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Inhibitory effect of short-chain fatty acids on growth and biofilm formation of oral streptococci

단쇄지방산에 의한 구강 연쇄상구균의 성장과 바이오필름 저해 효과

2021 년 06 월

서울대학교 대학원

치의과학과 면역 및 분자미생물학전공

박태환

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박태환의 이학석사 학위논문을 인준함
2021 년 06 월

위 원 장 박 주 홍

부위원장 한 승 현

위 원 심 재 원

Abstract

Inhibitory effect of short-chain fatty acids on growth and biofilm formation of oral streptococci

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Objectives

Oral streptococci are facultative anaerobic and gram-positive bacteria that contribute to oral diseases such as tooth decay and periodontal disease through their adhesion and biofilm-forming ability to the teeth and gum. The currently-available methods to remove bacteria or biofilm often accompany various adverse effects including increased antibiotic resistance, soft tissue damage, reduced tooth strength, and taste alterations implicating the need for an alternative, safe microbial control method. Short-chain fatty acids (SCFAs) such as acetate, propionate, or butyrate are metabolites produced by carbohydrate fermentation of commensal bacteria in the gut, and recent studies have shown that they can inhibit the growth of intestinal pathogens and modulate immune responses. However, the effects of SCFAs on oral streptococci are poorly understood. The purpose of this study was to investigate the effects of SCFAs, acetate, propionate, and butyrate, on biofilm

formation and growth of oral streptococci under various conditions mimicking oral environment.

Methods

Streptococcus gordonii was incubated in the presence or absence of various concentrations of SCFAs (acetate, propionate, and butyrate) under aerobic *versus* anaerobic and static *versus* shaking culture conditions. The bacterial growth was determined by measuring the optical density at 600 nm. The minimum inhibitory concentration/minimum bactericidal concentration test was performed under various conditions to investigate if propionate had bactericidal or bacteriostatic effects. The inhibitory effect of propionate on other oral streptococci was also investigated. On the other hand, the effect of SCFAs on the biofilm formation of various oral streptococci was examined by culturing in the presence of various concentration of SCFAs under aerobic static condition for 24 h. The biofilm formation was examined by a crystal violet assay. To determine the underlying mechanism of SCFAs on biofilm formation, *S. gordonii* was incubated with SCFAs in the presence of absence of AI-2 inhibitor which blocks its quorum sensing, and its mRNA expression was determined using real-time PCR.

Results

Of the SCFAs tested in this study, propionate most potently inhibited the growth of *S. gordonii* under anaerobic conditions. Propionate inhibited the growth of *S. gordonii* with bacteriostatic effect. The growth of five *S. gordonii* clinical isolates was potently inhibited by propionate under anaerobic conditions. The inhibitory

effect of propionate on oral streptococci was dependent on the growth conditions. *S. sanguinis* and *S. oralis* were inhibited by propionate under anaerobic conditions, whereas *S. sobrinus* and *S. mutans* were inhibited under aerobic conditions. *S. salivarius* was inhibited by propionate equally in all conditions described above. Propionate most strongly affected *S. mitis* growth in shaking conditions. SCFAs regulated oral streptococci biofilm formation. In particular, SCFAs inhibited *S. gordonii* biofilm formation even at early time points, but did not affect pre-formed biofilms. SCFAs also inhibited *S. gordonii* competence-stimulating peptide (CSP) gene expression level.

Conclusions

Propionate potently inhibited the growth of *S. gordonii* under anaerobic conditions. In addition, propionate inhibited the growth of all oral streptococci and the degree of inhibition was dependent on growth conditions. Among the oral streptococci, SCFAs most potently inhibited *S. gordonii* biofilm. Moreover, acetate inhibited *S. gordonii* biofilm through inhibition of CSP. Collectively, SCFAs may have the potential as a biocompatible anti-bacterial or anti-biofilm agent for the control of oral streptococci.

Keywords : Oral streptococci, Short-chain fatty acid, bacterial growth, biofilm
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CONTETS

Chapter I. Introduction

Oral diseases such as dental caries and periodontitis are among the most common diseases globally, putting tremendous burdens on public health sectors. At least 20% of dental caries are left untreated, and periodontitis cases soared up to 743 million worldwide [1]. Moreover, oral diseases even lead to chronic systemic disease such as diabetes mellitus, cardiovascular disease, cerebrovascular disease, and dementia [2]. Expenses used for treating oral diseases globally were predicted to be $442 billion US dollars per year [3]. Much of the blame for these relentless oral diseases are attributed to oral microorganisms in which *streptococcus* species are the main culprits [4, 5].

Oral streptococci are gram-positive bacteria that exist in the human body such as oral cavity and upper respiratory tract. *Streptococcus* species are classified based on their hemolytic property [6]. The oral streptococci, in particular, are grouped in the viridans group because they exhibit partial hemolysis when cultured on blood agar plates. Oral streptococci are facultative anaerobic bacteria which display exceptional survivability by forming biofilms in the oral cavity. In addition, oral streptococci form complex relationships among other bacterial species. For instance, *Actinomyces naeslindi* confers protection to *S. gordonii* by detoxifying hydrogen peroxide released from host cells [3]. In contrast, *S. sanuinis* and *S. gordonii* inhibit the growth of *S. mutans*

by secreting hydrogen peroxide [7]. Furthermore, *S. gordonii* physical interaction with *Porphyromonas gingivalis* reduced *P. gingivalis* adhesion to the oral niche [8].

Many infectious diseases such as periodontitis and dental caries are principally caused by biofilms [9]. Biofilms are aggregates of microorganisms in which cells are embedded in a matrix of extracellular polymeric substances (EPS) that adhere to numerous surfaces [10]. The EPS matrix permits aggregating with other bacteria [11]. Biofilm development are largely classified into three steps : attachment, maturation, and dispersion [12]. The major roles of biofilm are to provide antibiotics resistance, environmental stress tolerance, and immune response evasion [13]. In case of oral cavity, oral biofilms are formed by multispecies microorganisms. Oral streptococci are the early colonizers that initiate biofilm formation in the oral cavity. Matured biofilms provide conducive habitats for other oral microorganisms such as *Candida albicans, Fusobacterium nucleatum,* and *P. gingivalis*. This multispecies biofilm complex capitalizes the host by enhancing the aforementioned characteristic including antibiotic resistance, stress tolerance, and immune evasion [14, 15].

Quorum sensing is a bacterial cell to cell communication system that regulate the production and response to autoinducers (AIs) which are extracellular signaling molecules [16]. AIs regulate multiple bacterial behaviors such as bioluminescence, virulence

factors, and biofilm formation [17–19]. Of many known AIs, autoinducer–2 (AI–2) is one of most common signaling molecule shared among gram–positive and gram–negative bacteria which is synthesized by the LuxS system [20]. In oral streptococci, *Streptococcus gordonii, Streptococcus mutans,* and *Streptococcus salivarius*, have unique quorum sensing molecules called competence–stimulating peptide (CSP). Streptococci bacterial behaviors such as biofilm formation are mainly regulated by AI–2 and CSP [21–24].

As of today, some methods to remove oral microorganisms employ both physical and chemical techniques. Physical removal such as tooth brushing, scaling, and root planing are commonly used to physically detach oral microbes which are further subject to anti–caries agents [25, 26]. Chemical removal such as antibiotics, calcium hydroxide, and chlorhexidine are used in dental practice and root canal dressing, eliminating oral pathogens [27, 28]. However, the current methods leave unignorable side effects in which physical removal cause gingival recession [29] while chemical methods weaken dentine strength, generate antibiotic–resistant bacteria , cause dry mouth, and induce taste change [30–32].

Short–chain fatty acids (SCFAs) are bacterial metabolites which are the by–products of dietary fiber and carbohydrate fermentation in human [33]. The representative SCFAs, acetate, propionate and butyrate, account for more than 95 % of all SCFAs and exist in a

molar ratio of roughly 60:20:20, respectively, in the colon [34]. SCFAs concentrations in the human body range from 70 mM to 140 mM [35]. In oral cavity, 6.3–16.2 mM acetate, 1.2–3.1 mM propionate, and 0.0–0.4 mM butyrate are present [36]. Many commensal bacteria produce SCFAs in diverse pathways. The type of SCFAs produced depend on the specific bacterial species. For example, acetate and propionate are mainly produced by Bacteroidetes phylum, and butyrate is produced by Firmicutes phylum [37]. SCFAs are also produced by oral microorganisms. In the oral cavity, SCFAs play important roles in commensal dental biofilm formation [38]. SCFAs serve as energy sources for colonocytes subsequently leading to increased mucus and antimicrobial peptide production [39]. Moreover, SCFAs have antimicrobial and anti-biofilm effect on pathogenic bacteria. For instance, acetate inhibits the growth of *Escherichia coli* via forced acetate metabolism and methionine biogenesis [40, 41]. Butyrate limits the growth of *Helicobacter pylori* by destroying its cell envelope [42]. Propionate attenuates *Staphylococcus aureus* skin infection via growth inhibition [43]. In addition, SCFAs inhibit numerous biofilms such as biofilms formed by *Actinomyces naeslundii* [43] and *Staphylococcus epidermidis* [44]. However, the effect of SCFAs on the growth and biofilm formation of streptococci have not extensively been studied. In this study, we investigated the effects of SCFAs on the growth and biofilm formation of oral streptococci.

Chapter II. Materials and Methods

2.1. Reagent and chemicals

SCFAs, sodium acetate, sodium propionate, and sodium butyrate, and D-ribose were obtained from Sigma-Aldrich Inc. (St. Louis, MO, United States). SCFAs were dissolved in distilled water, and filtered with a syringe filter (0.2 um) obtained from Corning (Corning, NY, USA). CSP of *S. gordonii* was obtained from the Korea Atomic Energy Research Institute (Jeongeup, Korea). Todd-Hewitt (TH), yeast extract, and Bacto agar were obtained from BD Biosciences (Franklin Lakes, NJ, USA). Crystal violet was obtained from Sigma-Aldrich Inc.

2.2. Bacteria strains and culture conditions

S. gordonii CH1 was provided by American Type Culture Collection (Manassas, VA, USA). *S. gordonii* clinical isolates were obtained from the Korean Collection for Oral Microbiology (Chosun university, Gwangju, Korea). *S. sanguinis* KCTC 3284, *S. oralis* KCTC 13048, and *S. salivarius* KCTC 3960, and *S. mutans* KCTC 3065 were provided by Korean Collection for Type Cultures (Jeongeup, Korea). *S. mitis* SF-100 was obtained from the Korea Atomic Energy Research Institute (Jeongeup, Korea). *S. sobrinus* 6715-7 were obtained from professor Bong-Kyu Choi (Seoul national university, Seoul, Korea). These oral streptococci were cultured in Todd Hewitt broth containing 0.5% yeast extract at 37℃.

The anaerobic culture condition was carried out in the anaerobic workstation (Whitley DG250, Don Whitley Scientific, England, UK).

2.3. bacterial growth and biofilm formation

The experimental scheme is summarized in Fig. 1. single colony of bacteria was incubated overnight in each respective broth media. The culture was diluted 1:100 to fresh medium, in the presence of various concentrations of SCFAs (0, 12.5, 25, 50 or 100 mM) in 15 ml conical tube (SPL life science, Gyenggi, Korea). Bacteria were incubated 37℃, with or without shaking, and in the presence or absence of oxygen. Optical density at 600 nm was measured using a spectrophotometer (SPARK TECAN, Zurich, Switzerland). To determine the effect of propionate on pH, pH indicator strips (Whatman pH indicators, GE Healthcare, Chicago, USA) were used to measure the pH of culture media. For biofilm formation, the overnight bacteria were diluted 1:100 to fresh media and incubated until they reached the mid-log phase. The bacteria were cultured in the presence of various concentrations of SCFAs in 96-well plates (Eppendorf, Hamburg, Germany) at 37℃ for 24 h. The biofilm formation was evaluated by crystal violet assay. Briefly, Biofilms were washed with phosphate-buffered saline (PBS) once and stained with 1% crystal violet for 20 min. After incubation, biofilms were washed with PBS once and solubilized with the dissociation buffer. The dye intensity of solubilized biofilms was measured using a spectrophotometer at 600 nm.

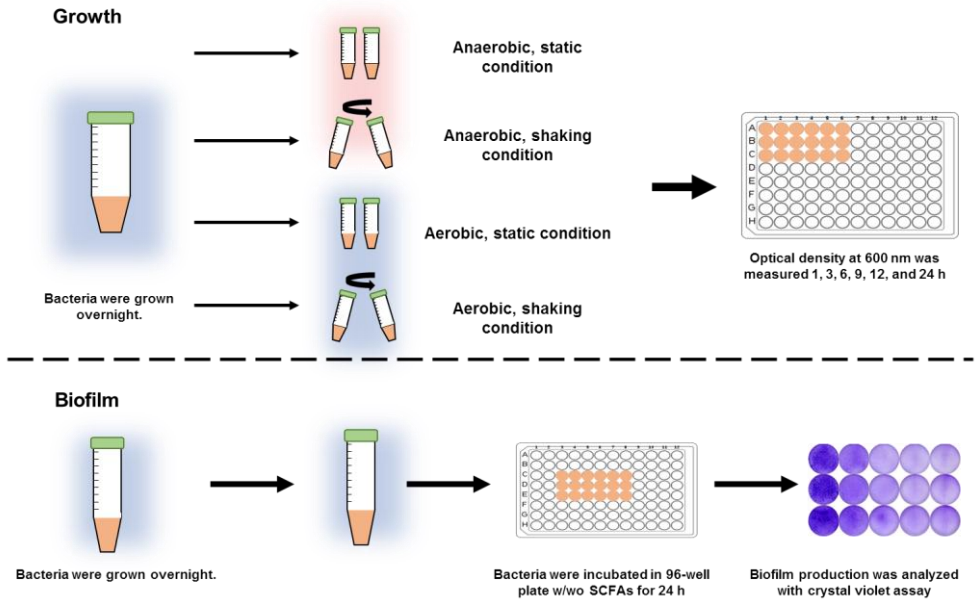

Fig. 1. Experimental scheme of bacterial growth and biofilm formation experiments. A single colony of bacteria was incubated overnight in respective media. Overnight bacteria were cultured in various concentrations of SCFAs under indicated conditions. Biofilm formation was determined by incubating the bacteria with various concentrations of SCFAs under anaerobic static condition. Bacterial growth and biofilm were determined by reading the optical density at 600 nm.

2.4. Minimum inhibitory concentration/minimum bactericidal concentration (MIC/MBC) test

The MIC/MBC test was performed as previously described at Clinical and Laboratory Standards Institute (CLSI) with minor modifications. Overnight bacteria were diluted 1:100 with fresh media and incubated in the presence of serially diluted SCFAs (0, 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, or 500 mM) for 24 h. MIC was defined as the minimum concentration of SCFA needed to inhibit visible growth of bacteria after 24 h. Bacteria that displayed no visible growth were reinoculated in fresh media free of SCFAs to further evaluate MBC. MBC was defined as the minimum concentration of SCFAs that inhibited visible growth of bacteria even after reinoculation.

2.5. Real-time PCR

S. gordonii was incubated in 6-well plates in the presence or absence of acetate (50 mM) at 37℃ for 24 h under aerobic static condition. The bacteria pellet was harvested, and RNA was isolated by using lysozyme, trizol, chloroform, and ethanol. Total RNA was cleaned up using RNeasy mini kit (Qiagen, Hilden, Germany). Complementary DNA (cDNA) was synthesized from total RNA using random hexamers and reverse transcriptase (Promega, Madison, USA). Real-time PCR was conducted with SYVR premix Ex Taq (Takara Bio, Shiga, Japan) using Step one plus (Applied Biosystems, Foster City, USA). Sequences of specific primers

(Table 1) were designed using sequence data acquired from the National Center for Biotechnology Information (NCBI)

Table 1. Primers used in this study

Gene name	Sequence
ComD (F)	5'-AAATGCACATCTTAATAGCTTTGCTAGT-3'
ComD (R)	5'-CATATTGTTCACGAGCAGACTTCAG-3'
ComE (F)	5'-TTGAGTCAGACGAGGTAAATCAACTT-3'
ComE (R)	5'-GCATAGGGATTATGTTGGCGTATA-3'
16s rRNA (F)	5'-AAGCAACGCGAAGAACCTTA-3'
16s rRNA (R)	5'-GGTTACAACAATCCGCTCGC-3'

2.6. Statistical analysis

All experiments were repeated at least three times. The mean value ± standard deviation (SD) were obtained from triplicate samples. Statistical significance was determined with student's *t*-test. Asterisk (*) indicates the treatment groups that were significantly different from the control group at $P<0.05$.

Chapter III. Results

3.1. The growth of *S. gordonii* is inhibited by SCFAs under anaerobic condition

To investigate the effect of SCFAs on the growth of *S. gordonii*, *S. gordonii* CH1 was incubated in the presence of various concentrations of acetate, propionate, or butyrate under anaerobic static condition, and the growth was determined by measuring their optical density. The growth of *S. gordonii* was inhibited by all SCFAs in which acetate had the lowest growth inhibition while propionate most potently inhibited the growth of *S. gordonii* (Fig. 2 A–C). Next, to identify if propionate's inhibitory effect was affected by the growth conditions, *S. gordonii* was cultured in the presence of propionate under anaerobic static or shaking and aerobic static or shaking conditions (Fig. 3A). In general, propionate more effectively inhibited the growth of *S. gordonii* under anaerobic conditions compared to aerobic conditions. To investigate if propionate had bacteriostatic or bactericidal effects, the MIC/MBC test was carried out with the microdilution method under various conditions. All conditions displayed MIC at 500 mM propionate (Fig. 3B). In addition, MBC test showed that propionate had no bactericidal effects (Fig. 3C). Moreover, the extracellular medium pH in the presence of propionate was maintained above 6 throughout the experiments. (Fig. 4). These results indicate that

propionate most potently inhibits the growth of *S. gordonii* among the SCFAs, and the growth inhibition by propionate was stronger under anaerobic condition than the aerobic condition.

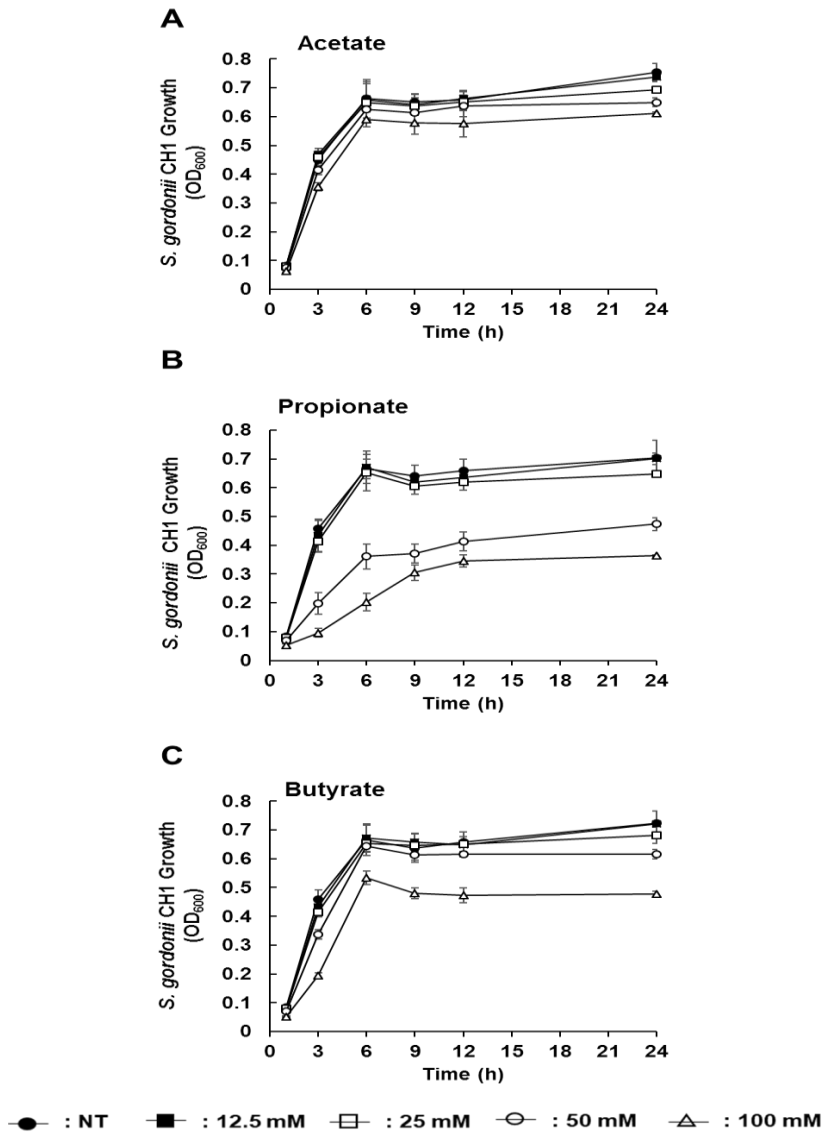

Fig. 2. Short-chain fatty acids inhibit the growth of *S. gordonii* under anaerobic condition. *S. gordonii* was incubated with the indicated concentrations of (A) acetate, (B) propionate or (C) butyrate under anaerobic condition. Optical density at 600 nm was determined at 1, 3, 6, 9, 12, and 24 h. Data shown are the mean values ± SD of triplicate samples and represent one of three similar results. NT, Non-Treated.

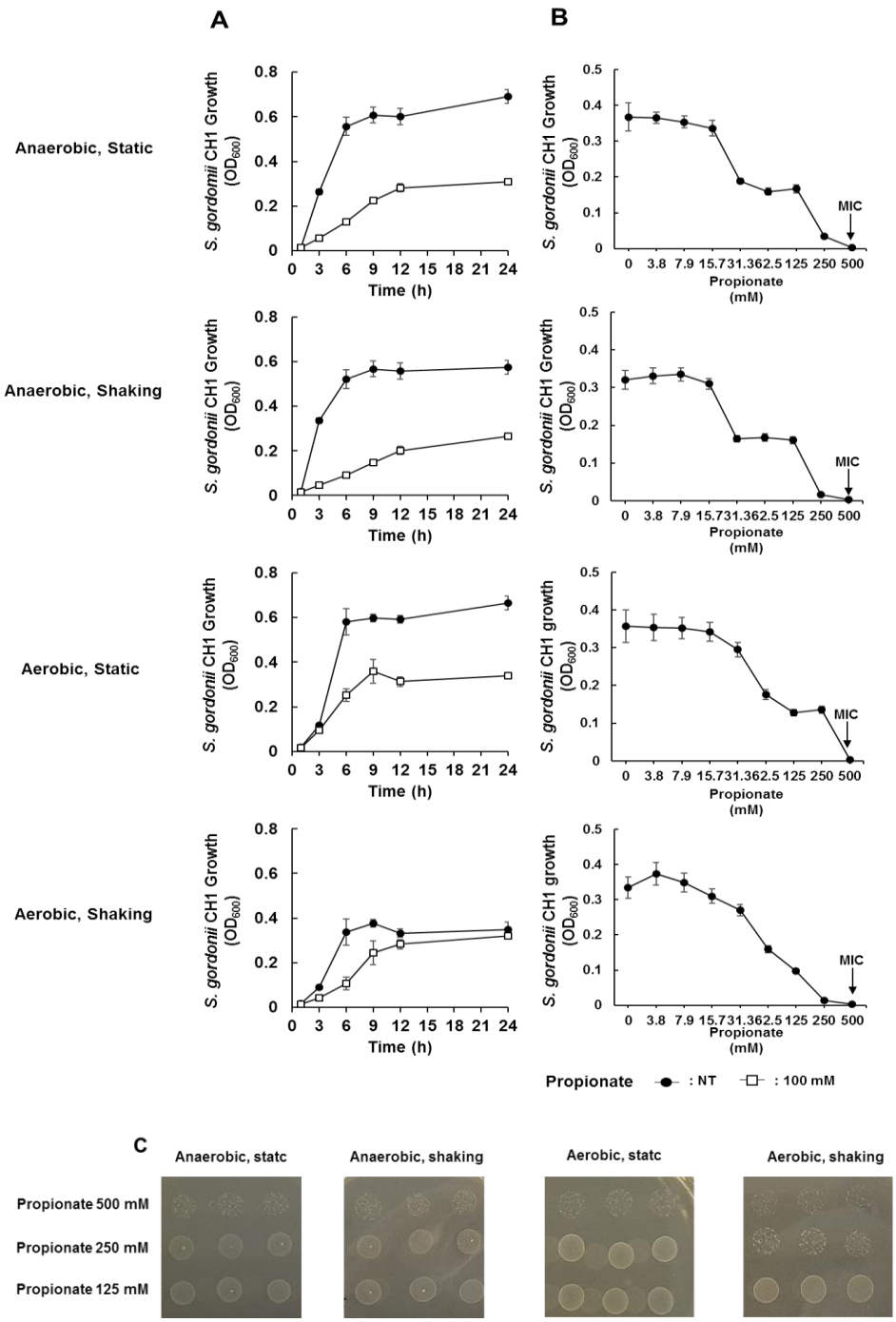

Fig. 3. Propionate potently inhibits the growth of *S. gordonii* under anaerobic condition as a bacteriostatic effect. *S. gordonii* was incubated in the presence or absence of propionate (100 mM) under (A) anaerobic static condition, anaerobic shaking condition, aerobic

static condition, and aerobic shaking condition. The MIC/MBC test was experimented using microdilution method with propionate under (B,C) anaerobic static, anaerobic shaking, aerobic static, and aerobic shaking conditions. (B) The MIC, which indicate complete growth inhibition, is shown with a black arrow. (C) The MBC was determined as 125, 250, or 500 mM of propionate after inoculation in propionate-free agar plate. Optical density was determined at 600 nm at 1, 3, 6, 9, 12, and 24 h. Data shown are the mean values ± SD of triplicate samples and represent one of three similar results. NT, Non-Treated.

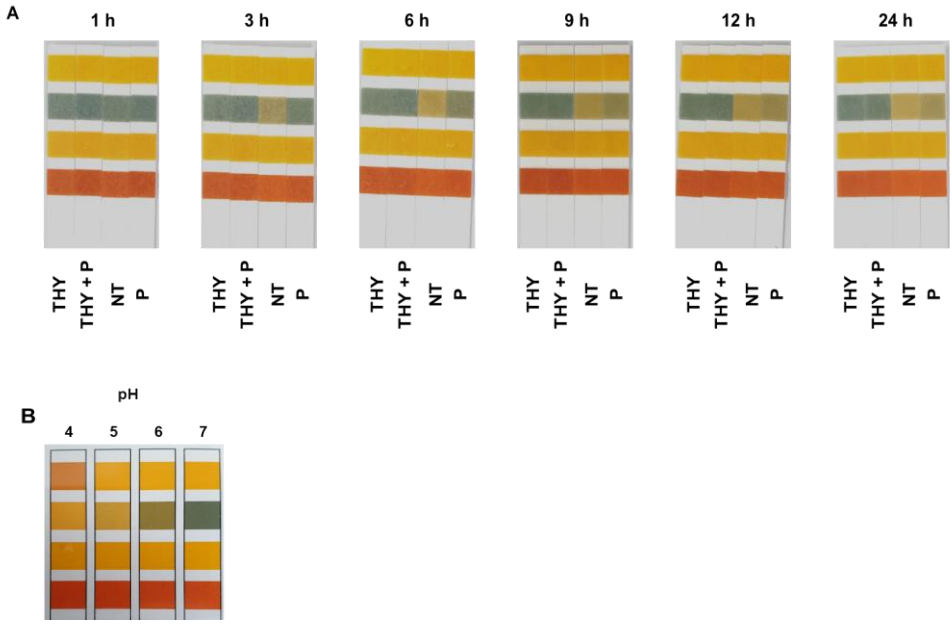

Fig. 4. Propionate does not affect the pH of the culture medium. The pH of extracellular medium determined (A) when *S. gordonii* was incubated in the presence or absence of 100 mM propionate under anaerobic conditions. (B) The reference pH strip indicates pH 4−7. Data represent one of three similar results. NT, Non−Treated. P, propionate.

3.2. Propionate most potently inhibits the growth of *S. gordonii* clinical isolates under anaerobic condition

To generalize the inhibitory effect of propionate on the growth of *S. gordonii, S. gordonii* clinical isolates were cultured in the presence of propionate under various conditions (Fig. 5A). The growth of *S. gordonii* clinical isolates were all inhibited by propionate under anaerobic condition. Interestingly, the inhibitory effect of propionate on the growth of *S. gordonii* clinical isolates was greatly reduced under aerobic conditions. Especially, *S. gordonii* KCOM 2867, *S. gordonii* KCOM 1851, *S. gordonii* KCOM 1967 were more resistant to propionate effect under aerobic conditions. As shown in Figure 5B, growth inhibition by propionate at 24 h was apparent under anaerobic conditions. These results suggest that the growth of *S. gordonii* is generally inhibited by propionate under anaerobic conditions.

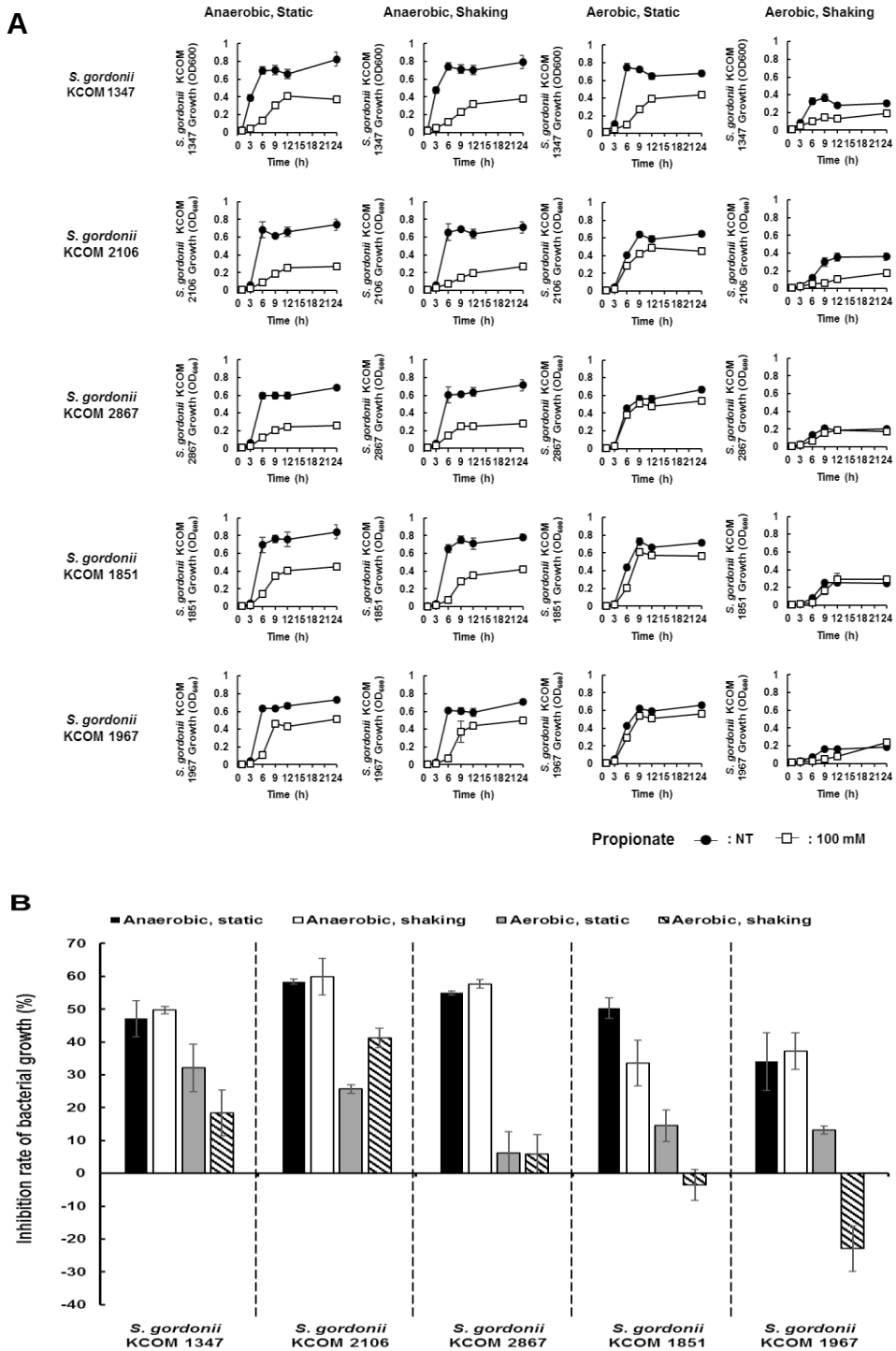

Fig. 5. Propionate potently inhibits the growth of *S. gordonii* clinical isolates under anaerobic condition. (A) *S. gordonii* clinical isolates were incubated in the presence or absence of propionate (100 mM)

under anaerobic static condition, anaerobic shaking condition, aerobic static condition, or aerobic shaking condition. Optical density was determined at 600 nm at 1, 3, 6, 9, 12, and 24 h. (B) The inhibition rate of the growth of *S. gordonii* clinical isolates at 24 h was calculated by comparing the control group and propionate-treated group. Data shown are the mean values ± SD of triplicate samples and represent one of three similar results. NT, Non-Treated.

3.3. Growth condition determines the growth inhibition by propionate on oral streptococci

The effect of propionate on *streptococcus* species was investigated using oral streptococci. The oral streptococci, (Fig. 6A) *S. sanguinis* KCTC 3284, (Fig. 6B) *S. oralis* KCTC 13048, (Fig. 6C) *S. salivarius* KCTC 3960 (Fig. 6D) *S. mitis* SF-100, (Fig. 6E) *S. sobrinus* 6715-7, and (Fig. 6F) *S. mutans* KCTC 3065 were cultured in the presence of propionate under various conditions. The inhibitory effect of propionate on the growth of oral streptococci was dependent on growth conditions. The growth of *S. sanguinis* and *S. oralis* were inhibited by propionate under anaerobic conditions, whereas *S. sobrinus* and *S. mutans* were inhibited under aerobic conditions. In addition, *S. salivarius* showed that the inhibitory effect of propionate on growth was similar under all conditions. In case of *S. mitis*, shaking conditions was the determining factor of propionate's growth inhibition where statically grown *S. mitis* was more affected by propionate. Interestingly, *S. sanguinis* and *S. mitis* growth in the presence of propionate under aerobic shaking condition were slightly increased. These results indicate that the growth inhibition by propionate in oral streptococci was dependent on growth conditions.

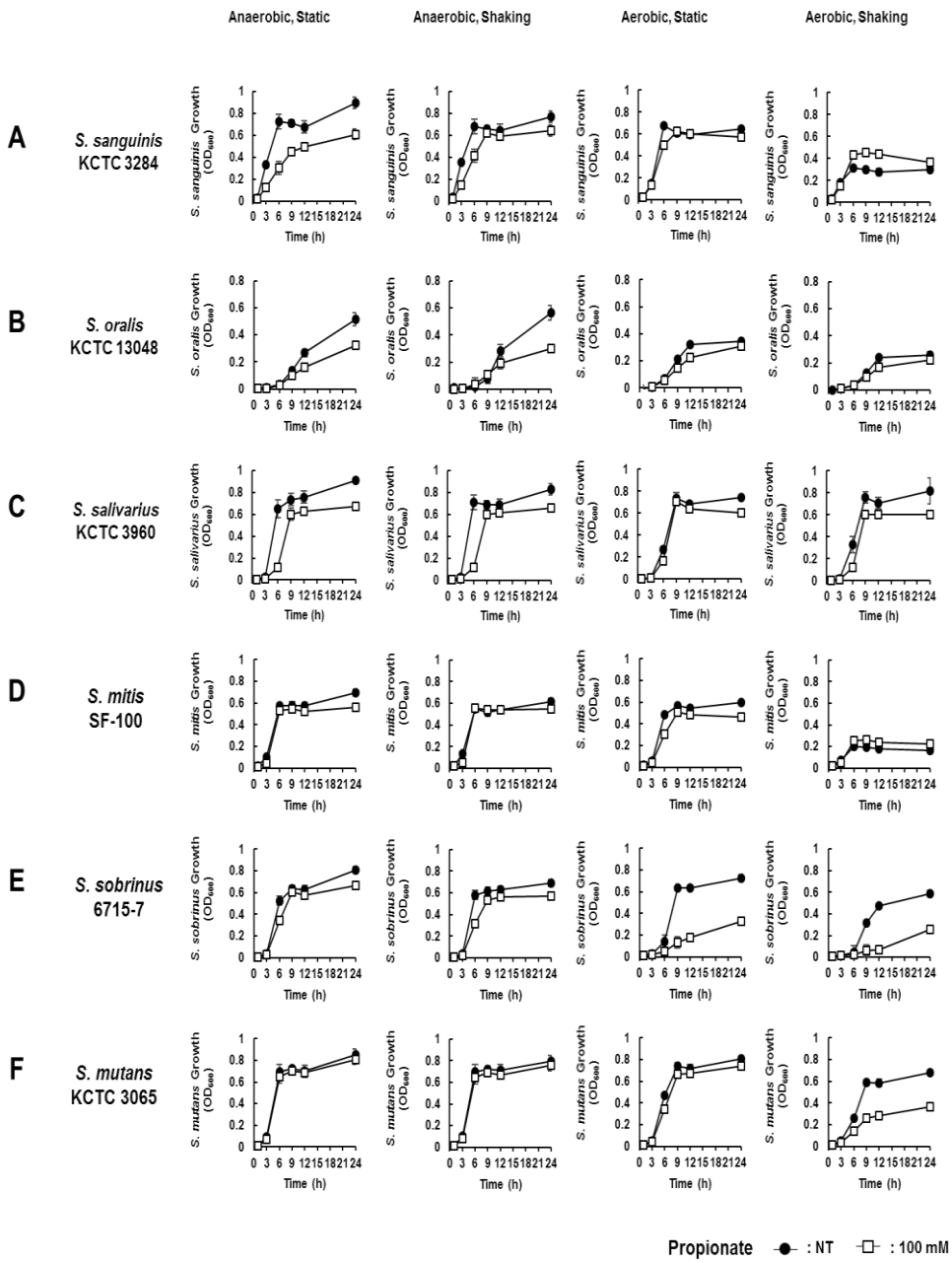

Fig. 6. Growth inhibition of oral streptococci is dependent on the growth condition. (A) *S. sanguinis* KCTC 3284, (B) *S. oralis* KCTC 13048, (C) *S. mitis* SF100, (D) *S. salivarius* KCTC 3960, (E) *S. sobrinus* 6715-7, and (F) *S. mutans* KCTC 3065 was incubated in the presence or absence of propionate (100 mM) under anaerobic

static condition, anaerobic shaking condition, aerobic static condition, or aerobic shaking condition. Optical density was determined at 600 nm at 1, 3, 6, 9, 12, and 24 h. Data shown are the mean values ± SD of triplicate samples and represent one of three similar results. NT, Non-Treated.

3.4. SCFAs inhibit *S. gordonii* biofilm formation

Next, the effect of SCFAs on *S. gordonii* biofilm formation was determined. *S. gordonii* CH1 biofilm formed in the presence of various doses of SCFAs under aerobic static condition in polystyrene plates at 37℃ for 24 h (Fig. 7A). The effect of SCFAs on the growth of *S. gordonii* under aerobic static condition for 24 h was determined (Fig. 7B). SCFAs inhibited *S. gordonii* biofilm formation in a dose-dependent manner. Collectively, S. *gordonii* biofilm formation was inhibited by propionate and butyrate more effectively than acetate. However, acetate did not affect the growth of *S. gordonii* under aerobic static condition, but the highest concentration, 50 mM, of propionate and butyrate slightly suppressed the growth of *S. gordonii*. The effect of SCFAs on oral streptococci, (Fig. 8A) *S. sanguinis,* (Fig. 8B) *S. oralis*, (Fig. 8C) *S. salivarius*, (Fig. 8D) *S. mitis,* (Fig. 8E) *S. sobrinus*, and (Fig. 8F) *S. mutans* biofilm formation was conducted. *S. sanguinis, S. oralis,* and *S. salivarius* biofilm formation was inhibited by acetate. In contrast, propionate enhanced *S. sanguinis, S. salivarius,* and *S. sobrinus* biofilm formation. Butyrate inhibited *S. sanguinis* and *S. oralis* biofilm formation while enhancing *S. mutans* biofilm formation. These results suggest that SCFAs most potently inhibited *S. gordonii* biofilm formation among the oral streptococci.

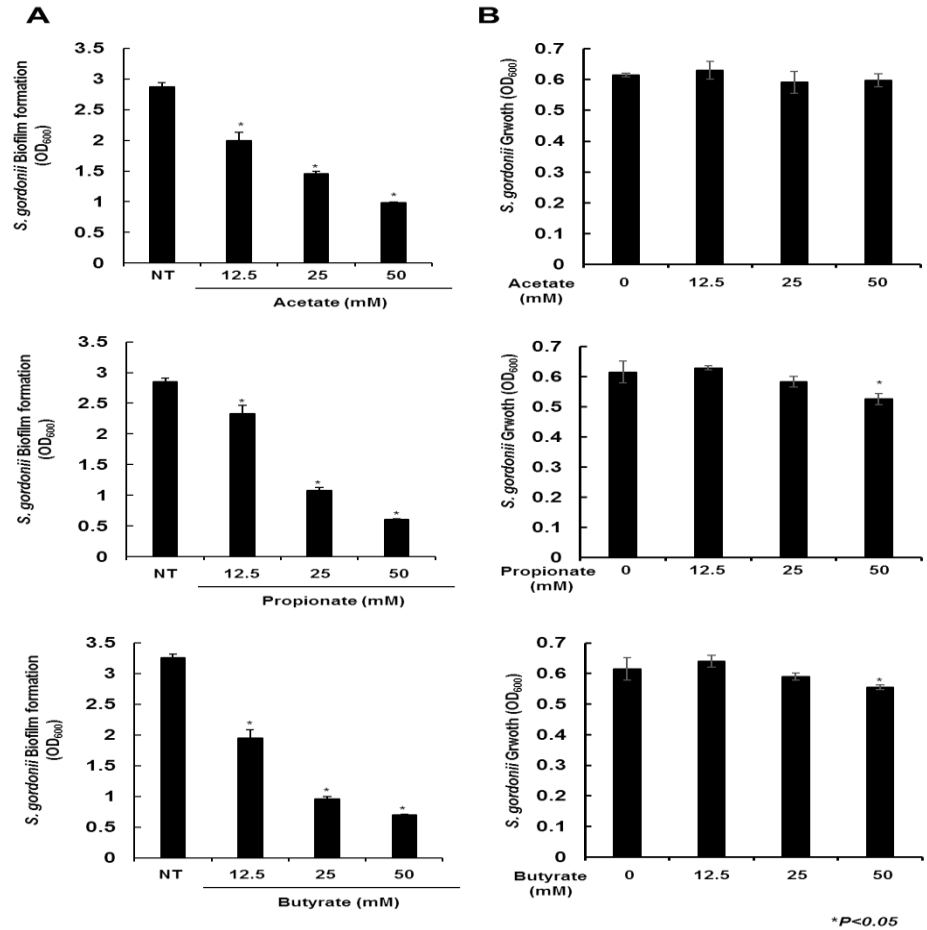

Fig. 7. Short-chain fatty acids inhibit *S. gordonii* biofilm formation. (A) *S. gordonii* (1×10^6 CFU/ml) was incubated in polystyrene 96-well plates at 37℃ for 24 h under aerobic static condition in the presence of various concentrations of acetate, propionate, or butyrate (0, 12.5, 25, or 50 mM). The biofilm formation was assayed by a crystal violet assay. (B) *S. gordonii* was cultured in the presence of various concentrations of acetate, propionate, or butyrate (0, 12.5, 25, or 50 mM) in conical tubes at 37℃ for 24 h. Data shown are the mean values ± SD of triplicate samples and represent one of three similar results. Statistical significance was calculated by paired student's *t*-test. *, $P<0.05$. NT, Non-Treated.

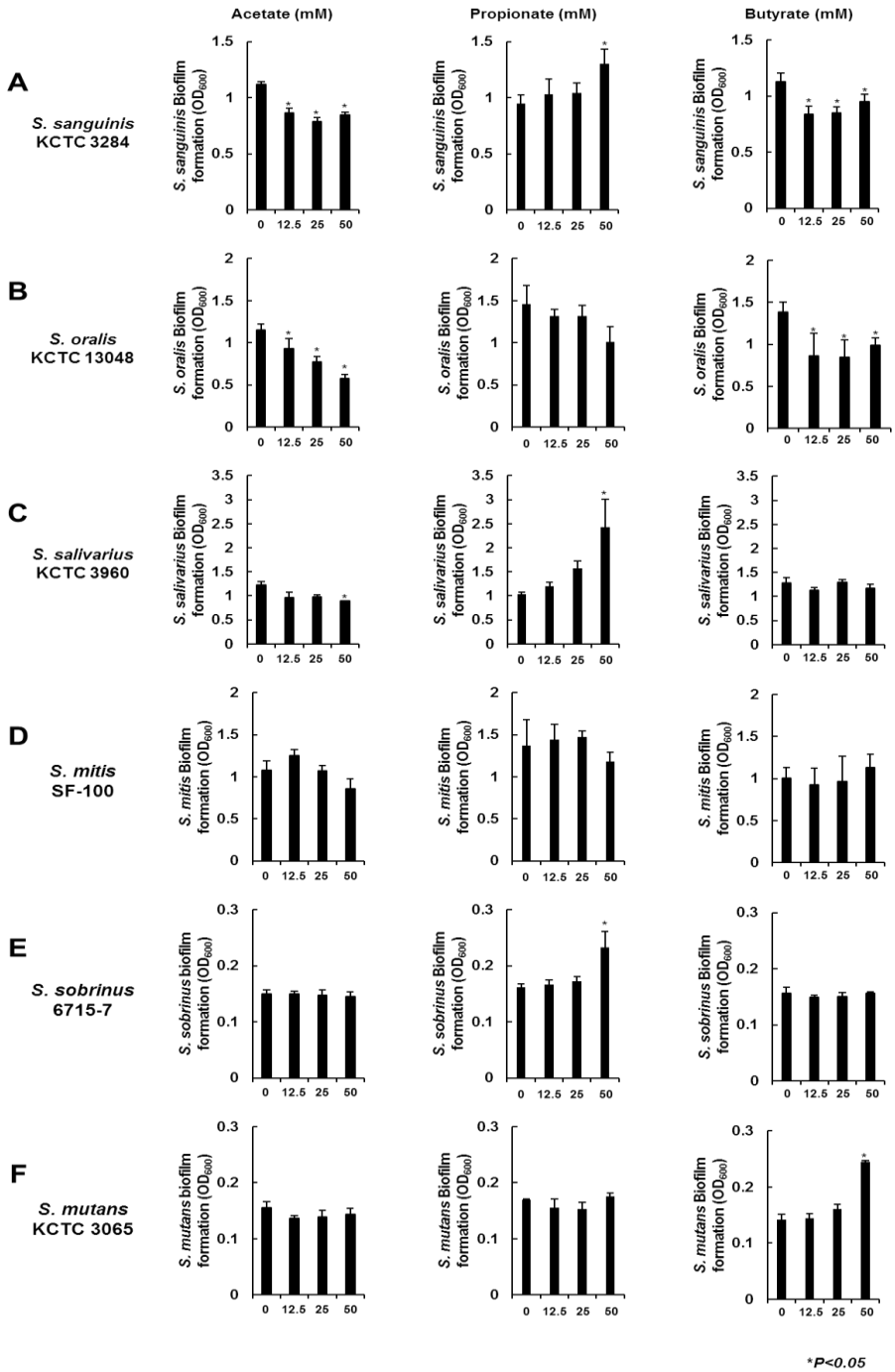

Fig. 8. The effect of SCFAs on biofilm formation is dependent on oral *streptococcus* species. Oral streptococci, (A) *S. sanguinis* KCTC 3284, (B) *S. oralis* KCTC 13048, (C) *S. salivarius* KCTC 3960, (D) *S. mitis* SF100, (E) *S. sobrinus* 6715-7, and (F) *S.*

mutans KCTC 3065 (1×10^6 CFU/ml) were cultured in the presence of various doses of acetate, propionate, or butyrate (0, 12.5, 25, or 50 mM). The amount of biofilm was assayed by a crystal violet assay. Data shown are the mean values ± SD of triplicate samples and represent one of three similar results. Statistical significance was calculated by paired student's *t*-test. *, $P<0.05$.

3.5. SCFAs inhibit *S. gordonii* biofilm formation at early time point and do not affect the disruption of biofilm

To determine when SCFAs affect the biofilm forming progress, *S. gordonii* biofilm formations were measured at various time points. *S. gordonii* biofilms were formed in polystyrene plates at 37℃ for 3, 6, 9, 12, or 24 h in the presence of various doses of SCFAs under aerobic static condition. The inhibitory effect of SCFAs on *S. gordonii* biofilm formation was observed at 6 h and lasted up to 24 h (Fig. 9A–C). In addition, to investigate if SCFAs induce biofilm disruption on pre–formed biofilms, pre–formed *S. gordoniii* biofilms were treated with SCFAs. *S. gordonii* biofilms were formed in the absence of SCFAs for 24 h. After incubation, the pre–formed *S. gordonii* biofilms were treated with various doses of SCFAs and incubated for 24 h. As shown in Figure 10A, B, and C, the pre–formed *S. gordonii* biofilms were not affected by SCFAs. These results indicate that SCFAs inhibited *S. gordonii* biofilm formation at an early time point and did not disrupt the pre–formed *S. gordoii* biofilms.

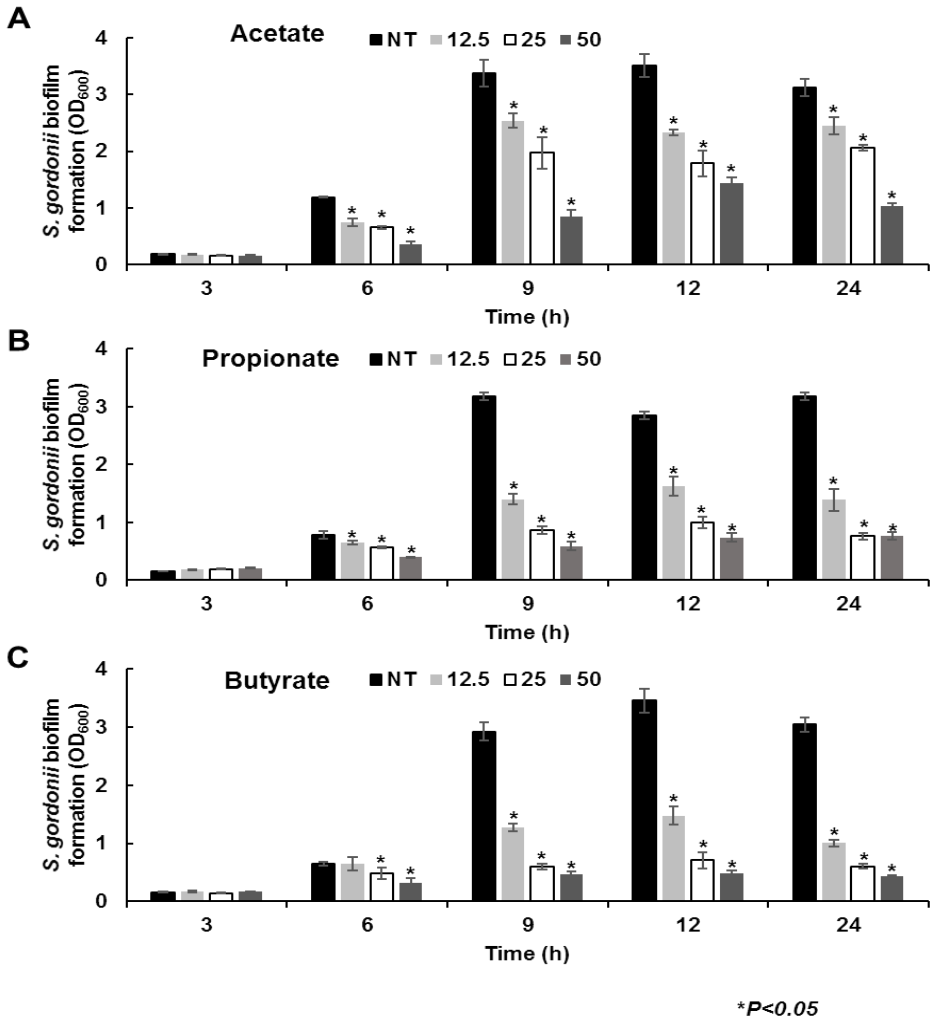

Fig. 9. Acetate inhibits the *S. gordonii* biofilm formation via inhibition at early stage. *S. gordonii* (1×10^6 CFU/ml) was incubated in the absence of SCFAs in 96-well plates at 37℃ for 3, 6, 9, 12, or 24 h in the presence of various concentrations of (A) acetate, (B) propionate, or (C) butyrate (0, 12.5, 25, or 50 mM). The amount of biofilm was assayed by a crystal violet assay. Data shown are the mean values ± SD of triplicate samples and represent one of three similar results. Statistical significance was calculated by paired student's *t*-test. *, $P<0.05$. NT, Non-Treated.

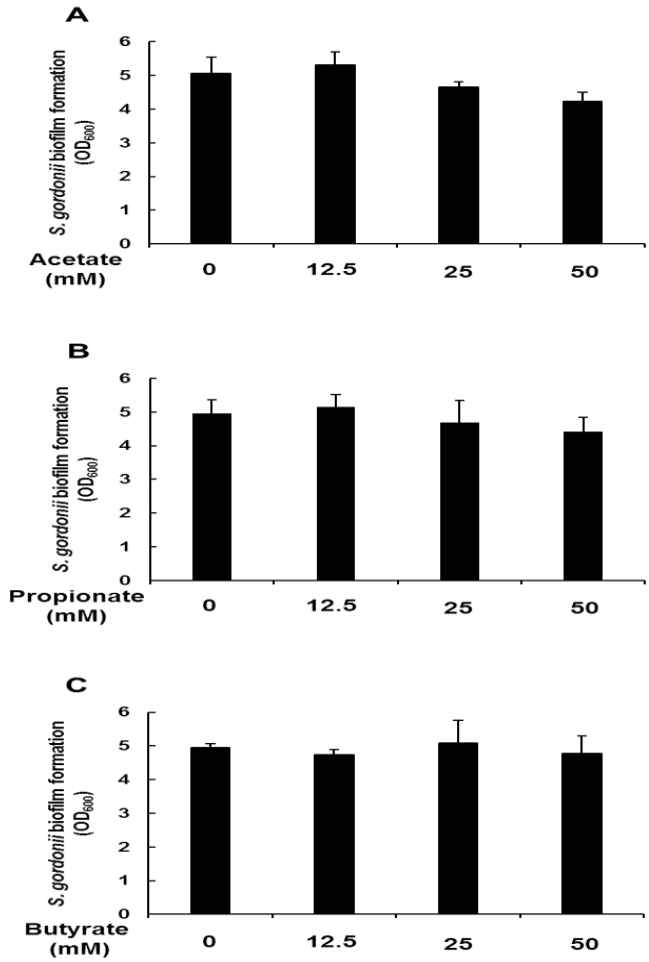

Fig. 10. SCFAs do not affect the disruption of *S. gordonii* biofilm. *S. gordonii* (1×10^6 CFU/ml) was incubated without SCFAs in 96-well plates at 37℃ for 24 h under aerobic static conditions. After incubation, pre-formed biofilms were washed using 1X PBS, and incubated in the presence of (A) acetate, (B) propionate, or (C) butyrate (0, 12.5, 25, or 50 mM) at 37℃ for 24 h. The amount of biofilm was assayed by a crystal violet assay. Data shown are the mean values ± SD of triplicate samples and represent one of three similar results. Statistical significance was calculated by paired student's *t*-test. *, $P<0.05$.

3.6. Acetate inhibits *S. gordonii* biofilm formation via inhibition of CSP pathway

Acetate was selected to investigate the anti-biofilm effect without affecting growth. To seek the underlying mechanism of biofilm inhibition of acetate, we tested if acetate would affect key quorum sensing system, AI-2 and CSP. AI-2 and CSP are well known to promote the biofilm formation of *S. gordonii* [23, 24]. To test if acetate inhibited biofilm formation through interference of AI-2 production, *S. gordonii* was incubated with acetate and D-ribose, an AI-2 inhibitor, for 24 h (Fig. 11A). D-ribose did not prevent acetate's biofilm inhibition indicating that acetate did not directly interact with AI-2. The mRNA expression levels of CSP-corresponding genes, *comD* and *comE*, were determined to see if acetate affected *S. gordonii's* gene regulation (Fig. 11B). The expression levels of *comD* and *comE* were decreased by acetate in a dose-dependent manner. Moreover, to check if decreased mRNA expression level reflect protein expression, *S. gordonii* was cultured in the presence or absence of CSP and acetate (Fig. 12). *S. gordonii* biofilm was increased by CSP in a dose-dependent manner. However, acetate treated *S. gordonii* biofilm was not significantly increased by CSP compared to non-treated group. These results indicate that acetate inhibited *S. gordonii* biofilm formation via inhibition of CSP pathway.

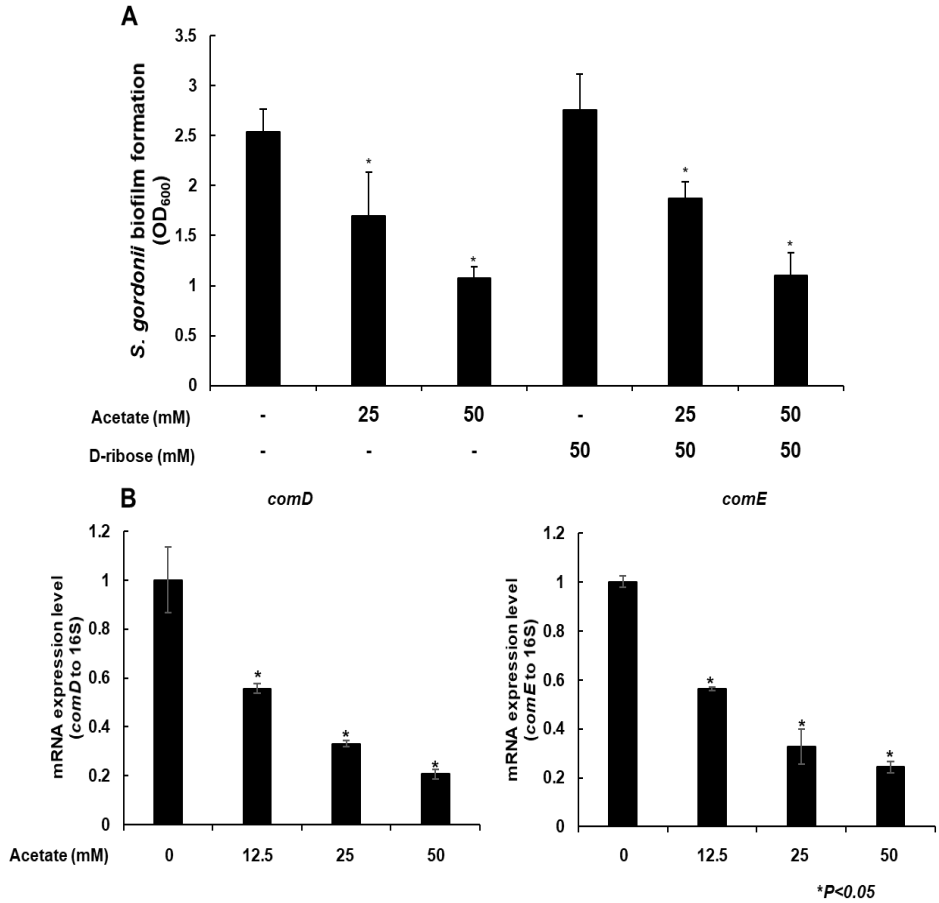

Fig. 11. Acetate inhibits expression of *comD* and *comE* which are involved in CSP pathway. (A) *S. gordonii* (1×10^6 CFU/ml) was cultured in the presence of various concentrations of acetate (0, 25, or 50 mM) and D-ribose (50 mM) in 96-well plates at 37℃ for 24 h under aerobic static conditions. (B) *S. gordonii* (1×10^6 CFU/ml) was cultured in the presence or absence of acetate (0, 12.5, 25, or 50 mM) in polystyrene 6-well plates at 37℃ for 24 h under aerobic static conditions. After incubation, biofilms in each well were harvested, and RNA was isolated from bacterial lysate using lysis buffer including lysozyme, ethanol, trizol, and isopropanol. mRNA expression levels of *comD, comE* and 16S rRNA genes were

measured by real-time PCR. The amount of biofilm was assayed by a crystal violet assay. Data shown are the mean values ± SD of triplicate samples and represent one of three similar results. Statistical significance was calculated by paired student's *t*-test. *, $P<0.05$.

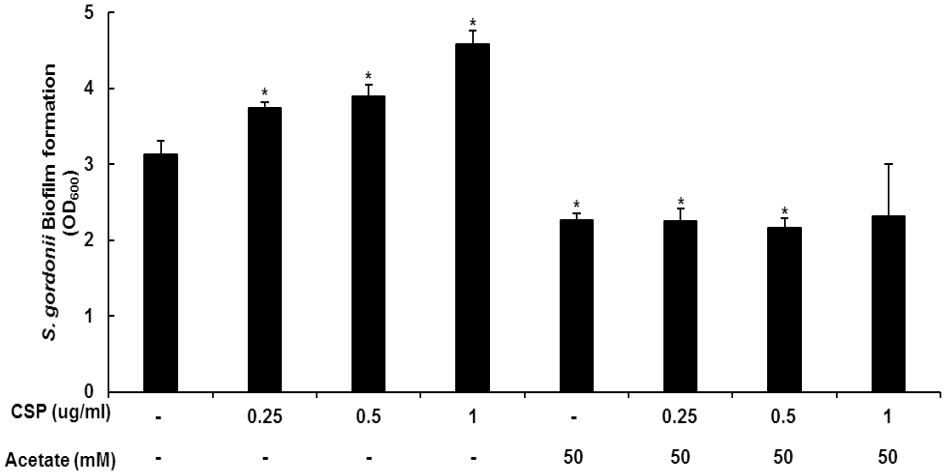

Fig. 12. Acetate inhibits *S. gordonii* biofilm formation via inhibition of CSP. *S. gordonii* (1 × 10^6 CFU/ml) was incubated in the presence or absence of CSP (0, 0.25, 0.5, or 1 ug/ml) and acetate (50 mM) in 96-well plates at 37℃ for 24 h under aerobic static conditions. The amount of biofilm was assayed by a crystal violet assay. Data shown are the mean values ± SD of triplicate samples and represent one of three similar results. Statistical significance was calculated by paired student's *t*-test. *, $P<0.05$.

Chapter IV. Discussion

The oral streptococci are the major bacteria that form vigorous biofilm [45]. As early colonizers, oral streptococci provide habitats for other oral microorganisms, thereby forming multispecies biofilm [46]. In the present study, propionate potently inhibited the growth of *S. gordonii* under anaerobic conditions, and these effects were generalized by the growth inhibition of clinical isolates. Moreover, the growth of oral streptococci was inhibited by propionate, but the inhibitory effect of propionate was strongly dependent on the growth conditions. In biofilm formation, SCFAs have successfully inhibited *S. gordonii* biofilm, whereas the effect on other oral streptococci were either weak or even enhanced their formation. Interestingly, acetate inhibited *S. gordonii* biofilm formation by interfering with the quorum sensing system such as CSP.

Propionate potently inhibited the growth of *S. gordonii* under anaerobic conditions. However, such inhibitory effect was weak in aerobic condition compared with that in anaerobic condition. *S. gordonii* can be grown in both aerobic and anaerobic conditions. In *S. gordonii*, oxygen level determines various phenotypic characteristic such as the hydrogen peroxide production, autolysis, and arginine deiminase system [47–49]. These phenotypic changes correlate with energy metabolism and regulation of intracellular pH [50, 51]. Moreover, propionate is known to have bacteriostatic effect on

various bacterial species. The growth of *Salmonella enterica, Listeria monocytogenes,* and *S. aureus* were inhibited by propionate [43, 52, 53]. Notably, the inhibitory effect of propionate on growth was caused by interference with energy metabolism, methionine biogenesis pathway, and increased intracellular pH [41, 54, 55]. Therefore, oxygen-induced phenotype changes may dictate the impact of propionate on *S. gordonii*.

In oral streptococci, the inhibitory effect of propionate depends on the growth conditions. The response to oxygen in oral streptococci varied among bacterial species. *S. sanguinis, S. mitis, S. oralis,* and *S. salivarius* show similar responses to oxygen, including hydrogen peroxide production, acid production from carbon metabolism, and oxygen uptake [56-58]. In contrast, *S. sobrinus* and *S. mutans* show differential adaptation effect by oxygen compared to other oral streptococci, such as superoxide dismutase activity, autolysis activity, and energy metabolism [59-61]. In addition, propionate does not affect the oxygen consumption in *L. monocyrogenes* [62]. The differential inhibitory effect of propionate may be attributed to the oxygen-induced differential adaptation in oral streptococci.

This study showed that SCFAs inhibited *S. gordonii* biofilm formation starting at 6 h and even at 24 h. In contrast, SCFAs did not affect pre-formed biofilms. Acetate inhibited *S. gordonii* biofilm without affecting its growth. Acetate inhibited gene expression involved in CSP pathway. Moreover, acetate inhibited the effect of

CSP in *S. gordonii* biofilm formation. In light of the fact that acetate inhibited CSP gene expression in *S. gordonii* biofilm, suppression of CSP might be involved in the action mechanism. The mechanism for anti-biofilm effect of SCFAs was investigated in other studies. SCFAs inhibited *S. enterica* biofilm formation by changing its gene expression[63]. Moreover, acetate inhibited *Pseudomonas aeruginosa* and *S. aureus* biofilm formation [64]. In contrast, *Bacillus subtilis* and *S. aureus* biofilm formation were enhanced by acetate. Acetate increased *B. subtilis* biofilm formation by promoting quorum sensing-like activity [65]. Moreover, acetate regulated quorum-sensing molecule of *Lactobacillus plantarum,* AI-2, via electrostatic interaction with quorum-sensing molecule receptor [64]. In *S. gordonii,* CSP mutant showed defective biofilm formation [66]. Collectively, among the SCFAs, acetate may regulate *S. gordonii* biofilm through regulation of quorum sensing activity.

We investigated the effects of SCFAs on the growth and biofilm formation of oral streptococci. SCFAs had bacteriostatic effect, and the degree of this effect was dependent on the growth conditions. Moreover, all SCFAs tested in this study inhibited *S. gordonii* biofilm formation, but only acetate did not affect its growth. These results suggest that SCFAs could be useful for controlling oral streptococci and serve as anti-biofilm agents to interfere oral biofilm formation.

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국문초록

단쇄지방산에 의한 구강연쇄상구균의 성장과 바이오필름 저해 효과

박태환

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1. 목적

구강 연쇄상구균은 그람양성세균으로 높은 부착능력과 생물막 형성 능력으로 구강 내 질환인 충치, 치주 질환 병소에서 주로 발견되는 박테리아이다. 구강 연쇄상구균는 호기, 혐기 환경 모두에서 생존하며 생물막 형성이 가능하다. 그러나, 현재의 구강 연쇄상구균을 비롯한 구강 내 미생물의 제어 방법은 치아의 강도 감소, 미각의 변화 등의 부작용을 내재하고 있어 차세대 미생물 제어 방법이 필요하다. 단쇄지방산은 미생물의 발효작용으로 생성되는 대사산물로 최근의 연구에서는 장내 병원균의 성장을 억제하고 면역반응 조절을 조절할 수

있다는 것이 밝혀져 있다. 그러나, 단쇄지방산인 acetate, propionate, 혹은 butyrate 가 구강 연쇄상구균에 대한 영향은 알려져 있지 않다. 이에 본 연구에서는 다양한 환경에서 구강 연쇄상구균이 성장할 때와 생물막형성에 대한 단쇄지방산의 영향을 알아보았다.

2. 방법

단쇄지방산이 구강 연쇄상구균 중 *Streptococcus gordonii* 의 성장에 미치는 영향을 알아보기 위해 여러 농도의 acetate, propionate, 혹은 butyrate 를 처리한 후 정적 호기성 환경에서 성장시킨 후 분광광도계로 흡광도를 측정하였다. *S. gordonii* 가 다양한 환경에서 성장할 때의 성장에 미치는 영향을 알아보기 위해 혐기와 호기 그리고 동적 또는 정적 상태에서 단쇄지방산을 처리 한 후 흡광도를 측정하였다. 또한, *S. gordonii* 임상 분리 균에 대한 단쇄지방산의 영향을 알아보기 위해 단쇄지방산을 처리 후 다양한 성장환경에서 성장시킨 후 흡광도를 측정하였다. 다른 구강 연쇄상구균들의 다양한 성장환경에서 단쇄지방산에 대해 어떠한 영향을 받는지 알아보기 위해 *Streptococcus sanguinis, Streptococcus oralis, Streptococcus mitis, Streptococcus salivarius, Streptococcus sobrinus,* 와 *Streptococcus mutans* 를 혐기와 호기 그리고 동적 또는 정적 상태에서 단쇄지방산을 처리한 후 성장시킨 후 흡광도를 측정하였다. 단쇄지방산이 정균적 혹은 살균적 작용을 하는지 보기 위해 *S. gordonii* 에서 minimum inhibitory concentration (MIC)/minimum bacteriacidal concentration (MBC) 테스트를 진행하였다. 이에 더해 생물막 형성에 대한 단쇄지방산의 영향을 알아보기 위해 경구 연쇄상구균을 여러 농도의 단쇄지방산과 함께 생물막형성을 진행하였다. 그 중 *S. gordonii* 를 대표로 하여 단쇄지방산이 *S. gordonii* 에 초기 부착, 생물막 형성 혹은 형성된 생물막에 대한 영향을 확인하기 위해 단쇄지방산이 처리된 *S. gordonii* 에 생물막을 각 시간별로 정량 하였고 생물막을 형성한 뒤

단쇄지방산을 처리하여 생물막을 정량 하였다. 단쇄지방산이 *S. gordonii* 의 생물막 형성 저해 기전을 알기 위해, 쿼럼센싱 인자인 AI-2 억제제를 단쇄지방산과 동시 처리하였고, 쿼럼센싱에 관련된 유전자에 대한 실시간 연쇄중합반응을 실시하였다.

3. 결과

단쇄지방산인 acetate, propionate, 혹은 butyrate 는 모두 *S. gordonii* 의 성장을 억제하였지만, propionate 가 *S. gordonii* 의 성장을 가장 효과적으로 억제하였다. 다양한 성장 환경 중 정적 혐기 상태일때의 억제효과가 가장 컸다. 또한, propionate 의 혐기환경에서의 억제효과는 *S. gordonii* 의 임상 분리 균 5 종에서도 동일한 효과를 보였다. 다른 구강 연쇄상구균에 대한 propionate 의 성장 억제 효과는 구강 연쇄상구균의 성장 환경에 따라 달라 졌다. *S. gordonii, S. sanguinis, 와 S. oralis* 는 혐기 환경에서 가장 크게 성장이 억제되었다. *S. salivarius* 의 경우에는 혐기와 호기 정적 또는 동적 상태 모두 균일한 성장 감소를 보였으며 *S. mitis* 는 동적 상태일 때 성장이 억제되었다. *S. sobrinus* 와 *S. mutans* 는 호기성 환경에서 성장이 억제되었다. 또한, MIC/MBC 테스트 결과 propionate 는 살균적 작용을 하지 않고 정균적 작용을 통해 성장을 억제함을 나타냈다. 단쇄지방산의 의한 경구 연쇄상구균의 생물막 형성에 대한 영향은 다양하게 나타났다. *S. gordonii* 의 생물막 형성의 경우에는 acetate, propionate, butyrate 모두에게서 억제 되었으나 성장에 영향을 주지 않고 생물막을 억제시키는 것은 acetate 였다. Acetate, propionate, butyrate 는 *S. gordonii* 의 생물막 형성을 초기단계에서부터 억제하였고, 이미 형성된 생물막에서는 아무런 효과를 끼치지 않았다. 또 한, acetate 의 *S. gordonii* 생물막 억제 기전은 쿼럼센싱 인자인 CSP 를 억제하는 것으로 나타났다.

4. 결론

본 연구에서는 단쇄지방산인 acetate, propionate, butyrate 가 경구 연쇄상구균인 *S. gordonii* 의 성장을 억제할 수 있음을 보여주었다. 그 중 propionate 가 가장 강하게 *S. gordonii* 의 성장을 억제하였다. Propionate 는 *S. gordonii* 뿐만 아니라 다른 구강연쇄상구균의 성장을 억제할 수 있고 이러한 억제 효과가 다양한 성장 상태에 따라 달라 질 수 있음을 보여주었다. 또 이러한 특징은 실제 환자에서 분리된 균주에서도 동일하게 나타났다. 구강 연쇄상구균의 생물막 형성에 있어서 단쇄지방산은 다양한 효과를 보였다. *S. gordonii* 생물막 억제에서는 쿼럼센싱 인자를 억제하였다. 따라서 단쇄지방산은 경구 연쇄상구균에 성장과 생물막형성의 저해제로서 사용될 수 있을 것이라고 예상된다.

주요어 : 구강 연쇄상구균, 단쇄지방산, 미생물 성장, 바이오필름
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