



Difference in formation of a dental multi-species biofilm according to substratum direction

Minh-Huy Dang^{a,d,1}, Jian-Na Cai^{b,d,1}, Hyeon-Mi Choi^c, Dongyeop Kim^d, Hyo-Won Oh^{e,*}, Jae-Gyu Jeon^{d,**}

^a Department of Odonto-Stomatology, Hue University of Medicine and Pharmacy, Hue University, Hue City, Viet Nam

^b Department of Oral Biology, School of Stomatology, Binzhou Medical University, Yantai, People's Republic of China

^c Department of Dentistry, Presbyterian Medical Center, Jeonju, Republic of Korea

^d Department of Preventive Dentistry, School of Dentistry, Jeonbuk National University, Jeonju, Republic of Korea

^e Department of Preventive and Public Health Dentistry, College of Dentistry, WonKwang University, Iksan, Republic of Korea

ARTICLE INFO

Keywords:

Dental caries

Multi-species biofilm

Substratum direction

Streptococcus mutans

ABSTRACT

Objectives: The aim of this study was to investigate the difference in dental biofilm formation according to substratum direction, using an artificial biofilm model.

Methods: A three-species biofilm, consisting of *Streptococcus mutans*, *Streptococcus oralis*, and *Actinomyces naeslundii*, was formed on saliva-coated hydroxyapatite (sHA) discs oriented in three directions: downward (the discs placed in the direction of gravity), vertical (the discs placed parallel to the direction of gravity), and upward (the discs placed in opposite direction of gravity). The biofilms at 22 h and 46 h of age were analyzed using microbiological and biochemical methods, fluorescence-based assays, and scanning electron microscopy to investigate difference in bacterial adhesion, early and mature biofilm formation.

Results: The biofilms formed in the upward direction displayed the most complex structure, with the highest number and biovolume of bacteria, as well as the lowest pH conditions at both time points. The vertical and downward directions, however, had only scattered and small bacterial colonies. In the 22-h-old biofilms, the proportion of *S. oralis* was similar to, or slightly higher than, that of *S. mutans* in all directions of substratum surfaces. However, in the 46-h-old biofilms, *S. mutans* became the dominant bacteria in all directions, especially in the vertical and upward directions.

Conclusions: The direction of the substratum surface could impact the proportion of bacteria and cariogenic properties of the multi-species biofilm. Biofilms in an upward direction may exhibit a higher cariogenic potential, followed by those in the vertical and downward directions, which could be related to gravity.

1. Introduction

Dental caries is a common and costly infectious disease that depends on biofilms in the oral cavity. It is mainly caused by the interaction of specific bacteria with diet constituents within dental biofilms. Microbial adhesion to solid surfaces is a crucial step in biofilm formation, including on tooth surfaces, where it serves as a base for subsequent colonization (Kolenbrander et al., 2010). The complex physical and chemical interactions between oral bacteria and tooth surfaces during

biofilm formation can significantly impact the composition and function of dental biofilms, ultimately affecting oral health (Bernabe et al., 2017). Therefore, understanding the interactions that influence dental biofilm formation is essential for developing strategies to control dental caries by modulating biofilms.

Various environmental factors, such as surface characteristics, salivary proteins, and oral conditions, can influence the interactions between tooth surfaces and microbial cells (Han et al., 2016). Among these factors, substratum-related factors such as surface charge, surface

* Correspondence to: Department of Preventive and Public Health Dentistry, College of Dentistry, WonKwang University, 460 Iksan-daero, Iksan, Republic of Korea.

** Correspondence to: Department of Preventive Dentistry, School of Dentistry, Jeonbuk National University, 567 Baekje-daero, Jeonju 54896, Republic of Korea.
E-mail addresses: dhdh@wku.ac.kr (H.-W. Oh), dentjik@jbnu.ac.kr (J.-G. Jeon).

¹ Minh-Huy Dang and Jian-Na Cai contributed equally to this work and should be considered co-first authors.

<https://doi.org/10.1016/j.archoralbio.2024.106002>

Received 27 March 2024; Received in revised form 9 May 2024; Accepted 11 May 2024

Available online 13 May 2024

0003-9969/© 2024 Elsevier Ltd. All rights are reserved, including those for text and data mining, AI training, and similar technologies.

hydrophobicity, and surface roughness have been extensively studied for their influence on microbial adhesion during the initial step of dental biofilm formation (Zheng et al., 2021). A recent study has also revealed that carious lesions are increasingly localized to specific tooth sites (Carvalho, 2014). In children and adolescents, occlusal surfaces are more vulnerable to dental caries compared to proximal surfaces, suggesting that the direction of tooth surfaces (substratum) can be an important factor affecting the interaction between microbial cells and tooth surfaces. Our previous study also reported that the direction of tooth surfaces significantly impacts the formation and pathogenicity of *Streptococcus mutans* biofilms (Dang et al., 2018). Specifically, the upward direction of substratum surfaces (occlusal surfaces of mandibular teeth) showed the highest colony-forming unit (CFU) counts, extracellular polysaccharide (EPS) amounts, biofilm density, and acid production of the biofilms. However, this study only examined biofilms composed of a single species, *S. mutans*, and did not simulate the complex microbial community found in dental biofilms.

The types and properties of bacteria also influence the interactions between tooth surfaces and microbial adhesion. Numerous studies have shown that bacteria adhere to surfaces based on their specific characteristics, such as hydrogen-bonding capability and ability to form ligand-receptor bonds (Gibbons et al., 1985; Nobbs et al., 2011). Moreover, a previous study demonstrated that initial colonizers of dental hard surfaces exhibited stronger adhesion forces to saliva-coated enamel than cariogenic strains (Mei et al., 2009), which may further impact the formation and microbial composition of dental biofilms. However, few studies have investigated the impact of substratum surface direction on the adhesion ability of different types of oral bacteria and their biofilm formation.

In this study, we investigated the differences in microbial adhesion and biofilm formation in dental biofilms according to the direction of tooth surfaces using a multi-species biofilm model. The multi-species biofilm model was composed of *S. mutans*, *Streptococcus oralis*, and *Actinomyces naeslundii* on three different saliva-coated hydroxyapatite (sHA) disc direction groups: downward (the discs placed in the direction of gravity), vertical (the discs placed parallel to the direction of gravity), and upward (the discs placed in the opposite direction of gravity) to mimic tooth surfaces in the mouth.

2. Materials and methods

2.1. Direction of substratum surface

Three different groups of hydroxyapatite (HA) discs were used in this study, each oriented in a different direction: downward, vertical, and upward. The surfaces of the HA discs were prepared as previously described (Dang et al., 2018). Briefly, HA discs (2.93 cm²; Clarkson Chromatography Products, Inc., South Williamsport, PA, USA) were placed in Teflon covers with a modified orthodontic wire wrapping and divided into three groups based on HA disc direction (Fig. 1): (a) the downward group, where the HA discs were placed horizontally in a downward position to allow biofilms to grow downwards; (b) the vertical group, where the HA discs were placed vertically to allow biofilms to grow laterally; and (c) the upward group, where the HA discs were placed horizontally in an upward position to allow biofilms to grow upwards.

2.2. Multi-species biofilm formation and experimental scheme

Multi-species biofilms were formed on saliva-coated HA (sHA) discs placed in different directions (Cai et al., 2021). In this study, *S. mutans* UA159, *S. oralis* KCTC 13048, and *A. naeslundii* KCTC 9013 were used to create three-species biofilms, as these organisms were observed in supragingival plaque in humans (Fig. 1). The sHA discs were generated by incubation with filter-sterilized (0.22-μm low protein-binding filter) human whole saliva for 1 h at 37 °C. For biofilm formation, the sHA discs were situated in Teflon covers and transferred to a 12-well plate containing 0.05% sucrose (w/v) ultrafiltered (10 kDa molecular weight) tryptone yeast-extract (UTE) broth with $5-7 \times 10^5$ colony forming units (CFUs)/ml *S. mutans*, $4-8 \times 10^6$ CFUs/ml *S. oralis* and $4-8 \times 10^5$ CFUs/ml *A. naeslundii* (6 ml/disc). The biofilms were grown undisturbed at 37 °C with 5% CO₂ for 22 h to allow bacterial adhesion and early biofilm formation. From this point, the culture medium was changed two times (at 22 and 31 h) until the biofilm was 46 h old, which was considered mature biofilms in this study. The 22- and 46-h-old biofilms were then analyzed via microbiological and biochemical methods, fluorescence-based assays, and scanning electron microscopy (SEM) (Fig. 1).

2.3. Biofilm bacterial cell count

The number of CFUs in the biofilms was determined as described elsewhere (Cai et al., 2021). Briefly, sHA discs containing the 22- and

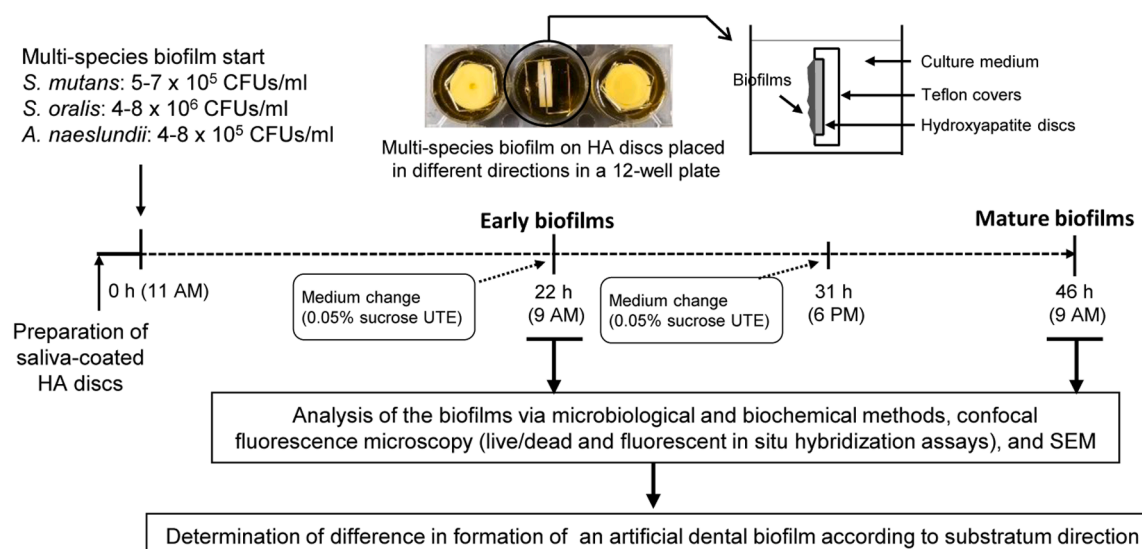


Fig. 1. Formation of an artificial dental biofilm on saliva-coated hydroxyapatite (sHA) discs placed in different directions and the experimental scheme.

46-h-old biofilms were released from Teflon covers and transferred into 2 ml of 0.89% NaCl. The discs were then sonicated in an ultrasonic bath (Power sonic 410; Hwashin Technology Co., Seoul, Korea) to disperse the biofilms. The resulting suspension was homogenized by sonication (VCX 130PB; Sonics and Materials Inc., Newtown, CT, USA) for 30 s after adding 3 ml of 0.89% NaCl. A 100 μ l aliquot of the homogenized suspension was serially diluted and plated onto selective agar plates.

2.4. Fluorescent in situ hybridization analysis

Fluorescence in situ hybridization (FISH) was performed as described previously (Thurnheer et al., 2004; Cai et al., 2021). Briefly, the oligonucleotide probe 5'-ACTCCAGACTTTCCTGAC-3' labeled with Cy3 was used to detect *S. mutans* (Paster et al., 1998). The oligonucleotide probe 5'-CTACACACGTGCTACAATGGCT-3' labeled with Cy5 was used to detect *S. oralis* (Jung et al., 2016). The oligonucleotide probe 5'-GCTACCGTCAACCCACCC-3' labeled with 6-FAM was used to detect *A. naeslundii* (Foster & Kolenbrander, 2004). For the sample preparation, the 22- and 46-h-old biofilms on sHA discs were first incubated in 100% ethanol for 15 min, and then fixed in 4% paraformaldehyde in phosphate-buffer saline (PBS) (pH 7.2) for 12 h at 4 °C. After fixation, all specimens were washed with PBS and incubated again in a solution containing 50% ethanol in PBS for 12 h. Subsequently, the specimens were incubated in a solution containing 10 mg/ml of lysozyme (1 M Tris-HCl pH 8) for 30 min at 37 °C. The samples were dehydrated and incubated with the oligonucleotide probes at a concentration of 20 ng each per 1 ml hybridization buffer. After hybridization, samples were incubated for 15 min at 48 °C in wash buffer and air dried. The CLSM imaging was observed with a LSM 880 with Airyscan microscope (Carl Zeiss, Jena, Germany). The excitation wavelengths for 6-FAM, Cy3 and Cy5 are 488, 561 and 633 nm, respectively. Three independent experiments were performed and five image stacks per experiment were collected (n = 15). The three-dimensional architecture of the biofilms was visualized using Imaris 8.0.2. The biovolume ($\mu\text{m}^3/\mu\text{m}^2$) was quantified from the confocal stacks using COMSTAT (Heydorn et al., 2000).

2.5. Live and dead biofilm bacterial cell analysis

To investigate difference in the biovolume of live and dead cells in the biofilms (Dang et al., 2018), the 22- and 46-h-old biofilms were stained at room temperature in the dark for 30 min using a FilmTracer LIVE/DEAD Biofilm viability kit L10316 (Invitrogen, Molecular Probes Inc., Eugene, OR, USA). The final concentrations of SYTO 9 and propidium iodide (PI) were 6.0 μM and 30 μM , respectively. The confocal laser scanning microscope (CLSM) images were observed with LSM 880 with Airyscan (Carl Zeiss, Jena, Germany) equipped with argon-ion and helium-neon lasers. The excitation wavelengths for SYTO 9 and PI are 488 and 561 nm, respectively. Three independent experiments were performed, and five image stacks per experiment were collected (n = 15). A stack of slices in 3.357 μm step sizes was captured from the top to the bottom of the biofilm. The biovolume of live and dead cells were quantified from the stacks using COMSTAT.

2.6. Scanning electron microscopic analysis

Scanning electron microscopic (SEM) study was performed as detailed elsewhere (Dang et al., 2018). Briefly, the 22- and 46-h-old biofilms were washed three times with 0.1 M cacodylate buffer and fixed with a 3% glutaraldehyde solution for 1 h, followed by post-fixation with a 1% osmium tetroxide solution for 1 h. The biofilms were dehydrated using a graded ethanol series (30–100%) and penetrated with nitrogen gas. Finally, the biofilm samples were sputter-coated with gold-palladium and observed by SEM (JSM-5900, Jeol, Japan).

2.7. Acid production analysis

Acid production in the 46-h-old biofilms was determined using the glycolytic pH drop assay as described elsewhere (Pandit et al., 2015). Briefly, the sHA discs containing the 46-h-old biofilms were released from Teflon covers. The discs were then transferred into 20 mM potassium phosphate buffer (pH 7.2) and incubated for 1 h to deplete endogenous catabolites and transferred to a salt solution (50 mM KCl + 1 mM MgCl_2 , pH 7.0). The pH was adjusted to 7.2. Glucose was then added to a final concentration of 1% (w/v). The decrease in pH was assessed using a glass electrode over a period of 240 min (Futura Micro Combination pH electrode, 5 mm diameter; Beckman Coulter Inc., CA, USA). The acidogenicity of the biofilms was evaluated using the initial rate of H^+ production and the total produced concentration of H^+ calculated by pH values of pH drop curves. The initial rate of H^+ production (y) was derived from the equation: $y = (\text{H}^+ \text{ concentration at } 30 \text{ min} - \text{H}^+ \text{ concentration at } 0 \text{ min})/30$. The total produced concentration of H^+ (y) was derived from the equation: $y = \text{H}^+ \text{ concentration at } 240 \text{ min} - \text{H}^+ \text{ concentration at } 0 \text{ min}$.

2.8. Statistical analysis

All experiments except CLSM and SEM studies were performed in duplicate, and at least three different experiments were conducted. The data are presented as mean $\pm$ standard deviation. Intergroup differences were estimated using one-way analysis of variance, followed by a post hoc multiple comparison (Tukey) test to compare multiple means. Values were considered statistically significant when the p value was < 0.05 .

3. Results

3.1. Changes in biofilm bacterial cell count

The total number and proportion of bacteria species in the multi-species biofilms were significantly influenced by the direction of substratum surfaces. As shown in Figs. 2a-1, 2a-2, 3a-1, and 3a-2, biofilms in the upward direction had the highest total number of bacterial cells (*S. mutans* + *S. oralis* + *A. naeslundii*) at both time points (22 and 46 h), compared to those in the vertical and downward directions ($p < 0.05$). However, as the biofilms developed, the proportion by bacterial species changed significantly according to the direction of substratum surfaces. In the 22-h-old biofilms, the proportion of *S. oralis* was similar to or slightly higher than that of *S. mutans* in all directions of substratum surfaces (Fig. 2a-3). On the other hand, the proportion of *S. oralis* in the 46-h-old biofilms in the vertical and upward directions was significantly lower than that in the downward direction (Fig. 2b-3). Moreover, in the 46-h-old biofilms, *S. mutans* became the dominant bacteria in all directions, especially in the vertical and upward directions ($p < 0.05$).

3.2. Changes in fluorescence in situ hybridization (FISH) study

To further investigate the effect of substratum direction on the adhesion ability and biofilm formation of oral bacterial species, a FISH study was conducted. The results of FISH study showed that biofilms in the upward direction were more structured and dense than the other test groups at both time points (22 and 46 h) (Figs. 3a-1 and b-1). Furthermore, the biovolume of bacterial species in the upward direction had the highest values at both time points, followed by that in the vertical direction (Figs. 3a-2 and b-2) ($p < 0.05$). At both time points, the biovolume in the downward direction was the lowest among those in the test groups ($p < 0.05$), showing the smallest size and scattered bacterial colonies (Figs. 3a-1 and b-1).

In the vertical direction, although the biovolume of *S. mutans* and *S. oralis* in the 22-h-old biofilms was similar to each other ($p > 0.05$), *S. mutans* in the 46-h-old biofilms clearly became the dominant species

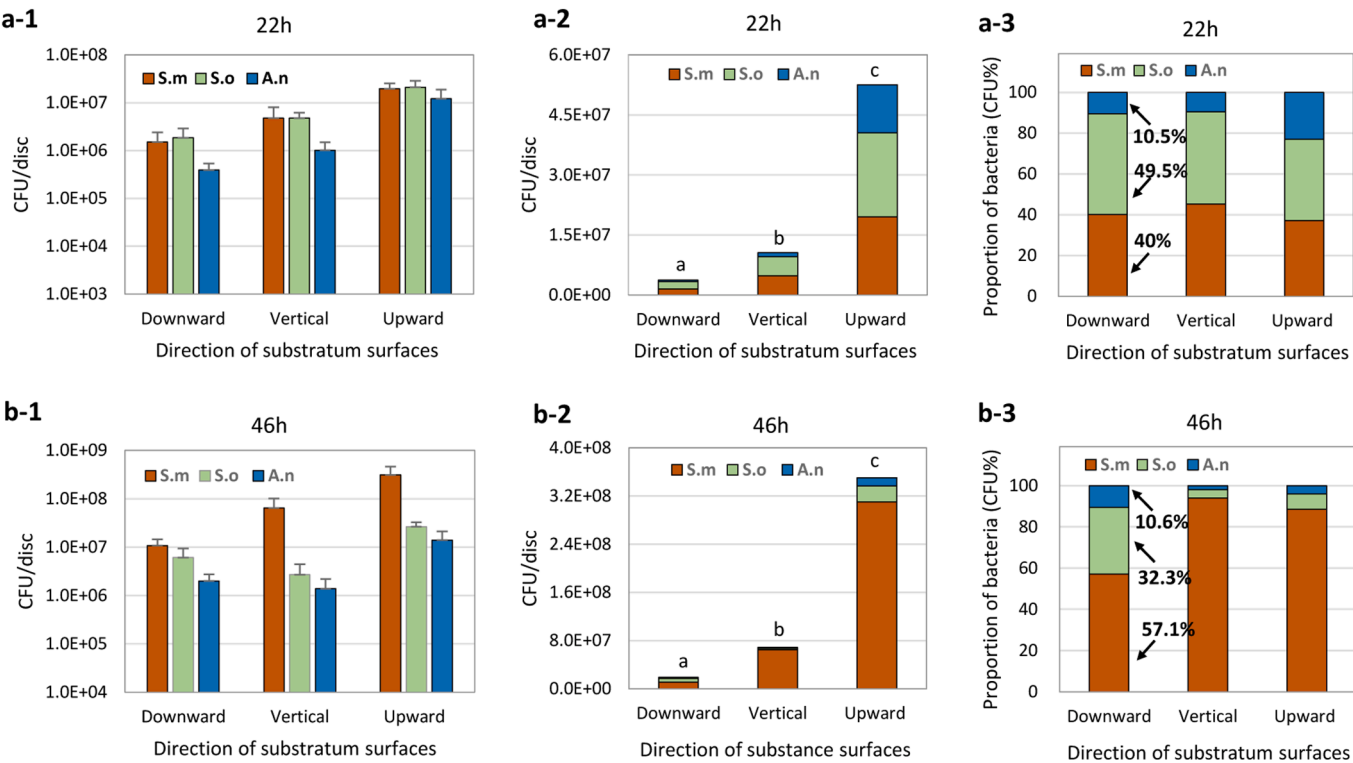


Fig. 2. Changes in bacterial counts of the multi-species biofilm formed in the downward, vertical, and upward directions. Each bacterial CFU count (a-1, b-1), total CFU count (a-2, b-2), and proportion of bacteria (a-3, b-3) in the 22- and 46-h-old biofilms. Values followed by the same superscript are not significantly different from each other ($p > 0.05$).

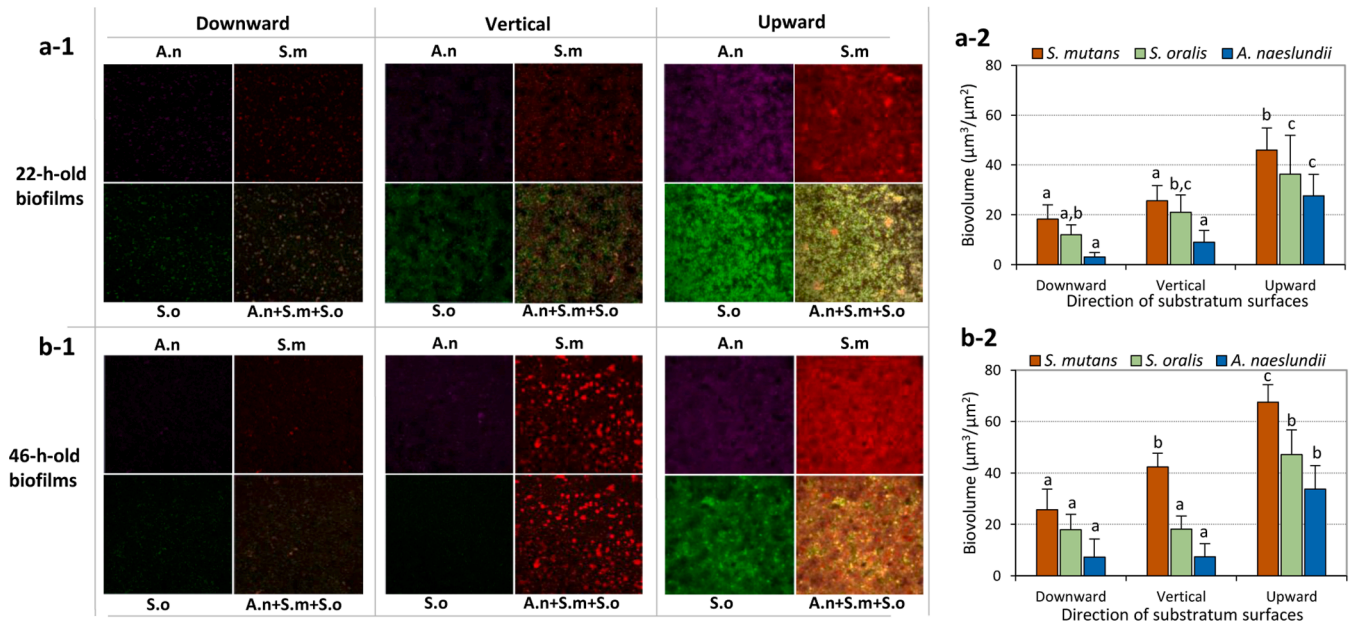


Fig. 3. Changes in fluorescence in situ hybridization (FISH) study on the multi-species biofilm formed in the downward, vertical, and upward directions. Representative confocal images of the 22- (a-1) and 46-h-old biofilms (b-1). Bacterial biovolume of the 22- (a-2) and 46-h-old biofilms (b-2). Values followed by the same superscript are not significantly different from each other ($p > 0.05$). A.n (*A. naeslundii*), S.m (*S. mutans*), and S.o (*S. oralis*).

($p < 0.05$), exhibiting higher densities, compared to that in the 22-hour-old biofilms. The biovolume of *A. naeslundii* in the upward direction was also higher than that in the other two directions, regardless of biofilm age (Figs. 3a-2 and b-2) ($p < 0.05$).

3.3. Changes in biofilm bacterial viability

Since the FISH study could only show the total volume (live + dead) of each bacterium, a live/dead biofilm bacterial viability assay was conducted to evaluate the biovolume of living and dead cells separately.

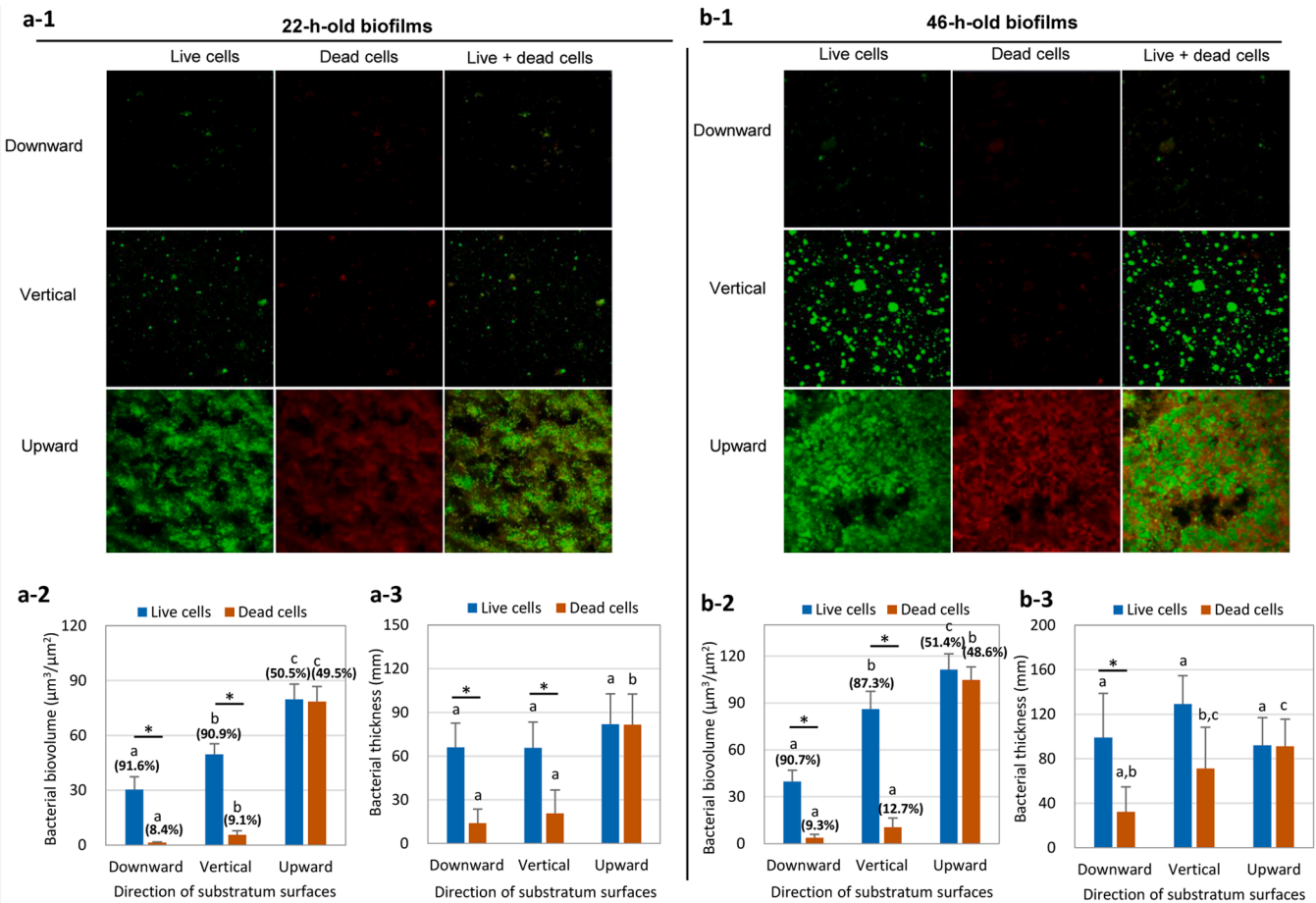


Fig. 4. Changes in biofilm bacterial viability of the multi-species biofilm formed in the downward, vertical, and upward directions. Representative 3-D images of live and dead cells in the 22- (**a-1**) and 46-h-old biofilms (**b-1**). Bacterial biovolume of live and dead cells in the 22- (**a-2**) and 46-h-old biofilms (**b-2**). Bacterial thickness of live and dead cells in the 22- (**a-3**) and 46-h-old biofilms (**b-3**) Values followed by the same superscript are not significantly different from each other ($p > 0.05$). * $p < 0.05$; significantly different.

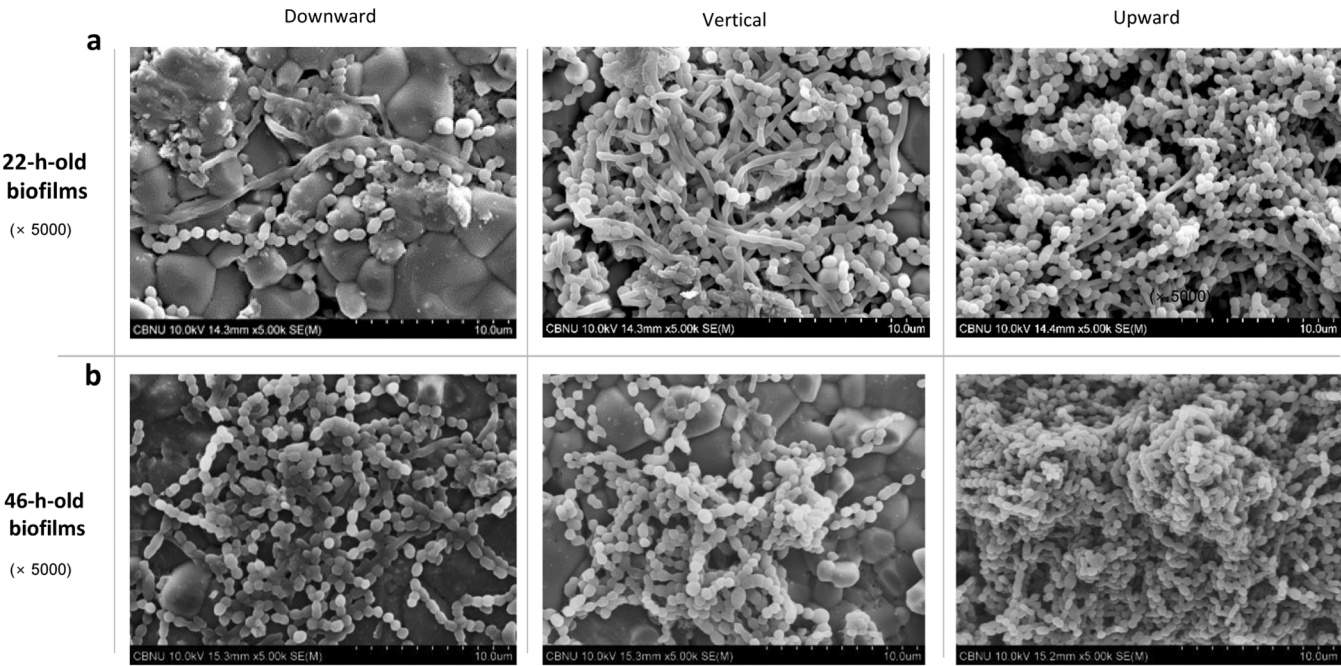


Fig. 5. Representative scanning electron microscopy (SEM) images ($\times 5000$) of the 22- (**a**) and 46-h-old biofilms (**b**) formed in the downward, vertical, and upward directions.

Figs. 4a-1 and b-1 show representative live/dead CLSM images, indicating that live or dead cells in the upward direction were more structured and dense than the other test groups at both time points (22 and 46 h). In contrast, biofilms in the other two directions had only scattered and small bacterial colonies, particularly dead cells that were difficult to detect.

As shown in Figs. 4a-2 and b-2, the biovolume of live or dead cells in the upward direction showed the highest values at both time points, followed by that in the vertical direction ($p < 0.05$). Interestingly, the biovolume percentage of live and dead cells in the upward direction were similar to each other ($p > 0.05$). On the other hand, the biovolume percentage of live cells was much higher than that of dead cells in the downward and vertical directions, regardless of biofilm age ($p < 0.05$). There was no significant difference in biofilm thickness of live cells in all directions at both time points ($p > 0.05$), but dead cells had higher values in the upward direction than in the vertical or downward directions ($p < 0.05$) (Figs. 4a-3 and b-3).

3.4. Changes in SEM study

As shown in Fig. 5a, the 22-h-old biofilms formed in different directions clearly showed micro-colonies of varying volume consisting of both branching filaments of *A. naeslundii* and typical long chains of Streptococci (*S. mutans* and *S. oralis*). However, in the 46-h-old biofilms, the dominance of *S. mutans* and lower numbers of *A. naeslundii* made it difficult to distinguish the latter (Fig. 5b). It was evident that the biofilms in the upward direction at both time points (22 and 46 h) exhibited more micro-colonies than those in vertical and downward directions, which was consistent with the results of the CLSM studies.

3.5. Changes in biofilm acid production

The acid production of the 46-h-old biofilms formed in different directions is shown in Figs. 6a and b. The biofilms in the upward direction showed the lowest pH values during 4 h experiment period, and the highest initial rate of H^+ production and total produced concentration of H^+ , among the three directions ($p < 0.05$). In addition, the initial rate of H^+ production and total produced concentration of H^+ of the 46-h-old biofilms in the vertical direction were significantly higher than those in the downward direction ($p < 0.05$).

4. Discussion

According to a recent study, the substratum direction has a significant influence on biofilm accumulation, acid production, and EPS formation in *S. mutans* biofilms (Dang et al., 2018). This implies that the

substratum direction may be an important factor affecting oral bacterial adhesion and cariogenic biofilm formation on tooth surfaces. However, there have been limited studies investigating the impact of the substratum direction on changes in cariogenic ability of dental biofilms during biofilm formation. In this study, our aim was to evaluate the differences in microbial adhesion and biofilm formation in an artificial dental biofilm according to the direction of tooth surfaces. To create the artificial dental biofilm, *S. mutans*, *S. oralis*, and *A. naeslundii* were chosen since they are commonly detected in human supra-gingival plaque. *S. mutans* is identified as a primary cariogenic bacterial pathogen associated with the initiation of dental caries, while *S. oralis* and *A. naeslundii* are regarded as the early colonizers of tooth surfaces and correlated with biofilm ecological balance (de Oliveira et al., 2020).

The bacterial composition of healthy dental plaque tends to remain stable over time. Previous research on species composition in healthy dental biofilms have revealed that a large majority (47–90%) of cultivable bacteria are non-cariogenic streptococci, such as *S. oralis*, and approximately one-third of the remaining bacteria are Actinomyces sp., such as *A. naeslundii* (Palmer et al., 2003). In this study, the 22-h-old biofilms were mainly composed of *S. oralis* and *A. naeslundii* (over 60%) (Fig. 2a-3), which suggests that they may reflect healthy dental biofilms. Furthermore, while the number of total bacterial CFUs in the 22-h-old biofilms varied depending on the substratum direction, the relative proportion of bacterial species remained consistent regardless of the substratum direction (Figs. 2a-2 and 2a-3). This indicates that the substratum direction may play an important role in influencing the number of bacteria during the adherent and early stages of biofilm formation, but does not affect the proportion of bacteria.

The results of this study revealed that the proportions of *S. mutans* in the 46-h-old biofilms increased rapidly compared to the 22-h-old biofilms, ultimately becoming the dominant species (Fig. 2b). This phenomenon could be attributed to the continuous exposure to sucrose in the biofilm model used, which has been known to stimulate the growth of *S. mutans* and disrupt the microbial balance between commensals and pathogens. Although the sucrose concentration used in this study was very low (0.05%), as biofilm aged (46 h), the accumulation acid could potentially interfere with the growth of *S. oralis* and *A. naeslundii*.

However, in the 46 h-old biofilms, both the number of total bacterial CFUs and the relative proportion of bacterial species changed depending on the substratum direction (Figs. 2b-2 and 2b-3). This suggests that the substratum direction can be a factor that affects both the abundance and composition of bacteria in the late stage of biofilm formation. Specifically, in this study, the proportion of *S. mutans* in the 46-h-old biofilms rapidly increased and became the dominant species, especially in the vertical and upward directions (over 90%) (Fig. 2b-3). However, the proportion of *S. mutans* in the downward direction was similar to that of

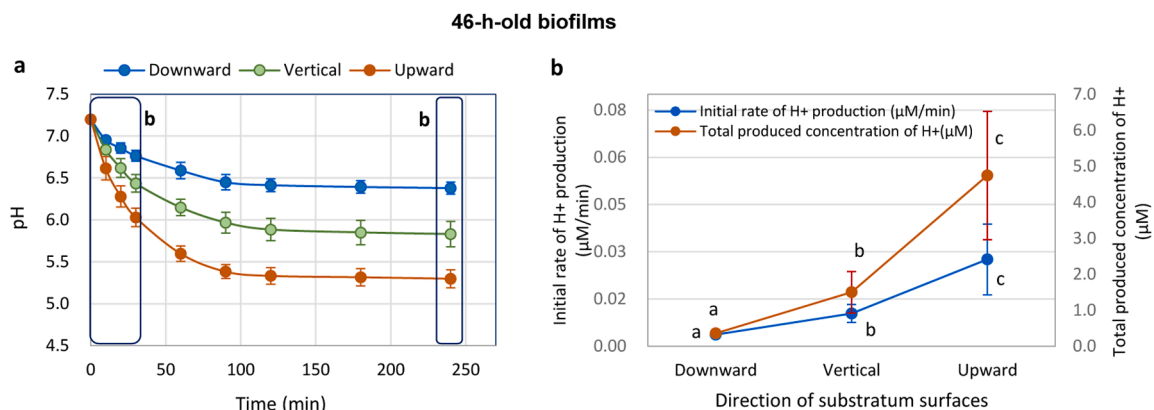


Fig. 6. Acid production in the 46-h-old biofilms formed in the downward, vertical, and upward directions. The result of biofilm pH drop assay (a) was used for the calculation of initial rate of H^+ production (0–30 min) and total produced concentration of H^+ (240 min) (b). Values followed by the same superscript are not significantly different from each other ($p > 0.05$).

the 22-h-old biofilms (Figs. 2a-3 and 2b-3), indicating that biofilms in the downward direction may still be in the stage of adherence and initial biofilm formation. This could be due to a lower number of bacteria in the 46-h-old biofilms in the downward direction compared to those formed in other directions, resulting in a delayed drop in pH within the biofilms (Fig. 6), which in turn prolongs the survival of *S. oralis* and *A. naeslundii*.

Acid production of dental biofilms is one of the main virulence factors that leads to the demineralization of the tooth enamel. In this study, we observed that the initial rate of H^+ production and total produced concentration of H^+ by the 46-h-old biofilms were highest in the upward direction, followed by the vertical and downward directions (Fig. 6). This finding suggests that the substratum direction plays a crucial role in biofilm acid production, particularly in the late stages of biofilm formation. The higher acid production ability of the 46-h-old biofilms in the upward direction may be related to the number of acidogenic and aciduric bacteria, such as *S. mutans*. As shown in Fig. 2b-2, *S. mutans* counts of the 46-h-old biofilms were higher in the upward direction compared to the other directions, indicating that biofilms in the upward direction can produce a large amount of hydrogen ions at a faster rate, thereby increasing the risk of dental caries development. Furthermore, acidification of the biofilms in upward direction may create an unfavorable environment for the survival of acid-sensitive bacteria, such as *S. oralis* and *A. naeslundii*, promoting the dominance of acidogenic and aciduric bacteria.

In this study, the 22-h- and 46-h-old biofilms in the upward direction (opposite to gravity) exhibited a higher number of live bacterial cells compared to those formed in the vertical (parallel to gravity direction) or downward (in the direction of gravity) directions (Figs. 2a-2 and 2b-2). This result may be attributed to the influence of gravity, as bacteria forming biofilms in the downward or vertical direction have to overcome the obstacle of gravity. The bacteria are easily dislodged from the surface, if they cannot overcome the obstacle of gravity. Our results of live/dead biofilm bacterial viability assay support this explanation. The biovolume of live cells in the downward and vertical directions in the 22-h- and 46-h-old biofilms was significantly lower than that in the upward direction, indicating that the environment associated with biofilm formation in the downward and vertical directions is less favorable due to the influence of gravity. However, regardless of biofilm age and the direction of biofilm formation, the thickness of live cells was similar among the directions (Figs. 4a-3 and 4b-3), suggesting a difference in biofilm density according to the direction of biofilm formation. Furthermore, gravity not only affects the biovolume of live cells in biofilms, but also the biovolume of dead cells. As shown in Figs. 4a-2 and b-2, the biovolume of dead cells in the upward directions was significantly higher compared to the downward or vertical direction, regardless of biofilm age, and was even comparable to the biovolume of live cell in that direction.

Considering bacterial count and the biovolume ratio of live to dead cells of the 46-h-old biofilms in the upward direction, dead cells as well as living cells may play an important role in dental caries development. The 46-h-old biofilms in the upward direction have the highest number of live cells, with a majority of them being *S. mutans* (Fig. 2b-2). This can increase the possibility of dental caries development as these live cells are capable of producing higher amounts of acid compared to biofilms in other directions. Furthermore, approximately 50% of the biovolume of the 46-h-old biofilm cells in the upward consists of dead cells, which is 4–5 times higher than that in the other directions (Fig. 4b-2). The increased biovolume of dead cells in the upward direction can influence the biovolume and density of the biofilms, further increasing the possibility of dental caries development. It has been well reported that diffusion of acid generated in the biofilm into the oral cavity and the penetration of saliva into the biofilm can raise the pH and inhibit the occurrence of tooth decay. However, if dead bacteria are not detached and a thicker biofilm is formed, it can hinder the diffusion of acid to the outside of the biofilms and the penetration of saliva, thereby increasing the possibility of dental caries development.

Collectively, the results of the present study showed that the direction of substratum surface could affect the bacterial proportion and cariogenic properties of the multi-species biofilm, especially in the 46-h-old biofilms. The 46-h-old biofilms formed in the upward direction exhibited the most complex structure with the highest number and biovolume of bacteria, especially *S. mutans*, and lowest pH condition, followed by that in the vertical and downward directions, which may be due to gravity. These findings may provide some insight into the clinical observation that occlusal surfaces of mandibular teeth are the most susceptible to initiation of dental caries, followed by proximal surfaces of mandibular or maxillary teeth, and occlusal surfaces of maxillary teeth.

Although the biofilm model used in this study simulated the clinical situation to some extent, it is important to consider other specialized models that simulate the oral environment. These include the artificial saliva-based flow cell model, which utilizes continuous artificial saliva as the growth medium to simulate the shear force and nutritional conditions of saliva in the oral environment, as well as microfluidic-based models. These models enable precise control over fluid flow, spatial gradients, and environmental conditions within microscale chambers, offering high-throughput screening capabilities and the ability to study biofilm formation under controlled microenvironments.

Furthermore, additional *in vivo* studies are required since observed site variations in caries susceptibility highlight the intricate interplay of numerous factors within the oral environment. Beyond the microbial composition of biofilms, other critical determinants such as stagnation, oxygen tension, substrate availability, and salivary clearance play pivotal roles in shaping caries risk at specific sites. Stagnation of saliva or food debris in anatomical niches can create localized environments conducive to biofilm formation and acidogenic activity. Variations in oxygen tension across different oral sites may also influence the metabolic activities of bacteria within biofilms, thereby impacting their cariogenic potential. Additionally, differences in substrate availability, including the presence of fermentable carbohydrates, can modulate biofilm composition and acid production. Moreover, the efficiency of salivary clearance mechanisms, such as saliva flow rate and buffering capacity, can profoundly affect local pH dynamics and remineralization processes. It's noteworthy to mention that our model lacked the presence of shear force, which is generated by saliva flow and masticatory movements and plays a crucial role in shaping biofilm architecture, microbial composition, and physiological responses.

Therefore, to comprehensively understand the differences in cariogenic biofilm formation according to substratum direction, future studies are needed to consider these multifaceted environmental factors since dental biofilms are composed of a complex microbial community, and the ecology of the mouth is complex and dynamic.

CRediT authorship contribution statement

Jaegyu Jeon: Writing – review & editing, Visualization, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis. **Hyo-Won Oh:** Writing – review & editing, Visualization, Supervision, Formal analysis, Conceptualization. **Dongyeop Kim:** Writing – review & editing, Visualization, Methodology, Formal analysis. **Hyeon-Mi Choi:** Methodology, Investigation, Formal analysis, Data curation. **Jian-Na Cai:** Writing – review & editing, Writing – original draft, Visualization, Funding acquisition, Formal analysis. **Minh-Huy Dang:** Validation, Methodology, Investigation, Formal analysis, Data curation.

Declaration of Competing Interest

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

Acknowledgments

This work was supported by the National Research Foundation of Korea grant (NRF) funded by the Korea government [RS-2023-00246619] and Scientific Research Foundation funded by Binzhou Medical University [2019KYQD40].

References

- Bernabe, E., MacRitchie, H., Longbottom, C., Pitts, N. B., & Sabbah, W. (2017). Birth weight, breastfeeding, maternal smoking and caries trajectories. *Journal of Dental Research*, 96(2), 171–178.
- Cai, J. N., Choi, H. M., & Jeon, J. G. (2021). Relationship between sucrose concentration and bacteria proportion in a multispecies biofilm. *Journal of Oral Microbiology*, 13(1), 1910443.
- Carvalho, J. C. (2014). Caries process on occlusal surfaces: evolving evidence and understanding. *Caries Research*, 48(4), 339–346.
- Dang, M. H., Jung, J. E., Choi, H. M., & Jeon, J. G. (2018). Difference in virulence and composition of a cariogenic biofilm according to substratum direction. *Scientific Reports*, 8(1), 6244.
- Foster, J. S., & Kolenbrander, P. E. (2004). Development of a multispecies oral bacterial community in a saliva-conditioned flow cell. *Applied and Environmental Microbiology*, 70(7), 4340–4348.
- Gibbons, R. J., Etherden, I., & Moreno, E. C. (1985). Contribution of stereochemical interactions in the adhesion of *Streptococcus sanguis* C5 to experimental pellicles. *Journal of Dental Research*, 64(2), 96–101.
- Han, A. F., Tsoi, J. K. H., Rodrigues, F. P., Leprince, J. G., & Palin, W. M. (2016). Bacterial adhesion mechanisms on dental implant surfaces and the influencing factors. *International Journal of Adhesion and Adhesives*, 69, 58–71.
- Heydorn, A., Nielsen, A. T., Hentzer, M., Sternberg, C., Givskov, M., Ersbøll, B. K., & Molin, S. (2000). Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology (Reading, England)*, 146(Pt 10), 2395–2407.
- Jung, J. E., Cai, J. N., Cho, S. D., Song, K. Y., & Jeon, J. G. (2016). Influence of fluoride on the bacterial composition of a dual-species biofilm composed of *Streptococcus mutans* and *Streptococcus oralis*. *Biofouling*, 32(9), 1079–1087.
- Kolenbrander, P. E., Palmer, R. J. Jr, Periasamy, S., & Jakubovics, N. S. (2010). Oral multispecies biofilm development and the key role of cell-cell distance. *Nature Reviews Microbiology*, 8(7), 471–480.
- Mei, L., Busscher, H. J., van der Mei, H. C., Chen, Y., de Vries, J., & Ren, Y. (2009). Oral bacterial adhesion forces to biomaterial surfaces constituting the bracket-adhesive-enamel junction in orthodontic treatment. *European Journal of Oral Sciences*, 117(4), 419–426.
- Nobbs, A. H., Jenkinson, H. F., & Jakubovics, N. S. (2011). Stick to your gums: mechanisms of oral microbial adherence. *Journal of Dental Research*, 90(11), 1271–1278.
- de Oliveira, R. V. D., Bonafé, F. S. S., Spolidorio, D. M. P., Koga-Ito, C. Y., Farias, A. L., Kirker, K. R., James, G. A., & Brighenti, F. L. (2020). *Streptococcus mutans* and *Actinomyces naeslundii* Interaction in Dual-Species Biofilm. *Microorganisms*, 8(2), 194.
- Palmer, R. J., Jr, Gordon, S. M., Cisar, J. O., & Kolenbrander, P. E. (2003). Coaggregation-mediated interactions of streptococci and actinomyces detected in initial human dental plaque. *Journal of Bacteriology*, 185(11), 3400–3409.
- Pandit, S., Cai, J. N., Jung, J. E., & Jeon, J. G. (2015). Effect of 1-minute fluoride treatment on potential virulence and viability of a cariogenic biofilm. *Caries Research*, 49(4), 449–457.
- Paster, B. J., Bartoszyk, I. M., & Dewhirst, F. E. (1998). Identification of oral streptococci using PCR-based, reverse-capture, checkerboard hybridization. *Methods in Cell Science*, 20, 223–231.
- Thurnheer, T., Gmür, R., & Guggenheim, B. (2004). Multiplex FISH analysis of a six-species bacterial biofilm. *Journal of Microbiological Methods*, 56(1), 37–47.
- Zheng, S., Bawazir, M., Dhall, A., Kim, H. E., He, L., Heo, J., & Hwang, G. (2021). Implication of surface properties, bacterial motility, and hydrodynamic conditions on bacterial surface sensing and their initial adhesion. *Frontiers in Bioengineering and Biotechnology*, 9, Article 643722.