

Antibiofilm and antibacterial properties of *Streptococcus* and *Staphylococcus* from the oral microbiome

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ABSTRACT

The human oral microbiome plays an important role in balancing beneficial and harmful microorganisms in the oral cavity. Certain oral bacteria are known to produce substances that inhibit the formation of microbial biofilm and growth of potentially pathogenic microorganisms. The oral microbiome of the Malaysian population is largely uncharacterized, and there is very little information on its diversity and potential as a source of probiotics or antibiofilm and antibacterial metabolites. The objectives of this study are to characterize the taxonomic composition of the oral microbiome in selected healthy Malaysian subjects; and to assess the capacity of bacterial isolates from oral microbiome to produce antibiofilm and antibacterial compounds. Metagenomic sequencing showed that the oral microbiome includes predominantly bacteria from the phyla Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria and Actinobacteria while *Streptococcus*, *Haemophilus*, *Neisseria*, *Phorphyromonas* and *Fusobacterium* are the most represented genera. Altogether, 117 bacterial cultures were successfully isolated from these microbiomes, where 14 (12.0%) isolates inhibited *Staphylococcus epidermidis* (ATCC 35984) biofilm formation at maturation stage with highest activity observed at 94.8%. Twelve (10.3%) isolates displayed antibacterial activity with the highest activity observed against *Streptococcus mutans* (ATCC 25175) at 76.9%, followed by *Bacillus subtilis* (ATCC6633) at 56.6%; but no activities were observed against *E. coli* (ATCC 25922) or *S. aureus* (ATCC 25923). Identification via 16S rRNA sequencing showed that the majority of the oral bacterial isolates displaying the antibiofilm and/or antibacterial activities were *Streptococcus salivarius* followed by strains of *S. aureus*. Two of these isolates, SA78 and SA10, produced both antibiofilm and antibacterial activities and were identified to be *S. salivarius* suggesting the potential of this species in treating oral infections and improving dental health. Understanding the oral

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microbiome composition and using resident beneficial bacterial species as oral probiotics may provide a novel strategy for addressing oral diseases.

INTRODUCTION

The oral microbiome is made up of microbial communities that live in various environments in our mouth, including the teeth, inner cheeks, tongue, gingiva, tonsils, and palates. These microbiomes are subjected to continuous exposure to exogenous foreign chemicals, which serve as defining elements for the establishment and persistence of microbes within this environment.

The human microbiome comprises two distinct components, the core microbiome and the variable microbiome. The core microbiome is universally shared among all individuals, while in contrast the variable microbiome is distinct to each individual based on their lifestyle choices and physiological variations (Dash & Das, 2022). In addition to host genetics and maternal transmission, environmental factors like food, dental hygiene, drugs, stress, and behaviors like smoking and drinking can affect the microbiota throughout life hence changing the oral microbiome ecology (Belstrøm, 2020; Dash & Das, 2022). These circumstances give rise to different interactions between microorganisms and their hosts, shaped by selective forces (Sedghi et al., 2021). Common bacteria found in the oral cavity include *Streptococcus*, *Porphorymonas*, *Haemophilus*, *Granulicatella*, *Gemella*, and *Veillonella*.

The microbiome community in the oral cavity is known to play a crucial role in general homeostasis of an individual. With over 700 distinct species, the microbiota of the oral cavity is known to be the second-largest and highly diverse population, encompassing a vast array of microbial organisms such as bacteria, fungus, viruses, and protozoa (Caselli et al., 2020; Deo & Deshmukh, 2019). Disruption of the balance in the oral microbiome can lead to pathological conditions including dental caries, periodontitis or even oral cancers (Dudek-Wicher et al., 2022). In addition, persistent oral infections may contribute to the development of systemic diseases such as cardiovascular disease, diabetes mellitus, rheumatoid arthritis, and Alzheimer (Wu et al., 2023).

Hence, considering their potential roles in oral and associated diseases, this large community of oral microbes is at the same time a promising resource of potential therapeutics adjuvants for oral disorders. Recent advances in the treatment of oral problems have shifted to more imaginative and less intrusive approaches aimed at restoring balance between the host periodontal tissues and mouth flora. Traditional broad-spectrum antibiotics are being reevaluated as periodontal infections become resistant to them. Among these new approaches is the use of chemical to limit biofilm formation (Haque et al., 2022) and the use of beneficial microorganisms as probiotic and oral microbiota replacement therapy (Parasuraman et al., 2023).

Some microorganisms in the oral microbiome have developed a distinctive mechanism for survival by forming biofilm communities. Many microorganisms can transit from a planktonic condition and congregate in a collective manner, referred to as "communities," to construct intricate matrix-like formations commonly referred to as biofilms (Berger et al., 2018). Early colonizers, or planktonic bacteria, are responsible for the formation of oral biofilms. Oral biofilms consist of a wide range of microbial communities that are encased inside an exopolysaccharide matrix. Oral bacterial biofilms are able to adapt to high cell density due to oral cavity features, creating multiple microenvironments that regulate pH, redox, and oxygen levels in their core (Radaic & Kapila, 2021). The fundamental etiology of dental caries, endodontic, and periodontal diseases can be attributed to the formation and growth of biofilms on the surfaces of dental hard or soft tissues (Wang et al., 2017).

Understanding germ-host relationships in dentistry is crucial due to oral bacteria's symbiotic relationship, with commensal populations preventing pathogenic species from adhering to the mucosa. Some oral bacterial species are able to produce protective compounds such as antibacterial and antibiofilm peptides (Pitts et al., 2021). For example *Streptococcus sanguinis*, *Streptococcus cristatus* and *Haemophilus parainfluenzae* are known to be able to produce substances to inhibit the growth of other microorganisms, and may contribute to the maintenance of oral health (Rajasekaran et al., 2024; Sedghi et al., 2021). However, studies on other potentially beneficial resident species of the oral microbiome are limited. Hence, the aim of this study is to evaluate the antibiofilm and antibacterial activities in selected group of bacteria isolated from the oral microbiomes of healthy young Malaysian individuals. Findings from this study would provide insights into the oral microbiome's capacity to produce antibacterial and antibiofilm compounds, which may potentially be employed to prevent or provide a fresh approach in treating oral infections.

METHODOLOGY

The study was conducted under the approval of the Research Ethics Committee of Universiti Teknologi MARA REC/06/2021 (MR/428). Salivary samples were obtained from eight healthy volunteers aged from 18 to 30 years, with no current or recent history of oral disease, and were not under antibiotic treatment. The saliva sample was selected for microbial isolation because it contains bacteria shed from the biofilm and surface of various oral tissue across multiple oral habitats. Consequently, the salivary microbiome may serve as a composite snapshot of the microbiota in the oral cavity and may provide biomarkers for monitoring the dental microbiota (Azevedo et al., 2023). The majority of the subjects were undergraduate students at the university where the study took place.

Sample collection

Subjects were asked to refrain from using oral hygiene products with antibacterial activity for 12 hours before sampling takes place. Each subject was requested to rinse their mouth briefly with sterile-filtered drinking water 30 minutes before saliva collection, and not to eat, drink, smoke or chew gum within this period. Then the subjects were asked to carefully expel approximately 5 mL of salivary sample into a sterile 50 ml Falcon tube without touching the mouth of the tube. From this, 2.0 ml saliva sample was removed to a fresh tube and added to 2.0 ml of DNA Shield solution (ZymoResearch, USA for metagenomic sequencing. These remaining sample portions were used for bacterial isolation. All samples were kept on ice and transported to the laboratory. Bacteria isolation was performed immediately, while samples for DNA extraction were stored at -20°C.

Metagenomic sequencing

A volume of 0.5 mL of eight samples of fresh saliva sample diluted 1:1 in DNA Shield solution was used for metagenomics sequencing. The samples were centrifuged at 13,000 rpm for 20 minutes, and the supernatant discarded. Total genomic DNA of each sample was extracted from the microbial pellet using Qiagen's Powersoil DNA extraction kit (Qiagen, USA) following the manufacturer's instructions. The extracted DNA samples were sent to a third-party sequencing facility (Genewiz, China) for metagenomics sequencing using Illumina (USA) (2 x 30bp) paired-end kit and primers to amplify the V3-V4 hypervariable regions of the bacterial 16S RNA gene. The data processing and bioinformatics analysis was conducted by using software routines embedded in the Multiple Taxonomic Profiling (MTP) pipeline (Schloss et al., 2009) on the EzBioCloud website (<https://ezbiocloud.net/>).

Bacteria isolation and preparation of sterile Cell-Free Medium (CFM)

The CFM that contains metabolites and secreted products of the bacteria culture was prepared according to the protocol by Shaaban et al., (2020) with minor modifications. Each of the saliva sample was inoculated into three different agar media i.e. Tryptone Soy Agar (TSA), Mannitol Salt Agar (MSA), and DeMan-Rogosa-Sharpe Medium (MRS) and incubated for up to 48 h at 37 °C. These media were specifically chosen to increase the recovery of streptococci, staphylococci and lactic acid bacteria, as these bacterial groups are known to be prolific producers of antibacterial and antibiofilm metabolites (Alam et al., 2022; Tang et al., 2023; Tuon et al., 2023). Single colonies were selected and purified by repeated dilution streaking on the same media. The purified isolates were then cultured in 2mL of Tryptone Soya Broth (TSB) in a 96-deepwell plate and grown for 48 h at 37 °C. Each culture medium was then individually filtered using a 0.45µm syringe filter. The resulting CFM were transferred to a new sterile 96-deepwell plate and stored at -20°C until further use.

Preparation of test bacteria

For the antibiofilm assay, *S. epidermidis* ATCC35984 was used as a positive biofilm producer while *S. epidermidis* ATCC12228 served as the negative control. The bacterial cultures were grown overnight at 37°C in TSB supplemented with 1% glucose (STSB). The following day, the cultures were diluted in fresh STSB at 1:100 and further grown until they reached the mid-log phase. The cultures' turbidity was adjusted to 0.08 - 0.13 at OD₅₇₀, which is equivalent to 1 X 10⁸ CFU/mL (Wiegand et al., 2008).

E. coli ATCC25922, *B. subtilis* ATCC6633, *S. mutans* ATCC25175, and *S. aureus* ATCC25923 used for the antibacterial assay were prepared in the same manner.

Antibiofilm assay

The antibiofilm assay was conducted according to the method by Razali et al., (2022) with minor modifications. For the attachment assay, 100 µL of prepared *S. epidermidis* ATCC35984 culture was inoculated into each of the wells of a microtiter plate. A volume of 50 µL of CFM was mixed with the *S. epidermidis* cultures, except for the control wells which receive 50 µl of diluted TSB at 1:10 instead. The plate was incubated at 37 °C for 4 h to allow bacteria cells to attach to the well surface and initiate the formation of biofilm. The contents were then discarded, and the plate was carefully washed with 300 µL of PBS and fixed in 150 µL of methanol for 20 minutes. The methanol was then discarded, and the microtiter plate was left inverted to air dry. Following that, the adherent bacterial cell layer was stained for 15 min with 150 µL of 1% crystal violet. The excess stain was then gently washed away with tap water and the stained biofilm was air-dried. The dye captured in the biofilm layer was then resolubilized in 150 µL of ethanol for 30 min, and the optical density was measured at a wavelength of 570 nm.

For the antibiofilm maturation assay, 100 µL of the *S. epidermidis* ATCC35984 culture was added into the wells of the microtiter plate and grown at 37 °C for one hour to allow the cells to attach to the well surface. Following that, except for the controls, 50 µL of the CFM was added to the wells and incubated at 37 °C for 24 h. The amount of biofilm formed was then measured as described in the antibiofilm attachment assay.

For the antibiofilm dispersion assay, 100 µl of the biofilm former *S. epidermidis* culture was incubated at 37 °C for 24 h to allow biofilm formation and maturation. Then, 50 µL of the CFM was added, and the incubation was continued at 37 °C for 24 h. Following that, the biofilm formation of *S. epidermidis* cells was evaluated in the same manner as described in the antibiofilm attachment assay.

Each of the experiments was performed in triplicates. The CFM samples were evaluated for antibiofilm activity using the following equation (Costa et al., 2018).

$$100 - [(S - N) / (P - N)] \times 100\%$$

whereby:

S = OD570 of *S. epidermidis* ATCC35984 + CFM (test samples)

N = OD570 of *S. epidermidis* ATCC12228 +TSB (negative control)

P = OD570 of *S. epidermidis* ATCC35984 + TSB (positive control)

Antibacterial assay

The CFM of each of the bacterial samples were further tested for their antibacterial activity against *E. coli* ATCC25922, *B. subtilis* ATCC6633, *S. mutans* ATCC25175 and *S. aureus* ATCC25923. Erythromycin (500ug/ml) was used as positive control for all the tested bacteria (Farzam et al., 2024; Folliero et al., 2022). Experiments were performed in triplicates.

A volume of 100 ul of the prepared test organisms were added into a microtiter plate and mixed with 50 uL of each of the CFM. For positive control, 100 uL of test culture was mixed with 50 uL of erythromycin (500ug/mL) instead while for negative control 50 μL of diluted TSB at 1:10 was used. The microtiter plate was incubated at 37 °C for 24 h before measuring the amount of growth inhibition at 600 nm.

The CFM samples were evaluated for antibacterial activity using the following equation (Paging et al., 2016);

$(A_c - A_+) / A_c \times 100$ whereby:

A_c = OD600 of positive control

A_+ = OD600 of sample

Identification of bacterial isolates

Bacterial isolates that produced antibiofilm and/or antibacterial activities were identified via 16S rRNA gene sequence analysis using the universal primers 27F (5' - AGA GTT TGA TCC TGG CTC AG -3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T -3') (Kai et al., 2019). The amplified PCR products were then sent to a third-party service provider (Apical, Malaysia) for sequencing. The sequencing results were used to search for similar sequences in the National Center for Biotechnology Information (NCBI) (<https://www.ncbi.nlm.nih.gov/>) nucleotide sequence database using BLAST (Wheeler & Bhagwat, 2007).

Statistical analysis

All experiments were conducted in triplicates and results were expressed as the mean ± standard deviation.

Flowchart of the research methodology

The workflow of the experiments is as shown in Figure 1 below.

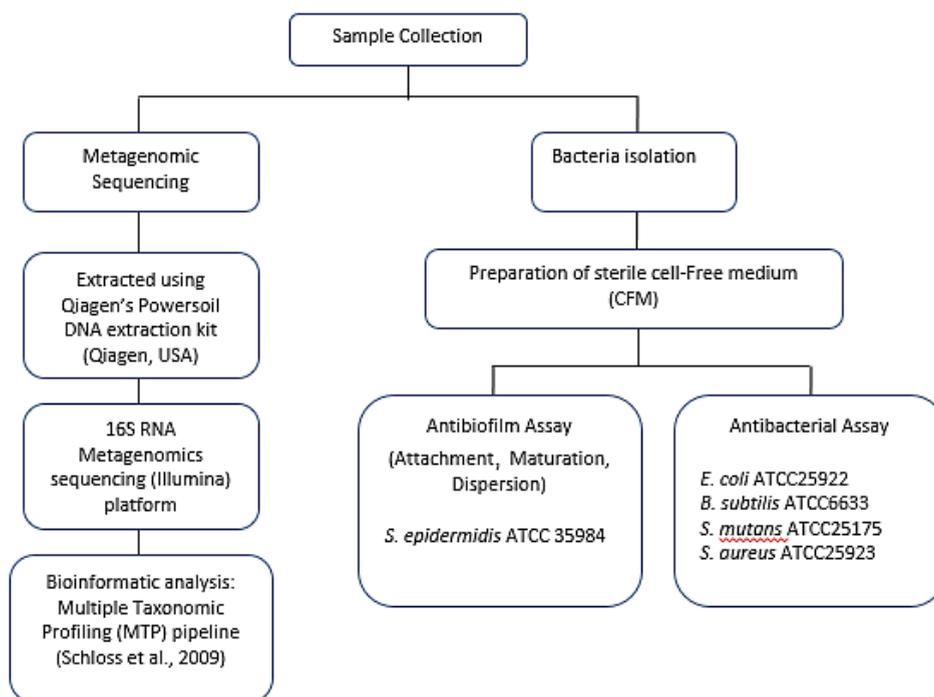


Fig. 1. Workflow of the study outlining sample collection, metagenomic sequencing, and bacterial isolation for antibiofilm and antibacterial assays. The 16S rRNA sequencing was analysed using the Multiple Taxonomic Profiling (MTP) pipeline as described by Schloss et al. (2009).

RESULTS

Microbiome diversity of the oral cavity

A pilot study of the oral microbiome diversity of the Malaysian population was performed using eight saliva samples. Figure 2 shows the relative abundance of different bacterial phyla. Members of Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria and Actinobacteria comprise the majority of the oral microbiome bacterial communities, with just 4% of the species belonging to other phyla. The ten most represented bacterial genera are *Streptococcus*, *Prevotella*, *Haemophilus*, *Neisseria*, and *Veillonella*, *Porphyromonas*, *Fusobacterium*, *Alloprevotella*, *Rothia*, and *Gemella* as shown in Figure 3. Species from the genus *Streptococcus* are known to produce bacteriocins which can inhibit the growth of other bacteria (Rajasekaran et al., 2024; Sedghi et al., 2021).

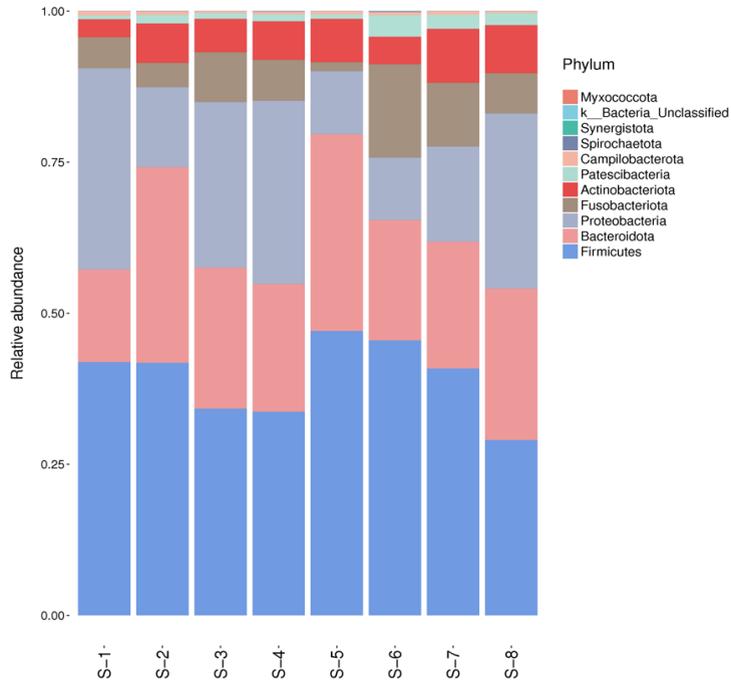


Fig. 2. Stacked bar plot of bacteria phyla distribution in saliva samples. The predominant phyla are Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria and Actinobacteria

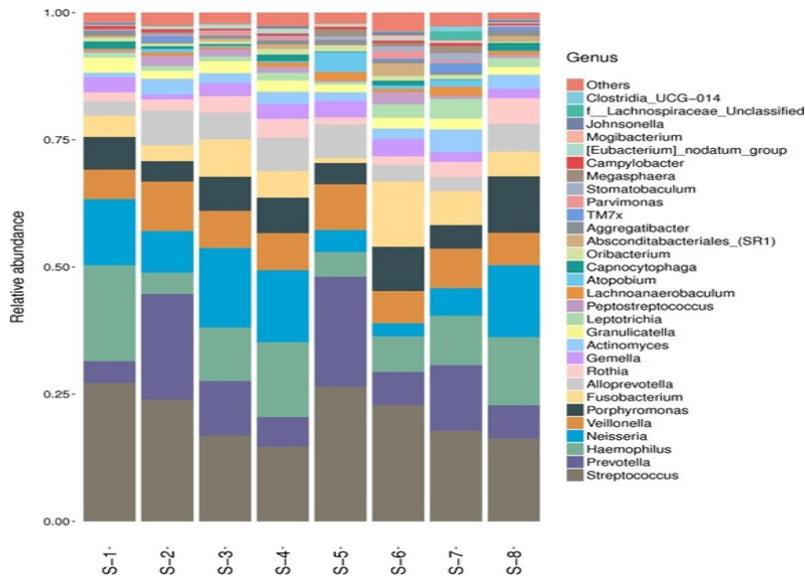


Fig. 3. Stacked bar plot of bacteria genera abundance in saliva samples. Predominant genera include *Streptococcus*, *Prevotella*, *Haemophilus*, *Neisseria*, and *Veillonella*, *Porphyromonas*, *Fusobacterium*, *Alloprevotella*, *Rothia*, and *Gemella*

Antibiofilm activity

A total of 117 non-duplicated oral bacteria were successfully isolated from the 8 saliva samples collected. These bacteria cultures were maintained in glycerol stock at -80°C for further study.

Antibiofilm activity of the oral bacterial isolates against *S. epidermidis* (ATCC35984) are as shown in Table 1. A total of 14 isolates (12.0%) displayed antibiofilm activities at the maturation stage with inhibition of biofilm activity ranging between 60% to 95%. The highest activity was observed in isolate SA108 which managed to inhibit 94.8% of biofilm formed by *S. epidermidis* ATCC35984. This is followed by SA69 at 92.0% and SA10 at 91.4% inhibition. Meanwhile, the lowest antibiofilm activity at the maturation stage was observed in SA68 with only 59.9% inhibition.

In contrast, antibiofilm activity at the attachment stage was observed in only one isolate, SA108, which managed to inhibit 94.4% of biofilm formation by *S. epidermidis* ATCC35984. Similarly, SA96 was the only sample that showed antibiofilm activity at the dispersion stage, resulting in loss of 75.3% of the biofilm formation. Overall, the results indicate the potential role of several of the oral bacterial isolates as antibiofilm agents.

Table 1. Antibiofilm activity of the oral microbiome in different stage of biofilm formation

No.	Samples	Antibiofilm activity					
		Attachment		Maturation		Dispersion	
		%	Strength	%	Strength	%	Strength
1	SA09	0.00	-	89.0 ± 28.55	++	0.00	-
2	SA10	0.00	-	91.4 ± 18.54	+++	0.00	-
3	SA67	0.00	-	89.9 ± 18.35	++	0.00	-
4	SA68	0.00	-	59.9 ± 14.93	+	0.00	-
5	SA69	0.00	-	92.0 ± 42.04	+++	0.00	-
6	SA70	0.00	-	83.7 ± 25.95	++	0.00	-
7	SA71	0.00	-	70.8 ± 19.36	++	0.00	-
8	SA72	0.00	-	78.3 ± 29.06	++	0.00	-
9	SA74	0.00	-	78.3 ± 34.04	++	0.00	-
10	SA78	0.00	-	79.7 ± 34.95	++	0.00	-
11	SA82	0.00	-	77.5 ± 28.42	++	0.00	-
12	SA96	0.00	-	86.2 ± 30.97	++	75.3 ± 26.56	++
13	SA99	0.00	-	60.9 ± 39.17	+	0.00	-
14	SA108	94.4 ± 19.11	+++	94.8 ± 22.21	+++	0.00	-

Note: +++, strong antibiofilm activity (>90%); ++, Moderate antibiofilm activity (>70%); +, Weak antibiofilm activity, (>50%); -, no antibiofilm activity (<50%)

Antibacterial activity

As presented in Table 2, the result demonstrated growth suppression effects only on *S. mutans* and *B. subtilis*. In total, 12 or 10.3% of the isolates exhibited positive antibacterial activity whereby three isolates were positive against *B. subtilis*, while nine isolates exhibited positive results against *S. mutans*. The highest antibacterial activity was observed in SA112 at 76.9% followed by SA57 at 66.0% against *S. mutans*. For *B. subtilis* highest antibacterial activity was observed in SA10 at 56.6% followed by SA58 at 55.0%. In contrast, none of the CFM showed any discernible antibacterial actions against *E. coli* and *S. aureus*.

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Identification of isolates

Table 3 shows the identity of the most active isolates based on antibiofilm and antibacterial properties. Identification was done using 16S RNA gene sequence analysis, with a minimum similarity of 97%. From the 14 oral bacterial isolates that displayed antibiofilm activity at the maturation stage, *Streptococcus* was the dominant group with six or 42.9% samples. The majority of these were preliminarily identified as *S. salivarius* except for one isolate of *Streptococcus australis*. This is followed by five isolates (35.7%) of *Staphylococcus* sp, four of which were identified as *S. aureus* while the remaining isolate was identified as *Staphylococcus argenteus*. A similar pattern was observed among the ten isolates with antibacterial activity whereby half or 50% of these were identified as *S. salivarius* while another four or 40% were of species of *Staphylococcus*.

Table 2. Antibacterial activity of the oral microbiome against tested bacteria

No.	Samples	Antibiofilm activity							
		<i>S. mutans</i>		<i>B. subtilis</i>		<i>E. coli</i>		<i>S. aureus</i>	
		%	Strength	%	Strength	%	Strength	%	Strength
1	SA04	65.0 ± 28.05	+	16.9 ± 5.20	-	0.0	-	0.0	-
2	SA10	0.0	-	56.6 ± 3.31	+	0.0	-	0.0	-
3	SA18	0.0	-	54.9 ± 6.77	+	0.0	-	0.0	-
4	SA49	57.4 ± 42.57	+	14.7 ± 9.01	-	0.0	-	0.0	-
5	SA57	66.0 ± 55.87	+	13.5 ± 6.69	-	0.0	-	0.0	-
6	SA58	0.0	-	55.0 ± 3.24	+	0.0	-	0.0	-
7	SA77	58.2 ± 20.85	+	0.0	-	0.0	-	0.0	-
8	SA78	53.3 ± 23.10	+	0.0	-	0.0	-	0.0	-
9	SA86	60.1 ± 23.40	+	0.0	-	0.0	-	0.0	-
10	SA89	52.1 ± 26.38	+	16.8 ± 11.90	-	0.0	-	0.0	-
11	SA108	55.7 ± 6.21	+	0.0	-	0.0	-	0.0	-
12	SA112	76.9 ± 54.78	++	25.8 ± 5.80	-	0.0	-	0.0	-

Note: +++, strong antibacterial activity (>90%); ++, Moderate antibacterial activity (>70%); +, Weak antibacterial activity (>50%); -, no antibacterial activity (<50%)

Isolate SA96, an unknown species, is unique in that it inhibits biofilm maturation and at the same time is able to disrupt mature biofilm. Advanced metagenomics and novel single-cell sequencing techniques may offer an efficient means to gain insights on uncultured bacteria (Liu et al., 2016). Isolate SA108, a strain of *S. aureus*, displayed the broadest spectrum of activity, being able to inhibit biofilm formation at the attachment and maturation stages, and also inhibit the growth of *S. mutans*. Two other isolates SA10 and SA78 also displayed dual activities of antibiofilm and antibacterial. The identity of three isolates i.e. SA70, SA112 and SA96 could not be adequately established using their partial 16S RNA sequence. It is possible that these may be novel bacterial species that are yet uncharacterized and thus are not identified in the NCBI database. Further biochemical characterization and whole-genome sequence analysis may provide an insight into this yet undescribed and untapped pool of microbial resources.

In summary, the study demonstrates that *Streptococcus* sp. especially *S. salivarius* as a potential bacterial responsible in inhibiting growth of other oral bacteria due to its antibiofilm and antibacterial activities. Other less well documented species of the oral microbiome were also shown to be able to produce antibiofilm and antibacterial compounds.

Table 3. Identification of selected oral microbiome by 16S rRNA sequence analysis

Isolates	Activity	Identity
SA69	AB ^M	<i>Streptococcus australis</i>
SA67	AB ^M	<i>Streptococcus salivarius</i>
SA09	AB ^M	<i>Streptococcus salivarius</i>
SA70	AB ^M	Uncultured bacterium
SA72	AB ^M	<i>Staphylococcus argenteus</i>
SA74	AB ^M	<i>Staphylococcus aureus</i>
SA82	AB ^M	<i>Staphylococcus aureus</i>
SA71	AB ^M	<i>Staphylococcus aureus</i>
SA99	AB ^M	<i>Streptococcus salivarius</i>
SA68	AB ^M	<i>Enterobacter asburiae</i>
SA96	AB ^M , AB ^D	Uncultured bacterium
SA112	AM ^S	Uncultured bacterium
SA57	AM ^S	<i>Streptococcus salivarius</i>
SA04	AM ^S	<i>Staphylococcus epidermidis</i>
SA77	AM ^S	<i>Streptococcus salivarius</i>
SA89	AM ^S	<i>Staphylococcus caprae</i>
SA58	AM ^B	<i>Streptococcus salivarius</i>
SA18	AM ^B	Staphylococcus sp.
SA10	AB ^M , AM ^B	<i>Streptococcus salivarius</i>
SA78	AB ^M , AM ^S	<i>Streptococcus salivarius</i>
SA108	AB ^A , AB ^M , AM ^S	<i>Staphylococcus aureus</i>

Note: AB^A, Antibiofilm (Attachment); AB^M, Antibiofilm (Maturation); AB^D, Antibiofilm (Dispersion); AM^S, Antibacterial (*S.Mutans*); AM^B, Antibacterial (*Bacillus subtilis*);

DISCUSSION

The Human oral microbiome database (HOMD) indicates that the global oral microbiome consists of several major phyla, namely Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Spirochaetes, and Fusobacteria. These phyla collectively make up 96% of the microbiome (Dewhirst et al., 2010). Similarly, many different studies also highlight the prominence of these phyla (Chaturvedi et al., 2025; Deo & Deshmukh, 2019; Li et al., 2022; Morrison et al., 2023; Tian et al., 2022; Zhang et al., 2018). In the Malaysian context, study from Mok et al. (2017) corroborates these findings.

The major genus shown in Figure 3 is similar to the HOMD findings pattern (Dewhirst et al., 2010). Several studies also reported that the microbiome of the saliva is dominated by the bacterial genera *Streptococcus*, *Prevotella*, *Veillonella*, *Haemophilus*, *Rothia* and *Neisseria* genera (Burcham et al., 2020; Caselli et al., 2020; Morrison et al., 2023).

Findings from metagenomics sequencing showed that the microbial diversity in a limited sample set of oral microbiomes in the Malaysian population is similar to that reported for several other geographical locations e.g. Qatar (Alqaderi et al., 2021; Murugesan et al., 2020). Analysis of data from the Human Oral Microbiome Database also indicates that these bacterial communities were prominent in the human oral microbiome from various locations (Chen et al., 2010). The predominant genus is *Streptococcus*. Various Streptococci species can be found in the oral cavity, including the oral pathogen *S. mutans*, which has been associated with dental caries. The other dominant genera – *Haemophilus*, *Neisseria* and *Porphyromonas* all include species which are potential pathogens. *Porphyromonas gingivalis* is the causative agent for

gingivitis and has been associated with Alzheimer's disease (Kanagasingham et al., 2020). *S. mutans* grows as a biofilm on the surface of the teeth, and this ability has been identified as a major pathogenic factor in the development of dental caries (Matsumoto-Nakano, 2018). In addition to *S. mutans*, *Scardovia wiggsiae*, *Parascardovia denticolens* and various *Lactobacillus* and *Bifidobacteria* have been identified in the context of caries development (Nivetha et al., 2025).

The *Streptococcus* genus is the most abundant genus in the oral cavity (Akimbekov et al., 2022). Study from Burcham et al., (2020) stated that, *Streptococcus* and *Haemophilus* are commonly found in healthy adults. Furthermore, other studies have also identified similar prevalent genera present in the oral microbiome (Barboza-Solis et al., 2020; Burcham et al., 2020; Caselli et al., 2020).

In this study, the antibiofilm activity of oral microbiomes was determined at three different stages of biofilm formation, attachment, maturation, and dispersion. Results indicate that most of the antibiofilm activities observed were targeted at the maturation stage, which prevents the formation of the biofilm after initial attachment. The maturation stage can be identified by the emergence of cell clusters that are many cells thick and embedded in the biofilm matrix, which later fully mature into microcolonies.

A number of these activities showed a high degree of more than 70.0% biofilm inhibition. These bacterial isolates thus have the potential to be developed as probiotics for the oral cavity and may help to prevent colonization of oral pathogens. The World Health Organization (WHO) defines probiotics as living bacteria that provide health benefits to the host when administered in adequate quantities (Albariqi, 2025). A study from Ben Taheur et al. (2016), also showed similar findings with the use of probiotic bacteria against oral pathogens. The study proposed that probiotic microorganisms may be suitable for the prevention and treatment of dental caries without adverse effects. For example, *Lactobacillus brevis* (90.0%) showed highest value for biofilm inhibition concentration against *Streptococcus oralis* (Taheur et al., 2016).

The antibiofilm compounds produced by these bacteria have the potential to be used as adjuvant to antibiotics for the treatment of oral infections. Antibiotics that are not able to kill microorganisms in a biofilm may now be more effective by preventing biofilm formation or by breaking down the biofilm, thus preventing the establishment of oral pathogens without the risk of antibiotic resistance development. An investigation conducted by Yin et al. (2019) examined the antibiofilm properties of butanolide, a potent anti-macrofouling chemical produced from a marine *Streptomyces* sp. The study showed that butanolide exhibited significant efficacy in both preventing the formation of biofilms and eliminating pre-existing biofilms. In addition, previous studies have demonstrated that culture supernatants obtained from several bacteria, including *Pseudomonas aeruginosa* (Qin et al., 2009) and *Bacillus licheniformis* (Rendueles et al., 2013), also have the ability to inhibit biofilm formation.

As shown in the result, SA108 or *S. aureus* has higher antibiofilm activity at attachment and maturation stage only but not at dispersion stage. The ability of this bacterium to inhibit attachment is interesting, as it could be potentially used to prevent biofilm formation from taking hold on surfaces that contact a patient, for example, medical devices. One possible mechanism is the production of metabolites by SA108 which can either inhibit or disrupt the cellular signaling pathway for cell attachment or metabolites that bind to the cell surface and prevent interaction with surfaces. Meanwhile, SA96 show moderate antibiofilm activity at maturation and dispersion stage, and will be a good candidate for the development of a biofilm dispersant to remove established biofilms (Kaplan, 2010). Unfortunately, the identity of this bacterium is unknown.

From this study, none of the samples showed any antibiofilm activities for all three stages of biofilm formation. However, most samples displayed antibiofilm activity at maturation stage. Antibiofilm

activities towards the attachment stage may be uncommon due to the vast range of extracellular proteins and ligands that can potentially interact. Similarly, dispersion of a mature biofilm will require the production of high amounts of lytic enzyme or disruptive metabolites. Both strategies will likely put a heavy strain on competing bacteria's resources. A combination of antibiofilm compounds with activities at different stages can lead to a new biological alternative for the prevention and removal of undesirable oral biofilm.

The antibacterial activity was assessed by employing specific bacterial strains, including *E. coli*, *B. subtilis*, *S. mutans*, and *S. aureus*. *Streptococcus mutans* inhabits the human buccal cavity, specifically dental plaque, a biofilm composed of multiple species that develop on the tooth's hard surfaces. The cariogenic potential of *S. mutans* is widely acknowledged to be based on three fundamental characteristics: (i) the capacity to produce substantial amounts of extracellular polymers of glucan from sucrose, (ii) the capability to transport and metabolize a diverse array of carbohydrates into organic acids, and (iii) the ability to flourish in the absence of oxygen (Lemos et al., 2019). Therefore, the focused elimination of *S. mutans*, specifically in the context of dental caries, is considered a viable strategy for preventing this disease. Several isolates in this study produced compounds which inhibit the growth of *S. mutans*. This may be due to the predominant presence of *S. mutans* in the oral cavity, and other bacterial strains evolved the ability to inhibit *S. mutans* as a competition strategy.

Meanwhile, antibacterial efficacy against *E. coli* and *S. aureus* yields no results. The absence of activity against *E. coli* (gram-negative bacteria) may be attributed to the outer membrane that is distinctive to gram-negative bacteria, differentiating them from gram-positive bacteria. That functions as a barrier against antibiotics and toxins (Breijyeh et al., 2020). Most studies indicated that nisin, bacitracin, and enterocin, which are peptides produced by bacterial species, can suppress the growth of *S. aureus* (Jensen et al., 2020). One possible mechanism is that *S. aureus* is known to possess efflux pumps that can actively and rapidly remove inhibitory metabolites out of the cell, thus allowing it to survive.

Based on the BLAST results (Table 3), the bacteria that have been identified are *S. salivarius* and *S. aureus*. Most of the identified bacterial isolated are commonly found in oral microbiome (Santacroce et al., 2023). *S. salivarius* is the most common isolate that displays antibiofilm and antibacterial activities. *S. salivarius* is one of the first colonizers of the human oral microbiome and gut after birth and hence may help the development of immunological homeostasis and modulation of host inflammatory responses (Kaci et al., 2014). Besides that, many studies have stated that *S. salivarius* is dominant in the oral microbiome. Apart from that, some strains have been shown to possess antibacterial capabilities, create bacteriocins, and function as antagonists to other bacterial species including *S. mutans*, *Streptococcus pyogenes* and *Streptococcus sobrinus* (Begić et al., 2023; Kaci et al., 2014; Tagg et al., 2023). Beside that, *S. Salivarius* can enhance the synthesis of anti-inflammatory cytokines and inhibit pro-inflammatory mediators, thereby fostering a balanced immune response in the oral cavity and contributing to oral health (Sali et al., 2025).

Several members of the Staphylococci are also found to display antibiofilm and antibacterial activities. *S. aureus* is an opportunistic pathogen that is commonly present in the oral microbiota. It can produce several enzymes to improve its pathogenicity and dispersion within the host; these include coagulase, hyaluronidase, deoxyribonuclease, and lipase (Tuon et al., 2023). Thus, the interaction of *S. aureus* and other pathogen could be antagonistic, for example *S. aureus* acts antagonistically towards *Candida glabrata* (Camarillo-Márquez et al., 2018).

Bacillus subtilis is a non-pathogenic bacterium that can be found in soil, food and *gastrointestinal tract of ruminants and humans* (Duanis-Assaf et al., 2020). *B. subtilis* has been reported to be commonly found in oral healthy people (Frid et al., 2020) and being utilized for many years in many diverse commercial applications, including farming, precision fermentation, and probiotic supplements (Jain et al.,

2013; Williams & Weir, 2024). Apart from that, there is study stated that, *B.subtilis* able to reduces periodontal pathogens (Tsubura et al., 2009). Briefly, *B. subtilis* offers more advantages than disadvantages. Thus, the potential of a different oral microbiota to suppress the growth of *B. subtilis* should be investigated to determine its relationship with oral diseases.

E. coli is a bacterium present in the intestines of humans and animals, as well as in the environment. Other than that, *E. coli* is the predominant pathogen causing simple cystitis and can also cause various extraintestinal disorders such pneumonia, bacteremia, and abdominal infections such as spontaneous bacterial peritonitis (Mueller & Tainter, 2024). Next, *S. aureus* is a prevalent bacterium present in the environment and in the typical human microbiota, particularly on the skin and mucosal membranes (Taylor & Unakal, 2024). Study from McCormack et al. (2015) stated that, *S. aureus* remains commonly found in the mouth and the area around the mouth, also suggested for a role in dental implant failure. In this study, none of the bacteria isolated is able to inhibit the growth of these bacteria.

It is interesting to note that of the dominant oral microbiome genera, only *Streptococcus* is prolific in producing antibiofilm and antibacterial compounds. These findings also supported by studies from Grover et al. (2025), Mihaylova-Garnizova et al. (2024) and Merritt & Qi, (2012). The other major genera are not shown in this study to produce these activities. It is probable that some of these genera are not amenable for cultivation on the media used in this study and are thus not recovered as positive isolates, or these genera may have evolved alternative strategies to compete for growth in the oral cavity. The other bacterial genera that is prolific in producing antibiofilm and antibacterial activities is *Staphylococcus*, which is a minor component of the oral microbiomes in this study and is seldom reported from other oral microbiome studies. The *Staphylococcus* strains isolated here may be transient members of the oral microbiomes, or they may be true inhabitants in niche micro-environments within the oral cavity.

Isolates SA10, SA78 and SA108 demonstrated favourable outcomes in terms of their antibiofilm and antibacterial activity and have the potential to be developed as oral probiotics or combination therapy for prevention and treatment of infectious oral diseases. As these isolates were obtained from the oral microbiome itself, they are expected to be able to establish easily as part of the microbiota.

CONCLUSION

The composition of the oral microbiome of the Malaysian population is similar to oral microbiomes characterized from different parts of the world. The predominant phyla include Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria and Actinobacteria; and their abundance are similar to other reported oral microbiomes. The oral microbiome contains bacterial species that could produce compounds with antibiofilm and antibacterial activities. The *Streptococcus* groups, particularly *Streptococcus salivarius* are the most prolific genus in producing these compounds with potent activities, potentially serving as probiotics or antibiotic adjuvants to improve oral health by preventing colonization and inhibiting the growth of pathogenic species. Further investigations into the properties of these bacteria species and the mechanism of actions of their antibiofilm and antibacterial compounds may provide new insights into the oral microbiome as a potential resource for oral hygiene and oral therapeutic products.

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ETHICAL STATEMENT

This study was approved by the Research Ethics Committee of Universiti Teknologi MARA.

CONFLICT OF INTEREST

The authors have declared no conflict of interest

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