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Original Article

구강내 그람 양성세균에 대한 프로바이오틱 후보인 *Streptococcus salivarius*의 항균 활성

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Antimicrobial activity of candidate probiotic *Streptococcus salivarius* against Gram-positive bacteria in oral cavity

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Objectives: The aim of this study is to investigate antimicrobial activity in isolated *Streptococcus salivarius* against Gram-positive bacteria related oral diseases.
Methods: *S. salivarius* was used in G2, G7, K12, and ATCC 7073 strains and tryptic soy broth supplemented with glucose was cultivated. *Actinomyces israelii*, *Actinomyces viscosus*, and *Enterococcus faecalis* were cultivated with brain heart infusion broth. *Streptococcus mutans* and *Streptococcus sobrinus* were maintained using tryptic soy broth. The antimicrobial activity of *S. salivarius* was performed by minimum inhibitory concentration using the spent culture medium.
Results: All *S. salivarius* have antimicrobial activity against Gram-positive bacteria in oral cavity. When comparing antimicrobial activity, *S. salivarius* G2 and G7 as isolated strain showed stronger antimicrobial activity against Gram-positive microbe than type K12 strain.
Conclusions: *S. salivarius* G2 and G7 have strong antimicrobial activity and may be prevent oral disease by Gram-positive bacteria in oral cavity.

Key Words: Antimicrobial activity, Gram-positive bacteria, Probiotics, Salivaricin, *Streptococcus salivarius*

Introduction

The infectious diseases in oral cavity can be divided two legions that it is a gingival and dental disease, and these infectious diseases are related oral biofilm that exists multi-species[1]. The biofilm of a healthy person is multi-species balance with high percentage of commensal bacteria. However, when this balance is disrobed and certain bacteria increase, it leads to oral disease[2]. *Actinomyces israelii* and *Actinomyces naeslundii* are considered with gingivitis basis on the epidemiological studies[3,4]. *Streptococcus mutans* and *Streptococcus sobrinus* are known to be cariogenic bacteria[5]. These bacteria have characteristics of acidogenesis and aciduricity, by which dental caries is induced[6]. Enterococcus faecalis, the predominant human enterococcus, has been related to oral diseases, such as endodontic infections, periodontitis, and peri-implantitis by characteristics of antibiotics resistance[7,8].

Streptococcus salivarius is Gram-positive facultative anaerobe[9], and act ac commensal bacteria that colonize mucosal surfaces of human[10]. Furthermore, this bacterium plays impor-

tant ecological roles that form a barrier against pathogens and reduce their adhesion and colonization[11]. Also, some strains are probiotic intended for use in the oral cavity[12-14]. Bacteria produce and use a substance called bacteriocin to compete for habitat between bacteria, and *S. salivarius* also produce bacteriocin-like inhibitory substances[15]. To compete better in the oral ecosystem, *S. salivarius* produce different kinds of lantibiotics such as salivaricin A, salivaricin B, salivaricin 9, and salivaricin G32[16-18]. The characteristics of these salivaricins contain lanthinonie and methyllanthionine, and *S. salivarius* is one of lantibiotics[17].

In this study, we investigated antimicrobial activity of different strains of *S. salivarius* isolated from healthy Korean subjects.

Materials and Methods

1. Bacterial species and cultivation

Streptococcus salivarius used in this study are two isolated, probiotic, and type strain. Isolated stains are *S. salivarius* G2 and G7 (formerly KCOM 2122 and 2137) and was kindly donated from Green store Inc. (Seongnam, Gyeonggi, Korea). As comparative strains, *S. salivarius* K12 and ATCC 7073 (type stain) were used.

2. Susceptibility assay

The antibacterial activity of *S. salivarius* against *A. israelii*, *A. viscosus*, *E. faecalis*, *S. mutans*, and *S. sobrinus* was evaluated by a minimum inhibitory concentration using a microdilution methods according to methods recommended by Clinical and Laboratory Standards Institute (CLSI)[19]. A milliliters of *S. salivarius* (1×10^7 bacteria/ml) was inoculated into 10 ml TSBG, and the bacterial suspension was incubated for 24 h in an aerobic condition. The suspension was centrifuged at 5,000 × g for 10 min, and the supernatant was transferred into a new 15 ml conical tube (SPL Life Sciences, Gyeonggi, Korea). The preparation was filtered with 0.22 μm of a polyvinylidene fluoride (PVDF) filter (Millipore, Billerica, MA, USA). The filtered supernatant as a spent culture medium (SCM) was used to susceptibility assay. 180 μl of TSBG was dispensed into 96-well plate (SPL Life Sciences, Gyeonggi, Korea). The SCM was added into 1st well containing the fresh medium and performed 2-fold serial dilution to the 11th column. *A. israelii*, *A. viscosus*, *E. faecalis*, *S. mutans*, and *S. sobrinus* were counted with a bacterial counting chamber (Marienfeld Superior, Lauda-Königshofen, Germany) and adjusted 2×10^6 bacteria/ml with the broth for each stain. The prepared *S. mutans* suspension (20 μl) was inoculated into the well containing the mixed media. The plate was incubated at 37℃ in an aerobic incubator. The bacterial growth was measured using optical density at 660 nm of wavelength by a microplate reader (BioTek, Winooski, VT, USA).

3. Statistical analysis

The data was obtained through experiment of three times in duplicate and analyzed Kruskal-Wallis test and Mann-Whitney U test using IBM SPSS statistics Ver. 23 (IBM, Armonk, NY, USA). *P*-values less than 0.05 were considered statistically significant.

Results

1. Antimicrobial activity against *A. israelii*

The antimicrobial activity of *S. salivarius* against *A. israelii* was investigated by minimum inhibitory concentration using a microdilution method according to recommended by CLSI. The SCM of *S. salivarius* type strain significantly inhibited the growth of *A. israelii* at 2-fold dilution ($P<0.05$). The SCM of *S. salivarius* G2 and G7 significantly inhibited the growth of *A. israelii* at 8-fold dilution and completely inhibited above 4-fold dilution ($P<0.05$). Finally, the SCM of *S. salivarius* K12 significantly inhibited the growth of *A. israelii* at 4-fold dilution and completely inhibited at 2-fold dilution ($P<0.05$) (Fig. 1).

2. Antimicrobial activity against *A. viscosus*

Next, in experiment of the susceptibility test of *A. viscosus*, The SCM of type strain significantly inhibited the growth of *A.*

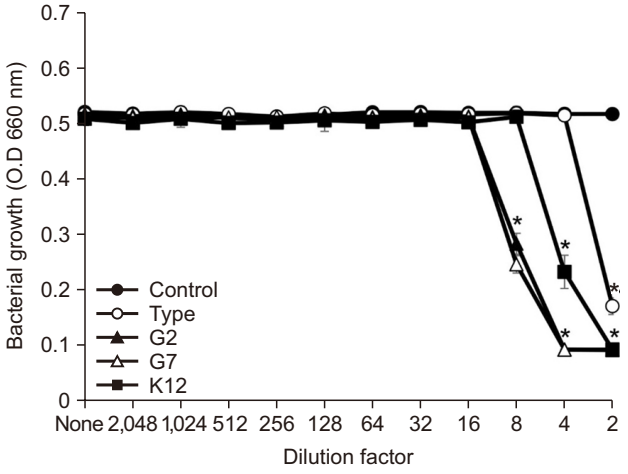

Fig. 1. Susceptibility assay of *A. israelii* for the spent culture medium of *S. salivarius*. *A. israeliis* was cultured in BHI broth and inoculated into TSB. After cultivating overnight, susceptibility test of *A. israelii* for the SCM of *S. salivarius* was performed according to the protocol of CLSI. The experiments were performed three times in duplicate and the representative data express mean and standard deviation. *Significance compared to untreated control bacteria ($P<0.05$).

viscosus at 2-fold dilution ($P<0.05$). The SCM of G2 and G7 strain significantly inhibited the growth of *A. viscosus* at 8-fold dilution and completely inhibited above 4-fold dilution ($P<0.05$). Finally, the SCM of K12 strain significantly inhibited the growth of *A. viscosus* at 4-fold dilution and completely inhibited at 2-fold dilution ($P<0.05$) (Fig. 2), As shown Fig. 2, the antimicrobial activity was strong in the order of G7, G2, K12 and type strain.

3. Antimicrobial activity against *E. faecalis*

When the SCM was investigated antimicrobial activity against *E. faecalis* as apical periodontitis related bacteria, The SCM of *S. salivarius* ATCC 7073 as type strain significantly reduced the growth of *E. faecalis* at 4-fold dilution and completely inhibited the growth of at 2-fold dilution ($P<0.05$), and the SCM of *S. salivarius* K12 significantly reduced the growth at 8-fold dilution and completely inhibited at 2-fold dilution ($P<0.05$). Finally, The SCM of *S. salivarius* G2 and G7 strain significantly reduced the growth of *E. faecalis* at 16-fold dilution and completely inhibited above 8-fold dilution ($P<0.05$) (Fig. 3).

4. Antimicrobial activity against *S. mutans*

In antimicrobial experiment using the SCM against *S. mutans* as a cariogenic bacterium, the antimicrobial activity of the SCM showed strong in the order of G2, G7, K12 and type strain (Fig. 4). The SCM of G2 and G7 showed similar antimicrobial activity against *S. mutans* growth. The SCM of *S. salivarius* ATCC 7073 as type strain significantly reduced the growth of *S. mutans* at 8-fold dilution and completely inhibited the growth of at 2-fold dilution ($P<0.05$), and the SCM of *S. salivarius* K12 significantly reduced the growth of *S. mutans* at 8-fold dilution and completely inhibited at 2-fold dilution ($P<0.05$). Finally, The SCM of *S. salivarius* G2 and G7 strain significantly reduced the growth of *S. mutans* at 16-fold dilution and completely inhibited above 8-fold dilution ($P<0.05$).

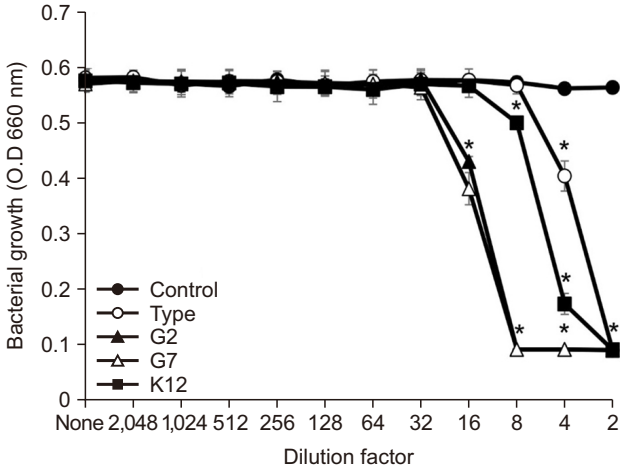

Fig. 3. Susceptibility assay of *E. faecalis* for the spent culture medium of *S. salivarius*. *E. faecaliss* was cultured in BHI broth and inoculated into TSB. After cultivating overnight, susceptibility test of *E. faecalis* for the SCM of *S. salivarius* was performed according to the protocol of CLSI. The experiments were performed three times in duplicate and the representative data express mean and standard deviation. *Significance compared to untreated control bacteria ($P<0.05$).

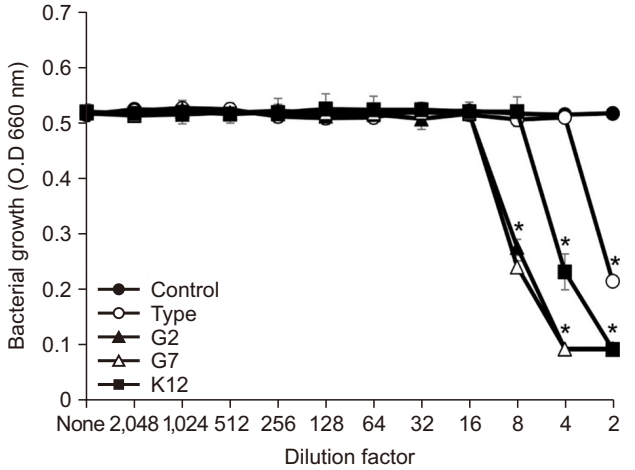

Fig. 2. Antimicrobial activity of the SCM of *S. salivarius* against *A. vicosus*. *A. viscosus* was cultivated in BHI broth and inoculated into TSB. After cultivating overnight, antimicrobial activity of the SCM of *S. salivarius* against *A. viscosus* was examined according to the protocol of CLSI. The experiments were performed three times in duplicate and the representative data express mean and standard deviation. *Significance compared to untreated control bacteria ($P<0.05$).

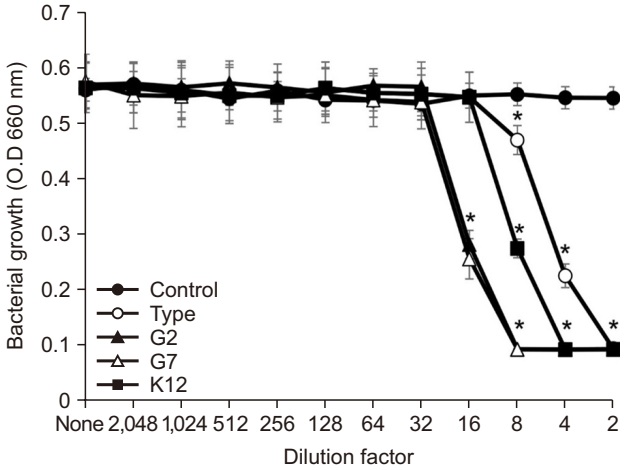

Fig. 4. Antimicrobial activity of the SCM of *S. salivarius* against *S. mutans*. *S. mutans* was cultivated in TSB. After cultivating overnight, antimicrobial activity of the SCM of *S. salivarius* against *S. mutans* was examined according to the protocol of CLSI. The experiments were performed three times in duplicate and the representative data express mean and standard deviation. *Significance compared to untreated control bacteria ($P<0.05$).

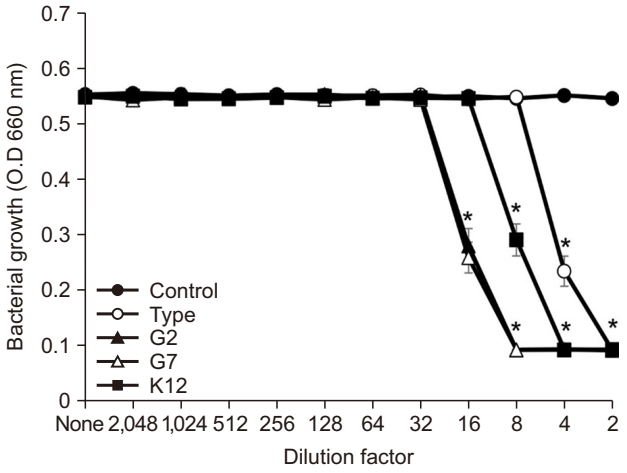

Fig. 5. Susceptibility assay of *S. sobrinus* for the spent culture medium of *S. salivarius*. *S. sobrinus* was cultured in TSB. After cultivating overnight, susceptibility test of *S. sobrinus* for the SCM of *S. salivarius* was performed according to the protocol of CLSI. The experiments were performed three times in duplicate and the representative data express mean and standard deviation. *Significance compared to untreated control bacteria ($P<0.05$).

5. Antimicrobial activity against *S. sobrinus*

The antimicrobial activity of *S. salivarius* against *S. sobrinus* was investigated by minimum inhibitory concentration using a microdilution method according to recommended by CLSI. The SCM of *S. salivarius* type strain significantly reduced the growth of *S. sobrinus* at 4-fold dilution and completely inhibited the growth at 2-fold dilution ($P<0.05$), and the SCM of *S. salivarius* K12 significantly reduced the growth of *S. sobrinus* at 8-fold dilution and completely inhibited at 2-fold dilution ($P<0.05$) (Fig. 5). The SCM of *S. salivarius* G2 and G7 significantly inhibited the growth of *S. sobrinus* at 16-fold dilution and completely inhibited above 8-fold dilution ($P<0.05$).

Discussion

S. salivarius is commensal bacteria in oral cavity and detected at high proportions in oral biofilm of healthy person. Also, this bacterium plays an important role in bacterial balance for healthy condition in oral cavity that form a barrier against pathogens and reduce their adhesion and colonization[11]. Furthermore, some strains are probiotic intended for use in the oral cavity[12-14]. In this study, the antimicrobial isolated *S. salivarius* was investigated against Gram-positive bacteria in oral cavity.

In this study, *S. salivarius* G2 and G7 are previously named KCOM 2122 and 2137, and ownership was changed from Korean collection for Oral Microbiology to Green Store Inc. These stains were isolated strains from healthy person of Korean. Also, comparative strains were used *S. salivarius* ATCC 7073 as a type strain and K12 as a known probiotic strain. All strains in this study showed the antimicrobial activity against *A. israelii*, *A. viscosus*, *E. faecalis*, *S. mutans*, and *S. sobrinus*. In comparing the antimicrobial activity against Gram-positive strains, *S. salivarius* G7, G2, K12, and ATCC 7073 showed strong in order. These data can be proven that the previous researchers predicted that the characteristics of *S. salivarius* may be different for each strain.

Most probiotics with antimicrobial activity are *Lactobacillus* species[20]. Among *Lactobacillus* spp, *L. rhamnosus*, *L. acidophilus*, and *L. casei* inhibit the growth of *S. mutans*[20]. The inhibition of *S. mutans* growth by SCM of *Lactobacillus* spp was several times higher than that of *S. salivarius*, indicating that *Lactobacillus* spp might secret stronger bacteriocins. Also, *Lactococcus* lactis inhibits cariogenic biofilm containing *S. mutans*[21]. However, in practice, it is difficult to apply to the oral cavity because it can cause dental caries due to the aciduricity of these bacteria.

The antimicrobial activity of most isolated *S. salivarius* was investigated using *Streptococcus pyogenes* as upper a respiratory disease related bacterium[15], and their antimicrobial activity was compared. Furthermore, this antimicrobial activity of *S. salivarius* is appeared by producing bacteriocin-like inhibitory substances which is called salivaricin[22]. The production of salivaricin type is different for each *S. salivarius* strain, and the antimicrobial activities and mechanisms of each salivaricin are also different[23]. The lantibotic nisin A and salivaricin 9 has antimicrobial activity through pore formation on bacterial surface. However, the salivaricin B inhibits formation of peptidoglycan layer[22]. The antimicrobial activity of *S. salivarius* was changed by culture condition[23]. In this study, TSBG was used for cultivating *S. salivarius*. The antimicrobial activity of *S. salivarius* was reduced by high glucose and low sucrose condition[15]. Also, extreme pH reduction inhibits producing salivaricin of *S. salivarius*[21].

In this study, isolated *S. salivarius* G2 and G7 satisfied one result to be probiotics for oral cavity. It is considered that additional examination such as antibiotic resistance and cytotoxicity should be investigated.

Conclusions

Based on previous studies and the data, *S. salivarius* used in this study may produce different salivaricin. Also, *S. salivarius* G2 and G7 may be a candidate bacterium for oral health.

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