

ANALYSIS OF THE INHIBITORY POTENTIAL OF *STREPTOCOCCUS SALIVARIUS* ISOLATED FROM ADULT SALIVA AND THE TONGUE DORSUM FOR THE GROWTH OF *CANDIDA ALBICANS*

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ABSTRACT

Objectives: The objective of this study is to analyze the effectiveness of *Streptococcus salivarius* and its protein for inhibiting the growth of *Candida albicans*.

Methods: The analysis was conducted using polymerase chain reaction, sodium dodecyl sulfate polyacrylamide gel electrophoresis, a Bradford test, deferred antagonism test, and well-diffusion agar.

Result: *S. salivarius*, isolated from saliva and the tongue dorsum, and its protein do not inhibit the growth of *C. albicans*. The morphology of *C. albicans* did not change after being exposed to protein produced by *S. salivarius*.

Conclusions: *S. salivarius* and its protein do not inhibit the growth of *C. albicans*. However, the bacterium has the capacity to maintain fungus morphology in the form of blastospore.

Keywords: Bacteriocins, *Candida albicans*, Probiotic, Protein, *Streptococcus salivarius*.

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INTRODUCTION

Oral health problems are common in Indonesia and have become more complex, resulting in the need for a variety of treatments. Such treatments can be conventional, such as using antibiotics, but overuse of antibiotic can lead to antibiotic resistance, which is a global health concern, both clinically and publically [1]. Therefore, probiotic agents have been developed as natural alternatives to oral health treatments. According to the World Health Organization, a probiotic is a living microorganism that is useful for maintaining health [2]. Probiotics kill or inhibit pathogenic bacterial growth by producing bacteriocins or other products that act antagonistically on pathogenic bacteria. One such probiotic is *Streptococcus salivarius* [3]. *S. salivarius* originates in the womb, is obtained from an infant's mother several hours after birth, and can be identified 2 days after birth [2]. This bacteria is usually isolated from saliva and almost all surfaces of the oral cavity, especially the tongue and mucus. Known strains include K12, M18, K58, and TOVE-R. K12 *S. salivarius* can bond with *Candida albicans* hyphae to prevent the bacteria from adhering to plastic substrate [4]. This strain has been used in New Zealand as an oral isolate probiotic to obstruct *in vitro* growth of *C. albicans* and has been shown to have the protective ability in an oral candidiasis model [5].

Oral candidiasis is an opportunistic and common fungal infection caused by *C. albicans* [6]. *C. albicans* is a component of normal microflora in the oral cavities of 30-50% of humans and is a dimorphic fungi, meaning it has two morphologies: A yeast type and a mold type (hyphae) [5]. Oral candidiasis caused by *C. albicans* can be treated by applying antifungal agents, such as nystatin and fluconazole [6]; however, there is a chance that probiotic agents can also decrease the growth of *C. albicans*. Protein secreted by *S. salivarius* is a possible natural alternative to antibiotics. Even though the probiotic effects of *S. salivarius* are known, no research has been conducted to identify the protein secreted by *S. salivarius* or inhibitory potential for *C. albicans* growth. Therefore, this research analyzes *S. salivarius* and its protein, isolated from the saliva and tongue dorsum of healthy adult subjects, to determine the inhibitory potential for *C. albicans* growth.

METHODS

Samples were swabbed from the dorsum of the tongue and saliva of 10 healthy adult subjects between 19 and 21 years of age [7]. A centrifuge tube (15 ml, 50 ml), cytobrush, 1000 ml tipped pipet, 200 ml tipped pipet, micropipette, Eppendorf tube, sterilized reaction tubes, 96-well microplate, incubator, anaerobic jar, vortex, mini centrifuge, autoclave, centrifuge, orbital shaker, enzyme-linked immunosorbent assay plater reader, analytic scale, water bath, round end ose berujung bulat, light lamp, Petri dishes, thermal cycler T100, electrophoresis, Gel Doc, Thermoblock, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) chamber, SDS-PAGE tank, and AxioCam ERc5s (Zeiss) microscope were the tools used to conduct this study. Additional materials included a brain-heart infusion (BHI) broth, BHI agar, sabouraud dextrose agar, bacto agar (Biomatic), mitis salivarius agar (MSA), sterilized aquadest, aquabides, cotton, aluminum foil, 70% alcohol, gas mixture (N₂: 10%; CO₂: 10%; H₂: 10%), phosphate buffer saline, primer Sal-AUS (5'-GTAGAAAATATTTACTACATACT) [8], primer Sal-ADS (5'-GTAAAGTATTCGTAATAACTGATG) [8], nucleus-free water, Dream Taq, Agarose, gel red, Tris-acetate-ethylenediaminetetraacetic acid buffer, 100 bp ladder, 10 mM Tris-HCl pH 7.4 liquid, 0.1 M Tris-HCl pH 6.8 liquid, phenyl methyl sulfonyl fluoride, glycerol, Coomassie brilliant blue, albumin protein standard, acrylamide 40%, stacking gel buffer, resolving gel buffer, SDS 10%, ammonium persulfate, tetra methyl ethylene diamine, marker protein (Pepqlab), MiliQ water, Laemmli buffer, β-mercaptoethanol, SDS running buffer, *C. albicans* ATCC 10231 glycerol stocks, and *S. salivarius* ATCC 13419 glycerol stocks.

Subjects were chosen using a non-probability, judgement or purposive sampling method, meaning that the researcher chose participants based on subjective assessments [9]. Samples of 10 ml were taken from stimulated saliva using parafilm M and from the dorsum of subjects' tongues using a cytobrush from the circumvallate papillae to the end of the tongue [10]. The samples were cultured in an MSA medium, and selective media for *Streptococci* were used to colonize *S. salivarius* strain of Barbour and Philip and Santagati *et al.* [11,12].

Morphology was identified by observing the size and surface consistency (i.e., soft or smooth) of colonies, which were confirmed as *S. salivarius* using the polymerase chain reaction (PCR) technique [13]. The presence of 118 base-couple DNA bands indicated that colonies were *S. salivarius*. These colonies were bred in a Mueller-Hinton infusion agar medium and BHI liquid for 18 hrs. The results were centrifuged, and pellets were made from the material separated from the spent medium protein using a cell lysate buffer and centrifugation. These were analyzed using SDS-PAGE (150 V; 80 mA for 60 minutes) [14]. Proteins with the same molecular mass were counted using a Bradford test [15]. *S. salivarius* inhibitory potential against *C. albicans* was tested using a deferred antagonism test [3], and secreted proteins were tested using the well-diffused agar method. This method used whole-cell and spent medium protein from clinically obtained *S. salivarius* and *S. salivarius* ATCC 13419 [16].

Independent variables were healthy adult saliva and dorsum tongue swabs. Dependent variables were *S. salivarius* inhibitory potential against *C. albicans* 10231, protein secreted by *S. salivarius*, and *C. albicans* morphological changes. The controlled variable was *S. salivarius* ATCC 13419. Data were analyzed by comparing *S. salivarius* colonies identified in isolated saliva and dorsum tongue swabs to colonies that did not contain *S. salivarius* from the same sources. Data were tested using a Fisher's test, and the means of *S. salivarius* inhibitory proteins for *C. albicans* growth in each isolated source were compared.

RESULTS

Colonies were identified by comparing the morphology of *S. salivarius* cultured from subject samples to the morphology of *S. salivarius* ATCC

13419 grown on an MSA medium. From six saliva and tongue dorsum samples, similar morphologies to *S. salivarius* ATCC were obtained (Table 1), and a PCR test was conducted to confirm this morphology. *S. salivarius* interaction with *C. albicans* was tested using the cross-streak method (Fig. 1) and a deferred antagonism test. Results for the inhibition zone diameter produced by *S. salivarius* against *C. albicans* were taken in millimeters (mm). From four subjects, only one subject's results could be interpreted; therefore, only saliva and tongue dorsum samples from this one subject were used.

Table 2 shows that in all isolated *S. salivarius* concentrations and the *S. salivarius* ATCC 13419 control, the inhibition zone mean was 0 mm. Protein secreted by *S. salivarius* had either whole-cell or spent medium protein inhibition potential against *C. albicans*, which was tested using the well-diffused agar method. Table 3 shows that the mean inhibition zone of *S. salivarius* concentrations from isolated sources was 0 mm.

Table 4 shows that the inhibition zone around each well of protein concentrate isolated from either source was 0 mm. *C. albicans* was taken using the well-diffused agar method and observed microscopically to obtain morphological images. Table 5 shows that *C. albicans* morphologic images without protein exhibited germ tube changes, while *C. albicans* exposed to the protein remained blastospores.

Table 1: *S. salivarius* isolate identifications

Isolated sources	<i>S. salivarius</i> colonies	
	+	-
Saliva	6 (60)	4 (40)
Tongue dorsum	6 (60)	4 (40)

S. salivarius: *Streptococcus salivarius*

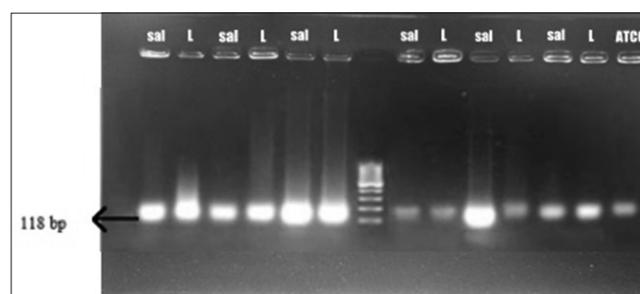


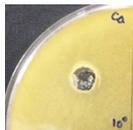
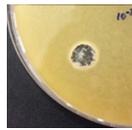
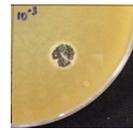
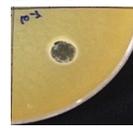
Fig. 1: Polymerase chain reaction results for *Streptococcus salivarius* isolated from the saliva and tongue dorsum. Sal: Saliva isolated; L: Tongue dorsum isolated; ATCC: *S. salivarius* ATCC 13419

Table 2: The mean for the inhibition zone of each isolate concentration based on a deferred antagonism test

Groups	Culture results			
<i>S. salivarius</i> ATCC 13419 (CFU/ml) concentration	1.7×10 ¹⁰	1.7×10 ⁹	1.7×10 ⁸	1.7×10 ⁷
<i>S. salivarius</i> ATCC 13419				
Inhibition zone mean	0 mm	0 mm	0 mm	0 mm
Clinical <i>S. salivarius</i> (saliva; CFU/ml) concentration	1.9×10 ¹⁰	1.9×10 ⁹	1.9×10 ⁸	1.9×10 ⁷
Clinical <i>S. salivarius</i> (saliva)				
Inhibition zone mean	0 mm	0 mm	0 mm	0 mm
Clinical <i>S. salivarius</i> (tongue dorsum) concentration (CFU/ml)	1.4×10 ¹⁰	1.4×10 ⁹	1.4×10 ⁸	1.4×10 ⁷
Clinical <i>S. salivarius</i> (tongue dorsum)				
Inhibition zone mean	0 mm	0 mm	0 mm	0 mm

S. salivarius: *Streptococcus salivarius*

Table 3: The mean inhibition zone for concentrations of whole-cell isolate using the well-diffused agar method

Groups	Culture results based on protein concentration			
Protein <i>S. salivarius</i> ATCC 13419 concentrations (µg/ml)	146.2	14.62	1.46	1.46×10 ⁻¹
<i>S. salivarius</i> ATCC 13419				
Inhibition zone mean (mm)	0	0	0	0
<i>S. salivarius</i> protein concentrations (saliva; µg/ml)	157.8	15.78	1.578	1.57×10 ⁻¹
Clinical <i>S. salivarius</i> (saliva)				
Inhibition zone mean (mm)	0	0	0	0
Clinical <i>S. salivarius</i> protein concentration (tongue dorsum; µg/ml)	264.4	26.4	2.64	2.64×10 ⁻¹
Clinical <i>S. salivarius</i> (tongue dorsum)				
Inhibition zone mean (mm)	0	0	0	0

S. salivarius: Streptococcus salivarius

Table 4: The mean inhibition zone for protein concentrations in a spent medium isolate using the well-diffused agar method

Groups	Culture results based on protein concentration			
Protein <i>S. salivarius</i> ATCC 13419 concentrations (µg/ml)	181.2	18.1	1.81	1.81×10 ⁻¹
<i>S. salivarius</i> ATCC 13419				
Inhibition zone mean (mm)	0	0	0	0
Clinical <i>S. salivarius</i> protein concentrations (saliva; µg/ml)	178.3	17.83	1.78	1.78×10 ⁻¹
Clinical <i>S. salivarius</i> (saliva)				
Inhibition zone mean (mm)	0	0	0	0
Clinical <i>S. salivarius</i> protein concentrations (tongue dorsum; µg/ml)	167.9	16.79	1.68	1.68×10 ⁻¹
Clinical <i>S. salivarius</i> (tongue dorsum)				
Inhibition zone mean (mm)	0	0	0	0

S. salivarius: Streptococcus salivarius

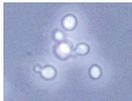
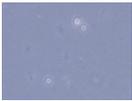
DISCUSSION

This research identified *S. salivarius* using cultured samples in an MSA medium using PCR tests. Results showed that from 10 samples, six subjects (p>0.05) were *S. salivarius* positive in both saliva and tongue dorsum samples. These results are consistent with the results of Ogawa et al. (2010) [22] and Wescombe et al. who stated that *S. salivarius* is a pioneer bacteria in the oral cavity and has predominant bacterial lifespan [4,16]. Although there was no significant statistical difference, low concentrations of *S. salivarius* found in this research were caused by certain factors. The absence of *S. salivarius* in four subjects was due to these subjects' diets. Ogawa et al. (2010) stated that diet can cause a specific microbiota composition due to the intrinsic capacity of each person to use consumed substrate. For example, consuming

polysaccharides causes different microbiota compositions. *S. salivarius* requires energy from sucrose to grow and colonize [16]; thus, another study found that sucrose influenced *S. salivarius* prevalence in adults. When sucrose is eliminated from the diet, concentrations of *S. salivarius* in saliva drastically decrease [17].

In addition, Roger et al. [17] stated that enzymes in saliva can influence *S. salivarius* growth in the oral cavity. These enzymes, including lysozyme, lactoperoxidase, and amylase, have antibacterial activities [17] that can reduce *S. salivarius* growth. In this research, subjects were allowed to eat before sampling, which was performed 3 hrs after a meal. Subjects also brushed their teeth beforehand; therefore, diet differences, enzymes contained in saliva, and sampling-taking procedures all affected the results obtained in this study.

Table 5: Microscopic images of *C. albicans* taken using the well-diffused agar method

Groups	Controlled	ATCC 13419	Saliva	Tongue dorsum
Whole-cell protein				
Spent medium protein				

C. albicans: *Candida albicans*

The specific protein secreted by *S. salivarius* colonies was identified using an SDS-PAGE test. Protein used in this test was presented as whole-cell protein (i.e., protein inside cellular membranes) and spent medium (i.e., protein secreted outside the cell). Barbour and Philip (2014) stated that 60-70% of bacteriocin are peptides that bond with bacterial cell-producer walls, while other inhibitory peptides are secreted extracellular in liquid mediums. Secreted peptides are dominant in antibiotic production because they act as communication molecules that accumulate during the growth process. In some concentrations, lantibiotic production is triggered [11]. According to Wescombe *et al.* [8], salivaricin A production can be detected in individuals' saliva in varying concentrations because there is a variety of salivary flow rates that dissolve salivaricin A and allow adsorption in normal cell flora [8]. SDS-PAGE results in the current study showed that from six *S. salivarius* samples, only four subjects produced protein with the same profiles, containing one band located inline. The absence of spent medium protein could be due to the communication mechanism not triggering protein production. An *S. salivarius* inhibition potential analysis was conducted to isolate it from the saliva and tongue dorsum samples from one subject, as well as *S. salivarius* ATCC 13419 and *C. albicans*, using the deferred antagonism test method and cross streak test. The results revealed that *C. albicans* growth was not inhibited by *S. salivarius* ATCC 13419 concentrations from either saliva or the tongue dorsum.

According to Morales and Hogan [18], bacteria and fungi have structural shapes, and the surfaces can interact to form communities known as biofilms. The interaction between *C. albicans* and oral *Streptococci* aggregates on cellular surfaces and forms a mixed-species biofilm [18]. According to Shirtliff *et al.* [19], the interaction between *C. albicans* and *Streptococci* gives synergistic results, in that *Streptococci* produces lactic acid that acts as a carbon source for *Candida* growth. In turn, *C. albicans* reduces oxygen pressure to a more suitable level for *Streptococci*, which stimulates bacterial growth due to nutritional metabolic support [19,20].

To analyze *S. salivarius* microbial activity further, inhibition potential tests were conducted on the protein secreted by *S. salivarius* against *C. albicans* using the well-diffusion agar method. *S. salivarius* produces bacteriocin, primarily lantibiotic, in the form of salivaricin [4]. The cell lysate method was used to obtain whole-cell protein, and spent medium protein was obtained by centrifugation. Similar to earlier results, protein inhibition potential tests showed that there was no *C. albicans* growth inhibition from either saliva-isolated or tongue-dorsum-isolated protein or ATCC 13419 concentrations of either whole-cell or spent medium proteins. Ishijima *et al.* [5] found that *S. salivarius* K12 is effective against *in vitro*-cultured *C. albicans* growth because *S. salivarius* K12 bonded directly to *Candida*, inhibiting adhesion to a plastic Petri dish. *S. salivarius* K12 bonded to *Candida* during germ tube formation, and no *Candida* hyphae form was found [5]. In the current research, microscopic images were obtained to identify *C. albicans* morphology, which showed that controlled *C. albicans* entered the germ tube stage, while *C. albicans* exposed to either whole-cell or spent medium protein

remained in the blastospore stage. This finding was supported by Ishijima *et al.* [5], who found that *S. salivarius* K12 reduced *C. albicans*' ability to maintain its blastospore shape. Thus, *S. salivarius* does not kill *C. albicans* but interacts and bonds with *C. albicans* hyphae and cannot inhibit *C. albicans* growth based on a deferred antagonism test, which showed that bacteriocin coded from the K12 strain did not target yeast [4,5]. MacDonald showed that *S. salivarius* K12 and M18 strains do not reduce yeast growth but inhibit hyphae formation and adhesion to surfaces of yeast [21]. Spent medium protein from *S. salivarius* K12 and M18 strains also significantly reduced *C. albicans* adhesion to wells and inhibit transition to hyphae form. This is consistent with results from Köhler *et al.* [9], in which *Lactobacilli* probiotic influenced the *C. albicans* metabolism and increased in relation to stress. Similarly, fungi were also challenged by *Lactobacilli* acid production, which reduced pH [9,21].

According to research by Köhler *et al.* [9], spent medium protein from probiotics have lower pH levels than *C. albicans* in normal growth media; thus, reducing pH can also reduce *C. albicans* adhesion. Stable spent medium pH does not reduce adhesion significantly, but decreasing spent medium pH decreases *C. albicans* adhesion. In addition, low pH levels support *C. albicans* yeast growth and inhibit hyphae formation [9,21]. In this research, protein secreted by *S. salivarius* was determined to have the potential to inhibit *C. albicans* morphological changes by maintaining the blastospore form, presumably due to low pH levels and related stress increases that influence *C. albicans* metabolic activity.

CONCLUSION

This research showed that *S. salivarius*, obtained from saliva or tongue dorsum samples, does not have a potential inhibitory effect on *C. albicans* growth. Either *S. salivarius* whole-cell or spent medium protein secreted from saliva or the tongue dorsum does have the potential to inhibit *C. albicans* morphological changes from blastospore to hyphae. It is suggested that purifying lantibiotic secreted by *S. salivarius* or using an auto-induction method could be used to obtain specific lantibiotic types. The K12 *S. salivarius* strain could be positively controlled in the future research, and further analysis of lantibiotic as a probiotic to prevent and treat oral candidiasis is necessary. The therapeutic effects of lantibiotic against *C. albicans* could be determined by conducting a candidiasis oral analysis.

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