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PRELIMINARY STUDY ON CHARACTERIZATION OF BACTERIOCIN-LIKE INHIBITING SUBSTANCES (BLIS) FROM ISOLATED ORAL BACTERIA

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Abstract – Oral cavity contains a wide range of bacteria species with dominant micro flora *Streptococcus*, other genus includes *Lactobacillus, Enterococcus* and *Staphylococcus*. The present study was aimed to isolate and identify oral bacteria using biochemical and molecular analysis respectively and to characterize oral bacteria for production of bacteriocin like inhibiting substances (BLIS). Oral samples were cultured on selective Mitis Salivarius Agar (MSA) plates and the colonies were isolated. The isolated strains were identified using biochemical tests including gram staining, catalase test, hemolysis and urease test where all the isolated strains were gram positive, catalase negative, urease negative and showed alpha hemolysis activity. The morphological and biochemical characterization confirmed that the isolated bacteria was from genus *Streptococcus*. It was further confirmed by molecular analysis where genomic DNA was isolated and verified qualitatively by nanodrop and quantitatively by gel electrophoresis. The concentration was within the range of 4.2 µg/mL – 17.6 µg/mL and purity 1.77-2.65. 16SrRNA gene was amplified and sequenced, three samples showed positive results where A1 was *Streptococcus salivarius* B0023-01R, A2 was *Streptococcus salivarius* YL456 and B1 was *Streptococcus salivarius* SAM3 with relatively 99% reference to query sequence using NCBI BLASTn. *Streptococcus salivarius* was also able to produce bacteriocin-like inhibiting substances (BLIS) which inhibited growth of *Staphylococcus aureus.*

INTRODUCTION

Human oral cavity is a dynamic niche for different species of bacteria to habituate with constant change in environment parameters of oral cavity (Rahman *et al*., 2015). It comprises of more than 700 species of bacteria found on different surfaces in the oral cavity (Aas *et al*., 2005). Due to this multispecies population, there is constant competition for survival, henceoral bacteria create a biofilm inside the oral cavity making microbiome commensal, symbiotic and pathogenic (Santagati *et al*., 2012).

The most dominant micro flora found in the oral cavity is the bacterial genus *Streptococcus*, followed by *Lactobacillus, Enterococcus, Staphylococcus, Veillonella* and *Bacteroids* (Rahman *et al*., 2015). They have both beneficial and harmful effects; oral bacteria have the ability to create a biofilm for adherence of the bacteria and the ability for bacteriocin production to inhibit the growth of unwanted flora. However, other bacteria can become destructive to the host if they migrate from superficial oral tissue to the blood stream which may in turn cause diseases (Rahman *et al*., 2015). *Streptococci* are gram positive bacteria that are round in chains, some are facultative and obligate anaerobes and have ability of adherence to tissue and high efficiency of survival (Patterson, 1996).

Owing to the abundant bacteria population in the oral cavity, there is constant survival risks due to the heterogeneous environment and competition to colonize the oral cavity, hence the production of peptides that act against other bacteria. Bacteriocin production is a way in which oral bacteria survives the harsh nature of the oral cavity. Bacteriocins are toxins produced mainly by gram positive bacteria, the toxins are harmful to other bacteria hence allowing for survival of the producer strain in the

oral cavity (Rahman *et al*., 2015). Bacteriocins function as colonizing peptides by introducing dominance of a producer strain against already existing bacteria in the niche. The toxins also act as antimicrobial peptides through inhibiting bacterial growth and finally bacteriocins as signalling peptides within the bacterial niche between bacterial species and signalling cells of the immune system of the human host (Dobson *et al*., 2011)

According to World Health Organization (2018), the use of antibiotics to treat certain bacterial diseases increases the chance of the host developing resistance towards the antibiotics due to development of different resistance mechanisms by bacteria. This in turn allows bacterial growth and infection and poses a risk on global health as there is an acceleration in the use of antibiotics and the oral bacteria are no exception. Therefore, in this study, oral bacteria samples were isolated, tested its ability to produce BLIS which have the potential and ability to act against bacteria that is existent in the oral cavity niche that can otherwise be a threat to the host with risk of causing infections and diseases (Rahman *et al*., 2015).

MATERIALS AND METHODS

Sample collection and culture

Volunteers were selected according to the following parameters set, 2 males and 2 females, ages ranging from 20-25 years, volunteers had not consumed antibiotics prior collection and had no history of gum inflammation or redness. They were informed to brush teeth twice every day for three months and had no consumption of antibiotics three months's prior collection. Samples were collected from the dorsum of the tongue and molar tooth surfaces where sample A1, B1, C1 and D1 were tooth samples and samples A2, B2, C2 and D2 were tongue samples. 100 µL of samples were spread on Mitis Salivarius Agar (MSA) plates and incubated overnight. The distinct blue colonies were selected and sub-cultured into new MSA plates.

Biochemical Characterization

Isolated bacteria were subjected to various biochemical test that were physical tests, media based and enzyme based. Tests included Gram staining (Gram, 2001), Catalase (Aryal, 2015) and Urease test (Brink, 2010), Hemolysis test using 5% sheep ds blood agar plates, (Santagati *et al*., 2012) and antibiotic susceptibility test (Hombach *et al*., 2014).

Antibiotic Susceptibility Test

All samples were tested for sensitivity against three antibiotics and distilled water used as a negative control. Bacitracin, Streptomycin and Ciproflaxin were used to test for antibiotic sensitivity. Sensitivity was expressed as resistant (R) and (Sensitive) based on the zone of inhibition measured.

Molecular Analysis

QIAGEN DNeasy kit was used to isolate bacterial DNA from bacteria by following manufacturer's manual and analysed quantitatively and qualitatively using Nanodrop (Thermo Scientific) and gel electrophoresis. Extracted DNA was used for PCR using 16SrRNA primer pair, Forward primer: U16SRT-F:5'AGAGTTTGATCATGGCT CAGG3' and Reverse primer: U16SRT R:5'GGACTA CCAGGGTATCTAATT3' (Clifford *et al*., 2012). The amplified PCR products were then purified and sequenced. Sequences obtained were subjected to homology search using BLASTn against NCBI 16SrRNA sequence database.

Bacteriocin-like Inhibiting Substance (BLIS) Assay

Bacteria samples were cultured in Todd Hewitt broth and incubated at 37 °C for 24 hrs. Broth was centrifuged and sterilized using a filter membrane to obtain crude BLIS. Nutrient agar (NA) plates were cultured with indicator strains and disc infused with crude BLIS was placed on cultured NA plates at 37 °C for 24 hrs. Zone of inhibition was recorded.

RESULTS AND DISCUSSION

Isolation of Bacterial Strains and Biochemical Characterization

Bacteria were isolated using selective media, MSA. Genus of bacteria were identified using biochemical tests performed on pure strains which supported the genus as *Streptococcus* (Leslie, 2019). All strains were gram positive, catalase negative, urease negative and hemolysis test identified most strains as being alpha hemolytic supported by findings by Arya *et al*. (2016). A summary of biochemical tests is presented in Table 1.

Antibiotic Sensitivity of Isolated Bacteria

Strains from tooth samples A1 and B1 were sensitive

Table 1. Biochemical characterization of tooth sample, A1-D1 and tongue sample, A2- D2

ID	Gram's Stain	Urease Test	Catalase Test	Hemolysis test
A1	+	-	-	α
B1	+	-	-	α
C1	+	-	-	β
D1	+	-	-	α
A2	+	-	-	β
B2	+	-	-	α
C2	+	-	-	β
D2	+	-	-	γ

Keys: + = positive result, **-** = negative result, α = alpha hemolysis, β = beta hemolysis and γ = gamma hemolysis

to all antibiotics. All strains were sensitive to Streptomycin and Ciproflaxin but strains D1, A2, B2, C2 and D2 were resistant to Bacitracin (Table 2). *Streptococcus* species showed high susceptibility to antibiotics even at low concentrations but may still acquire resistance over a period of time. Thus, the test detects susceptibility as well as resistance of bacteria isolates. Results were qualitative by using disc diffusion method where an isolate can be susceptible or resistant to the antibiotic according to the zone of inhibition (Reller *et al.*, 2009).

Table 2. Antibiotic sensitivity test of tooth sample, A1-D1 and tongue sample, A2- D2

Sample	A	B	C	D
A1	S	S	S	-
B1	S	S	S	-
C1	NG	NG	NG	NG
D1	R	S	S	-
A2	R	S	S	-
B2	R	S	S	-
C2	R	S	S	-
D2	R	S	S	-

Keys: S= Susceptibility, R= Resistant and NG= NO Growth, A= Bacitracin, B = Streptomycin, C= Ciproflaxin and D= water.

Quantitative results were obtained through zone of inhibition (Refer Table 3). According to results obtained, the isolates were susceptible to antibiotics and it can be concluded that the bacteria isolated belonged *Streptococcus* species Molecular Characterization of bacterial isolates

Molecular Characterization of bacterial isolates

Molecular identification identified three strains of *Streptococcus*, sample A1 was *Streptococcus salivarius* B0023-01R, A2 was *Streptococcus salivarius* YL456 and B2 was *Streptococcus salivarius* SAM3 with 99% of homology to the query. *S. salivarius* is an alpha haemolytic bacterium that has the ability to colonize and interfere with other pathogenic bacteria (Santagati *et al.*, 2012). *S. salivarius* is predominant in the oral cavity along with *S. mitis*. *S. salivarius* is characterized as having low pathogenic potential and therefore each strain has to be assessed to be used for commercial use when BLIS is characterized and purified. Santagati *et al.* (2012) explain that FDA requires identification of genus, antibiotic sensitivity patterns, virulence patterns, toxicity and haemolytic activity of strain to be identified before it can be granted as safe. The *S. salivarius* strain is in close relation with *S. thermophilus* hence have the same

Table 3. Zone of inhibition by antibiotics measured in mm for tooth sample, A1-D1 and tongue sample, A2- D2

Sample	A	B	C	D
A1	0.45 mm	1.3 mm	1.65 mm	-
B1	0.45 mm	1.1 mm	1.55 mm	-
C1	NG	NG	NG	-
D1	-	1.2 mm	1.65 mm	-
A2	-	1.2 mm	1.55 mm	-
B2	-	0.5 mm	1.60 mm	-
C2	-	1.0 mm	1.50 mm	-
D2	-	1.1 mm	1.50 mm	-

Keys: A= 1g/10 mL Bacitracin, B= 1g/10 mL Streptomycin, C= 10ug Ciproflaxin disc, D = sterile distilled water and NG= No growth

ribosomal properties and can be considered to be used to make probiotics from produced BLIS.

BLIS Inhibiting Activity

It was also hypothesized that BLIS have the ability to inhibit growth of other oral bacteria with working mechanism like "probiotics"° and thus have the potential of replacing constant use of antibiotic which can reduce development of antibiotic resistant bacteria. All isolated strains were characterized for production of BLIS against indicator strains, *S. aureus, E. coli* and *B. cereus*. Indicator strains were selected as strains found in oral microbiome and low pathogenicity. All strains showed no BLIS activity towards *E. coli* and *B. cereus* such that no growth was inhibited. Meanwhile only one strain from tongue sample C2 inhibited growth of *S.aureus* with minimal inhibition of 0.5mm and maximum inhibition 0.6mm. Hence, oral *Streptococcus* have the ability to produce BLIS which can inhibit growth of other bacteria.

CONCLUSION

In conclusion, oral bacteria were isolated from human cavity specifically from dorsum of the tongue and molar tooth plaque and identified as *Streptococcus salivarius*. Bacteriocin-like inhibiting substances were characterized to inhibit the growth of oral bacterium *Staphylococcus aureus* using an antagonistic test which gave a minimum zone of inhibition. Antimicrobial properties of BLIS can be further characterized to determine optimal working parameters of BLIS. Partial purification can also be done to optimize temperature, pH and oxygen concentration in order to determine the optimal working conditions of the bacteriocin molecules produced by *Streptococcus* sp.

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