivarius* on other oral-bacterial pathogens.

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