Antibiofilm Properties of Bacteria Isolates from the Oral Microbiome

Nurul Hasanah Dawis[1], Nik Airin Syazlin Azhan[1], Mohd Shafiq Aazmi[1], Aziyah Abd Aziz[1], Mohammad Faiz Foong Abdullah[1*]

[1]Faculty of Applied Science, Universiti Teknologi MARA, Shah Alam, Malaysia
mohdf184@uitm.edu.my

Abstract. The oral microbiome consists of diverse microorganisms inhabiting the mouth cavity. An imbalanced oral microbiome may lead to periodontitis and other oral diseases. Certain species in the oral microbiome may be able to produce antibiofilm compounds and play a protective role. Bacterial cultures were isolated from saliva samples of individuals and investigated for the ability to prevent biofilm formation in *Staphylococcus epidermidis* at different stages. A total of 14 isolates (11.97%) showed antibiofilm activities, primarily at the maturation and attachment stages. The results demonstrate the potential of these bacteria as probiotics for the improvement of oral health.

Keywords: Antibiofilm activity, biofilm, oral microbiome.

1 Introduction

1.1 Background of the study

The oral microbiome is made up of microbial communities that live in various environments in our mouth, including our teeth, inner cheeks, tongue, gingiva, tonsils, and palates. The microbiota of the oral cavity is known to be the second largest and highly diverse, encompassing a vast array of microbial organisms such as bacteria, fungus, viruses, and protozoa, with a total of over 700 distinct species [1,2]. The human microbiome is comprised of two distinct components, namely the core microbiome and the variable microbiome. The core microbiome is universally shared among all individuals, while the variable microbiome is distinct to each individual based on their lifestyle choices and physiological variations [3].

The oral microbial ecology is subject to continuous exposure to exogenous foreign chemicals, which serve as defining elements for the establishment and persistence of microbes within this environment. These circumstances give rise to different connections between microorganisms and their hosts, which are shaped by selective forces [4].

Salivary microbiota, consisting of bacteria shed from oral surfaces, has been shown to be individualized, temporally stable, and influenced by diet and lifestyle [5]. Common bacteria genera found in the oral cavity are *Eubacteria, Streptococcus*, *Granulicatella, Gemella,* and *Veillonella*. It is reported by previous study that the

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majority of the bacteria that inhabit the mouth cavity are symbiotic and beneficial to the host.

Microorganisms 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 [6]. Oral biofilms consist of a wide range of microbial communities that are encased inside an exopolysaccharide matrix. The fundamental etiology of caries, endodontic, and periodontal diseases can be attributed to the formation and growth of biofilms on the surfaces of dental hard or soft tissues [7].

Understanding germ-host relationships is a priority in dentistry and industry. This is due to oral bacteria's symbiotic relationship. Commensal populations prevent pathogenic species from adhering to the mucosa and maintain control. Once bacteria cross the commensal barrier, they become pathogenic and cause illness. Hence, the examination of microbial interactions within the human mouth cavity holds potential for future research endeavors.

1.2 Problem statement

The oral microbiome and its host have a significant relationship in maintaining oral health. However, certain species in the oral microbiome can behave pathogenically toward its host when there is an imbalance or dysfunction in the microbiome environment. This could lead to a number of oral diseases such as dental caries, periodontitis, and oral cancer. On the other hand, some members of the oral microbiome may provide a protective effect by producing molecules such as peptides that antagonizes the growth and colonization of pathogens. There are little studies on the ability of beneficial species in the oral microbiome in producing protective compounds such as antibiofilm peptides.

1.3 Significant study

The study’s findings provide insights into the oral microbiome’s capacity to produce antibiofilm compounds, which may potentially be employed to prevent or provide a fresh approach to treating oral infections.

1.4 Objectives

1. To evaluate the antibiofilm activity from oral microbiomes
2. To analyze the types of biofilm inhibition activities

1.5 Scope and limitation of the study

1. The samples were collected from a group of people with the same geographical niche.

2. Antibiofilm activity was studied by using only one test organism which is *Staphylococcus epidermis* as it is a common strain for biofilm assays.

2 Literature Review

2.1 The oral microbiome

A colony of microorganisms with up to 1000 different species, including bacteria, fungi, viruses, archaea, and protozoa, that reside in the mouth cavity is referred to as the oral microbiome. They have an incredible range of anticipated protein activities when compared to other body locations. Human microbiome has core and varied microbial communities. All people share the same core microbiome; however, their lifestyle and physiology determine their microbiome. Bacteria can invade the oral mucosa and teeth's hard and soft tissues. The hard and soft palates, tonsils, gingival sulcus, teeth, tongue, and cheeks offer a hospitable environment for microbes to thrive [2].

Normally, the womb is a sterile environment in which the fetus develops. Recent research suggests that up to 70% of pregnant women have oral bacteria in their amniotic fluid. The newborn is exposed to environmental and maternal uterine and vaginal microbiota during birth. The mouth of an infant is usually sterile, but *Fusobacterium nucleatum* soon colonizes it. These microorganisms spread through saliva and other channels. They can also transmit passively from the mother and through water, milk, and environmental bacteria, which help develop the baby's native oral microbiota [2].

Environmental factors like food, dental hygiene, drugs, stress, and systemic factors can affect the oral microbiota throughout life, in addition to host genetics and maternal transmission. The mouth is a dynamic microbial habitat, hence the oral microbiota changes throughout time. Primary deciduous tooth emergence produces new microbial colonization surfaces, changing the oral microbiome ecology. The oral microbiome changes with a person's food choices, age, hygiene practices, and behaviors like smoking and drinking [4].

2.2 Host-microbiome interaction

Typically, the oral microbiota lives as a biofilm. It is essential for preserving oral homeostasis, safeguarding the oral cavity, and halting the progression of illness. For a mechanistic understanding of the major actor, knowledge about the identification of the microbiome and the nearby neighbors with whom they frequently interact is required. Critical physiological, metabolic, and immunological processes carried out by the microbial communities present in the human body include food and nutrition digestion, energy production, differentiation and maturation of the host mucosa and its immune system, regulation of fat storage and metabolism, processing and detoxification of environmental chemicals, maintenance of the immune system, and the balance between pro- and anti-inflammatory cytokines [2].

National Institute of Health launched Human Microbiome Project in 2008. The Human Microbiome Project (HMP) has produced tools, approaches, and findings that

link microbiome-human interactions to health outcomes over 10 years in two phases (The Integrative Human Microbiome Project, 2019). According to previous study, the Human Oral Microbiome Database (HOMD) has sequenced over 600 16S RNA gene libraries and 35,000 clone sequences. The samples came from healthy people with over a dozen diseases, including oral cancer, endodontic infections, caries, and periodontal disease.

According to reference [2], approximately 700 species of prokaryotes have been identified in oral cavity. These species contain 185 genera and 12 phyla, 54 percent of which have official names, 14 percent are nameless but cultivated, and 32 percent are uncultivated phylotypes. There are 185 genera and 12 phyla. Firmicutes, Fusobacteria, Proteobacteria, Actinobacteria, Bacteroidetes, Chlamydiae, Chloroflexi, Spirochaetes, SR1, Synergistetes, Saccharibacteria (TM7), and Gracilibacteria are the 12 phyla (GN02).

Other than that, research from the NIH Human Microbiome Project (HMP) demonstrated that, compared to other habitats like skin and gut, the oral microbiome has the highest number of bacteria that are shared by unrelated people. Typical oral bacteria found in the oral cavity are *Streptococcus mutans*, *Porphyromonas gingivalis, Staphylococcus, revotella, Capnocytophaga, Nisseria, Haemophilis, Treponema, Peptostreptococcus* and *Lactobacillus*. However, the relationship of oral microbiome with its host is still understudied as compared to the stomach at the time of writing, a pubmed search (2017-2022) with “oral Microbiome” resulted in 7801 articles as compared to 36301 with “gut microbiome”.

2.3 Oral bacterial biofilm

Oral bacterial biofilms adapt to high cell density due to oral cavity features, creating a microenvironment that regulates pH, redox, and oxygen levels in their core. Early colonizers, or planktonic bacteria, are responsible for the formation of oral biofilms. Streptococcus species make up 80% of early colonizers, which are facultative anaerobes that feed on oral glycoproteins and salivary mucins.

Numerous populations of microorganisms, including viruses, mycoplasmas, bacteria, Archaea, fungi, and protozoa, can develop in the mouth. These communities create the resident oral microbiome, which persists on all surfaces as multispecies biofilms and typically coexists with the host. It provides significant advantages that support general health and well-being.

Oral biofilms' microbial residents coexist close, resulting in synergistic or antagonistic interactions. Oral environment affects microbiome makeup. Changes in the local environment can affect microbial interactions in oral communities and determine whether the oral microbiome and host have a symbiotic or dysbiotic relationship, increasing the risk of caries and periodontal diseases.

2.4 Antibiofilm compounds

One of the most prevalent infectious illnesses affecting people is dental caries, which is directly associated with cardiovascular disease in people. It is brought on by the oral

microbiota's homeostatic imbalance. *S. mutans* is thought to be one of the main cariogenic bacteria in the oral cavity, despite the fact that other bacteria are also linked to the pathogenesis of dental caries. *S. mutans* may colonize the hard tissues on the surface of teeth and facilitate the production of cariogenic biofilms, which helps them survive in hostile conditions [8].

Water-insoluble (-1,3-linked) and soluble (-1,6-linked) glucans are crucial for the development of biofilms in *S. mutans*. They were created from sucrose by glucosyltransferases (GTFs). In addition to making up the majority of the biofilm's polymeric matrix and supporting its structural stability and integrity, glucans also help *S. mutans* attach to tooth surfaces and improve bacterial cell aggregation and coaggregation [8]. Numerous dairy products contain probiotic bacteria from the *Lactobacillus* genus to promote consumer health. Inhibiting pathogen infection is one of the advantages of *Lactobacillus* species as a probiotic [9]. As part of the mechanism, organic acids, H_2O_2, bacteriocins, and adhesion inhibitors are produced, the function of the epithelial barrier is improved, and pathogens' ability to adhere to epithelial cells is inhibited. It has also been shown that *Lactobacillus* has beneficial benefits on the mouth. For instance, *Lactobacillus acidophilus* culture supernatant can reduce gingivitis and treat periodontitis [8].

3 Methodology

3.1 Ethical approval

The approval of the study protocol was requested from the Research Ethics Committee of Universiti Teknologi MARA. The subjects were undergraduate students of Universiti Teknologi MARA.

3.2 Sample collection

Salivary samples were collected from volunteer who consented to join the study. Subjects were asked to refrain from using oral hygiene products with antimicrobial activity for 12 hours before samples 5 ml of saliva are collected in a sterile tube.

3.3 Preparation of sterile conditioned medium (scm)

The swab samples were inoculated into three different media which are Tryptone Soy agar (TSA), Mannitol Salt Agar (MSA), and DeMan-Rogosa-Sharpe Medium (MRS). Different media were used to promote the growth of gram-positive and gram-negative bacteria according to their preference. Then, the culture will be incubated for 24 hours at 37 °C. Single colonies were selected and purified by repeated streaking. Purified isolates were then cultured in 2ml of tryptone soya broth in a 96-deepwell plate and incubated for 48 hours at 37 °C. The culture media inside the 96-deepwell plate was then filtered individually with 0.45 μm membrane and transferred to a new deepwell plate; and stored at -20°C until further use.

3.4 Antibiofilm assay

The samples were tested against *S. epidermidis* ATCC35984, a known biofilm producer. *S. epidermidis* ATCC12228, a non-biofilm producer, was used as the negative control. Cultures of the test organism and negative control were prepared by first growing them overnight at 37 °C in Tryptone Soy Broth supplemented with 1% glucose (STSB), then diluting in fresh STSB at 1:100 and further incubating until they reach the mid-log phase. The cultures' turbidity was adjusted to 0.08 - 0.13 at OD_{570}, which is equivalent to 1 X 108 CFU/ml.

For the antibiofilm attachment assay, about 100 µl of the prepared *S. epidermidis* ATCC35984 culture was dispensed into the wells of a microtiter plate. A volume of 50 µl of cell free medium was mixed with the bacteria 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 hours to allow bacteria cells to attach to the well surface and initiate formation of a 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 biofilm layer was stained for 15 minutes with 150 µL of 1% crystal violet. The excess stain was then gently washed away with tap water before being air-dried and resolubilized in 150 µL of ethanol for 30 minutes. The optical density of the resolubilized dye 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 incubating it at 37 °C for one hour to allow cells to attach to the well surface. Following that, except for the controls, 50 µl of cell free medium secretion was added to the wells and incubated at 37 °C for 24 hours. The amount of biofilm formed was then measured as described in the antibiofilm attachment assay above.

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

The readings were evaluated by using a formula to compute the percentage of antibiofilm activity.

$$100 - ([(S - N) / (P - N)] \times 100\%) \quad (1)$$

Whereby:
S = OD_{570} of *S. epidermidis* ATCC35984 + cell free medium (test samples)
N = OD_{570} of *S. epidermidis* ATCC12228 + diluted TSB (negative control)
P = OD_{570} of *S. epidermidis* ATCC35984 + diluted TSB (positive control)

4 Findings

4.1 Antibiofilm activity

Antibiofilm activity of samples from oral microbes was tested against *Staphylococcus epidermidis* (35984) and shown in Table 1. The samples showed different antibiofilm activity at different stages of attachment, maturation and dispersion. A total of 14 isolates (11.97%) showed antibiofilm activities at the maturation stage, while only one isolate each showed activities at the attachment and dispersion stages. Isolate SA108 inhibited biofilm formation at both the attachment and maturation stages, while SA96 showed inhibitory activities at both the maturation and dispersion stages. The average percentage of biofilm inhibition for attachment is 94.4%, maturation is 80.89% and dispersion is 75.3% as shown in Figure 4.

Table 1. Antibiofilm activity of oral microbes

Sample	Antibiofilm activity		
	Attachment	Maturation	Dispersion
SA09	-	++	-
SA10	-	+++	-
SA67	-	++	-
SA68	-	+	-
SA69	-	+++	-
SA70	-	++	-
SA71	-	++	-
SA72	-	++	-
SA74	-	++	-
SA78	-	++	-
SA82	-	++	-
SA96	-	++	++
SA99	-	+	-
SA108	+++	+++	-

(+++) strong biofilm producer; (++) Moderate biofilm producer; (+) Weak biofilm producer

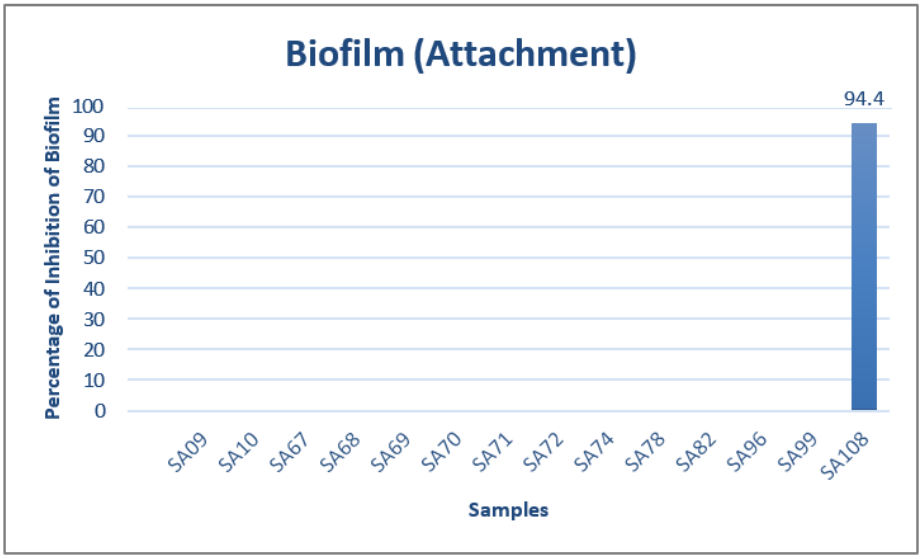

Fig. 1. Bar graph representing the percentage of inhibition of biofilm at attachment stage

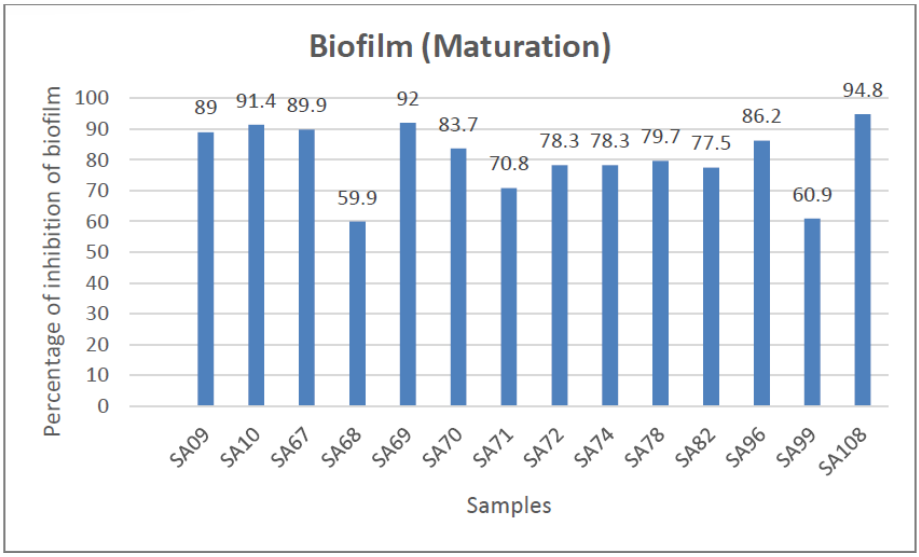

Fig. 2. Bar graph representing the percentage of inhibition of biofilm at maturation stage

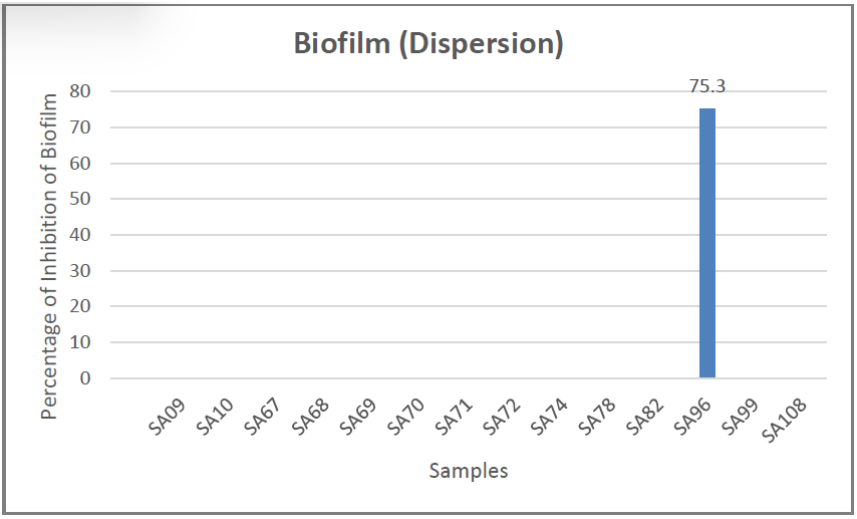

Fig. 3. Bar graph representing the percentage of inhibition of biofilm at dispersion stage

Antibiofilm activity at the attachment stage is observed only in isolate SA108, which inhibited biofilm formation by 94.4%. Isolate SA96 was the only sample that worked at the dispersion stage, resulting in loss of 75.3% biofilm. the majority of the positive samples exhibited antibiofilm action during the maturation stage. Several of these showed good potential as antibiofilm compounds, resulting in the inhibition of up to 90% biofilm formation

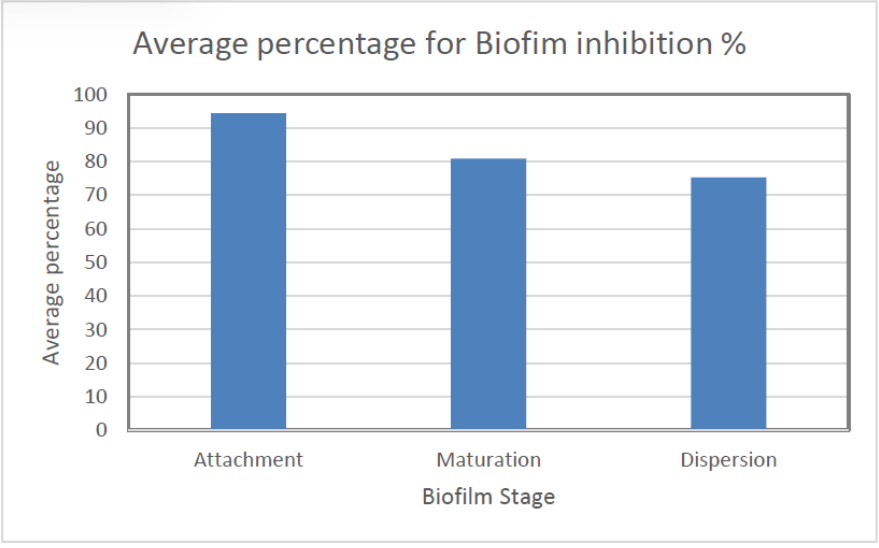

Fig. 4. Bar graph representing the average percentage of inhibition of biofilm at attachment, maturation and dispersion stage

5 Discussion

In this study, oral biofilms were investigated on the antibiofilm activity of oral microbes and the percentage of biofilm inhibition. The antibiofilm activity was analyzed with three different stages of biofilm formation which are attachment, maturation, and dispersion.

A related study from reference [10], also showed positive findings with the use of probiotic bacteria against oral pathogens. The study proposed that the probiotic microorganism may be suitable for the prevention and treatment of dental caries without adverse effects. For example, *Lactobacillus brevis* (90.00%) showed the highest value for biofilm inhibition concentration against *Streptococcus oralis* [10].

Based on the result, the maturation stage shows the highest antibiofilm activity compared to the other stages. Meanwhile, the attachment and dispersion stage only show one positive result. 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 as depicted in Figure 1 [11].

The antibiofilm compounds produced by these bacteria has the potential to be use 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 due to the presence of a compound that prevent biofilm formation or breaks down the biofilm. An investigation previous study examined the antibiofilm properties of butanolide, a potent anti-macrofouling chemical produced from a marine *Streptomyces* sp. The study shown 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* and *Bacillus licheniformis*, has the ability to inhibit biofilm formation.

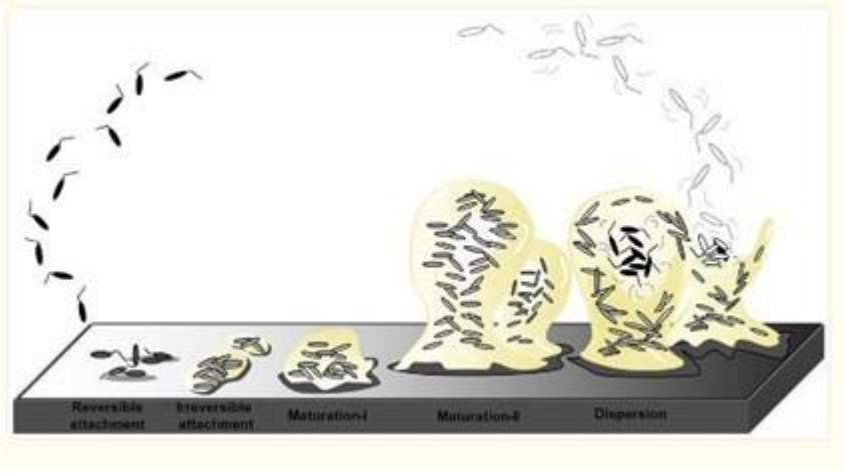

Fig. 5. The stages of biofilm development (Sauer et al., 2022)

Next, based on table 1, antibiofilm activity at the attachment stage shows strong biofilm activity from SA108. The percentage of the antibiofilm activity at attachment stage is 94.4% meanwhile at maturation stage is 94.8% which categorized as a strong biofilm producer.

As shown in the result, SA108 has higher antibiofilm activity at attachment and maturation stage only but not at dispersion stage. The ability of SA108 to inhibit attachment is interesting, as it could be potentially used to prevent biofilm formation from taking hold on surfaces that contact a patient eg. medical devices. Meanwhile, SA96 shows 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 [12].

From this study, none of the samples showed high antibiofilm activities for all stages. However, most samples show positive antibiofilm activity at maturation stage. Thus, the use of biological approach can be further study as it can lead to a new biological alternative for the treatment of oral biofilm.

6 Conclusion

In conclusion, the results have shown that certain members of the oral microbiome can produce compounds with antibiofilm activities against the various stages of biofilm formation. Thus, a 'good' oral microbiome may play major roles in maintaining oral health by preventing colonization by pathogenic species. These antibiofilm compounds has the potential to be developed as probiotics or antibiotic adjuvants for the improvement of oral health.

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