

Characteristics of antimicrobial activity of *Streptococcus salivarius* K12 by culture condition

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Purpose: The purpose of this study was to examine effects of culture conditions on the growth and antibacterial activity of *Streptococcus salivarius* K12. **Materials and Methods:** *S. salivarius* K12 was cultivated in medium containing animal and plant protein or in medium of neutral and acidic conditions. The growth of *S. salivarius* K12 was measured every 2 hours by a spectrophotometer. The antimicrobial activity of *S. salivarius* K12 against *Streptococcus mutans* and *Porphyromonas gingivalis* was investigated by the susceptibility assay using the spent culture medium. **Results:** the growth of *S. salivarius* K12 showed faster in medium containing plant protein and neutral pH condition. The antimicrobial and antifungal activity of *S. salivarius* K12 appeared stronger in medium containing plant protein than animal proteins. **Conclusion:** For application of *S. salivarius* K12 to bacterial oral disease, co-substances may be needed for *S. salivarius* K12 to colonize in the oral cavity and enhance the antimicrobial activity. (*J Dent Rehabil Appl Sci* 2021;37(4):244-50)

Key words: *S. salivarius* K12; probiotics; prebiotics; antimicrobial activity

Introduction

Major diseases induced by pathogenic bacteria are dental caries and periodontitis, and the most common fungal disease in oral cavity is candidiasis.^{1,2} According to epidemiological studies, dental caries is related with *Streptococcus mutans* and *S. sobrinus*, and periodontitis is caused by obligate anaerobic Gram-negative bacteria such as *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*,³ and oral candidiasis is related with *Candida albicans*.² Although various research have been studied on the characteristics of these pathogens and antibiotics and physical therapy to treat oral diseases, the studies on

prevention of oral disease are still lacking. Recently, Oral biofilms are considered to be more important than individual characteristics of these bacteria in oral diseases, and comparative studies on characteristics between healthy biofilm and pathogenic biofilm is in progress.^{4,6} Oral biofilms are classified according to the distribution of bacteria in the biofilm, and when the distribution of pathogenic bacteria is high levels, it is recognized as a disease-related biofilm, and when the distribution of normal flora is high levels, it is considered as a healthy biofilm.¹ Therefore, the ideal prevention of oral disease is to maintain a high distribution of commensal bacteria such as viridans streptococci except mutans streptococci within the biofilm.

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Probiotics are live microorganisms and provide a health benefit to the host.⁷ Due to concerns about the emergence of antibiotics-resistant bacteria, many studies have been conducted on treatment and prevention of infectious disease using probiotics.⁸ Lactobacilli plays an important role to maintain human healthy by stimulating native immune system and protection against infection of pathogenic bacteria.⁹ However, Since Lactobacillus species have characteristics of abundant production of lactic acid and aciduricity, it is careful when applying it to oral disease. Among probiotics, *Streptococcus salivarius* K12 is Gram-positive and facultative anaerobic bacteria. This bacterium, which is isolated from healthy person, is a pioneer colonizer of oral cavity.¹⁰ *S. salivarius* K12 is also known to be a probiotic bacterium and produces bacteriocin of two class such as salivaricin A2 and salivaricin B.¹¹ Above all, *S. salivarius* K12 don't have aciduricity like Lactobacillus species. The selection of prebiotics as well as probiotics is important for treatment and prevention of disease. Prebiotics are defined "dietary prebiotics" as "a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health".¹² Eventually, prebiotics for oral cavity can selectively influence oral microbiota, and it is necessary to find suitable prebiotics to enhance the growth and antimicrobial activity of *S. salivarius* K12

Although, *S. salivarius* K12 showed antimicrobial activity against oral bacteria,¹³ There are insufficient information on the culture conditions optimized to apply *S. salivarius* K12 to the oral cavity. The purpose of this study was to investigate effects of culture conditions on the growth and antibacterial activity of *S. salivarius* K12

Materials and Methods

S. salivarius K12 was donated from Green store Inc. (Seongnam, Korea) and used in this study after isolating from Bactoblis[®] tablet. *S. salivarius* K12 was cultivated with M17 broth (BD bioscience, Sparks, USA). For susceptibility assay, a species of Gram-positive and Gram-negative bacteria were selected, respectively, and *Streptococcus mutans* ATCC 25175, *Porphyromonas gingivalis* ATCC 33277, and *Candida albicans* ATCC 10231 was selected for dental caries, periodontitis, and candidiasis, respectively. *S. mutans* was cultivated with Tryptic soy broth (BD bioscience, Spark, USA), and *P. gingivalis* was cultured with brain heart infusion (BHI; BD bioscience) supplemented with hemin (0.5 µg/ml; Sigmaaldrich Co., San Jose, USA) and vitamin K (0.2 µg/ml; Sigmaaldrich Co.). *C. albicans* was cultivated in tryptic soy broth (TSB; BD bioscience), The bacteria and fungus were maintained at 37°C in an aerobic condition (H₂ 5%, CO₂ 10%, and N₂ 85%) and an aerobic condition, respectively. In investigate characteristics of *S. salivarius* K12 by culture conditions, *S. salivarius* K12 was cultured with media of two types (medium containing animal or plant protein), and the components of two media is shown in Table 1. In another experiment, the medium containing plant protein was added with 10 mM sodium phosphate dibasic and 2 mM potassium phosphate, monobasic and then adjusted the level of pH 7.0 and pH 5.5 with lactic acid. *S. salivarius* K12 was cultured in the two conditions. The growth of *S. salivarius* K12 cultured in various conditions was measured optical density at 660 nm of wavelength every 2 hours. After collecting spent culture medium (SCM) of *S. salivarius* K12 cultured in various conditions, the SCM from various condition was investigated

Table 1. The component of media containing animal and plant protein

	Medium containing animal protein	Medium containing plant protein
Formula	Peptic digest of animal tissue Pancreatic digest of casein Yeast extract Sodium chloride Glucose Sodium phosphate dibasic	Peptic digest of soybean meal Pancreatic digest of casein Yeast extract Sodium chloride Glucose Sodium phosphate dibasic

antimicrobial activity against oral bacteria according to Clinical and Laboratory Standard Institute (CLSI) guidelines. Briefly, 180 μ l of the specific medium for each oral microbe was dispensed into 96-well plate (SPL Lifescience, Pocheon, Korea), and the SCM was added into the first row. The SCM was performed 2-fold serial dilution with micropipette. The bacterial suspensions were adjusted 2×10^6 cells/ml for *S. mutans*, 3×10^6 cells/ml for *P. gingivalis*, and 1×10^6 cells/ml of *Candida albicans* after counting the bacteria using a bacterial counting chamber (Hausser Scientific, Horsham, USA). 20 μ l of the bacterial suspension was inoculated into the prepared well with diluted SCM, and the plate was incubated at 37°C in an aerobic condition (H₂ 5%, CO₂ 10%, and N₂ 85%). The growth of bacteria was measured optical density at 660 nm of wavelength by a spectrophotometer (BioTek, Winooski, USA). The significant difference among each group was analyzed by non-parametrical analysis such as Kruskal Wallis test and Mann-Whitney U test using IBM SPSS statistics Ver. 23 (IBM, Armonk, USA). *P*-values less than 0.05

were considered statistically significant.

Results

When the growth of *S. salivarius* K12 using medium of animal protein and plant protein was investigated, although the growth of *S. salivarius* did not show significant difference between medium containing animal and plant protein, the its growth was slightly faster in presence of plant protein than animal protein (Fig. 1). The growth of *S. salivarius* K12 in Also, when the effects of pH level on the growth of *S. salivarius* K12 were examined, *S. salivarius* K12 was cultured very slow and did not reach stationary phase in the medium of pH 5.5 (Fig. 2). On other hand, the growth of *S. salivarius* K12 in pH 7.0 condition showed S shaped growth curve (Fig. 2). In case of *S. salivarius* K12 growth in two pH levels, there were a lot of growth differences to compare the antimicrobial activity of *S. salivarius* K12. Therefore, the antimicrobial activity of *S. salivarius* K12 cultured in medium containing animal or plant pro-

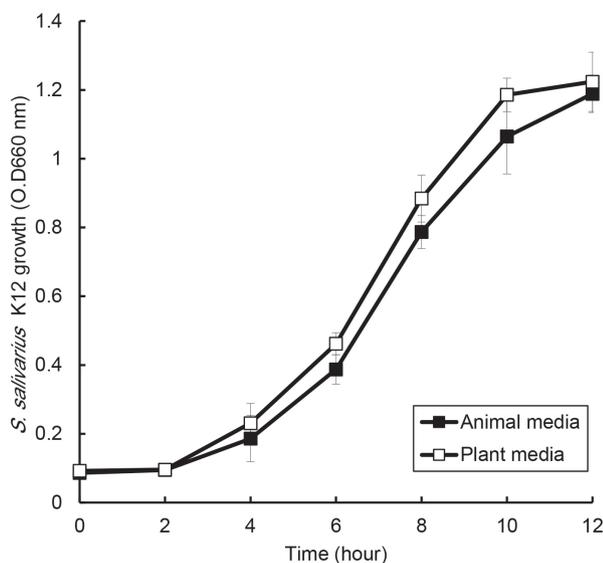


Fig. 1. Comparison of effects of animal and plant protein on the growth of *S. salivarius* K12. *S. salivarius* K12 was cultured with medium containing animal and plant protein, and the growth of this bacterium was measured every 2 hours by optical density using a spectrophotometer. The experiments were carried out three times in triplicate, and data are expressed as mean \pm standard deviation.

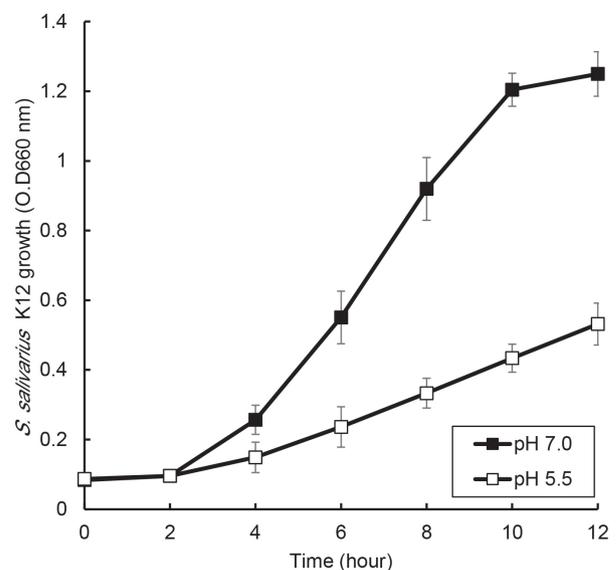


Fig. 2. Comparison of effects of pH condition on the growth of *S. salivarius* K12. *S. salivarius* K12 was cultured in medium with pH 7.0 and pH 5.5, and the growth of this bacterium was measured every 2 hours by optical density using a spectrophotometer. The experiments were carried out three times in triplicate, and data are expressed as mean \pm standard deviation. * indicates statistically significant differences between the two conditions.

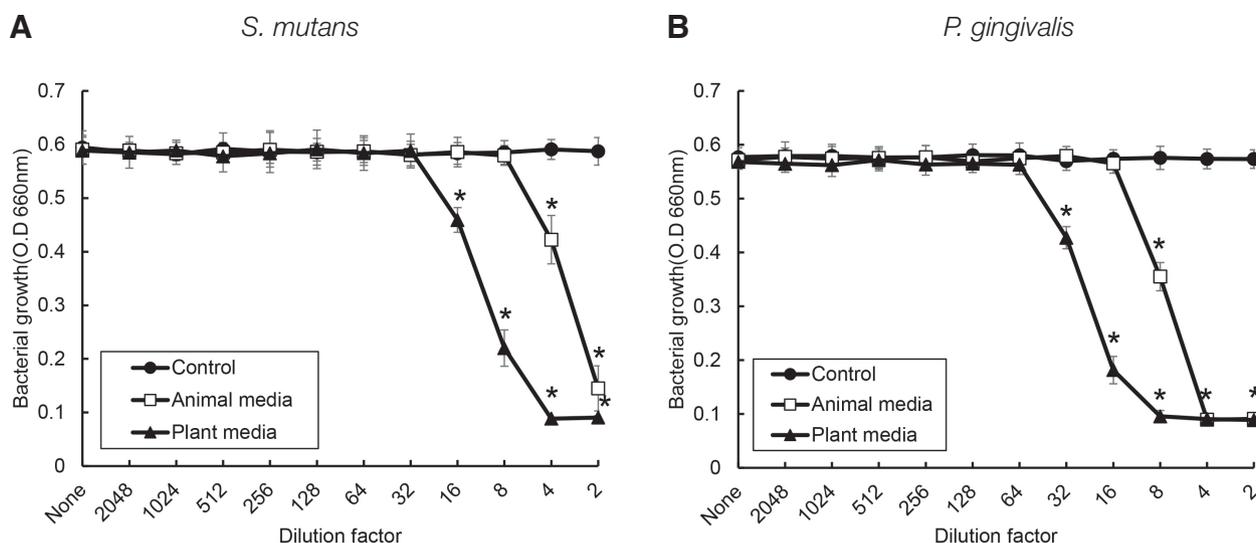


Fig. 3. Antimicrobial activity of *S. salivarius* K12 against oral bacteria. The spent culture medium was collected from *S. salivarius* K12 cultured with medium containing animal and plant protein. *S. mutans* (A) and *P. gingivalis* (B) was incubated in the presence or absence of the SCM of *S. salivarius* K12. Bacterial growth was measured by a spectrophotometer. The experiments were carried out 4 times in duplicate, and data are expressed as mean ± standard deviation. * indicates statistically significant differences compared control group.

teins was compared using SCM. The SCM of *S. salivarius* K12 cultured in medium containing animal and plant proteins significantly inhibited the growth of *S. mutans* at or above 4-fold diluted and 16-fold diluted concentration, respectively ($P < 0.05$) (Fig. 3A). The difference in antimicrobial activity of *S. salivarius* K12 cultured in the two media was approximately 4 times. Also, in case of examination of *P. gingivalis*, the SCM of the probiotics cultured in medium containing animal and plant protein significantly reduced the growth of *P. gingivalis* at or above 8-fold and 32-fold diluted concentration, respectively ($P < 0.05$) (Fig. 3B). Similar to the case of *S. mutans*, the two SCM of *S. salivarius* K12 showed 4 times difference in the antimicrobial activity against *P. gingivalis*. The SCM of *S. salivarius* K12 cultured in medium containing plant proteins significantly inhibited the growth of *C. albicans* at or above 4-fold diluted concentration ($P < 0.05$) (Fig. 4). The SCM from medium containing animal protein slightly inhibited the growth of *C. albicans*.

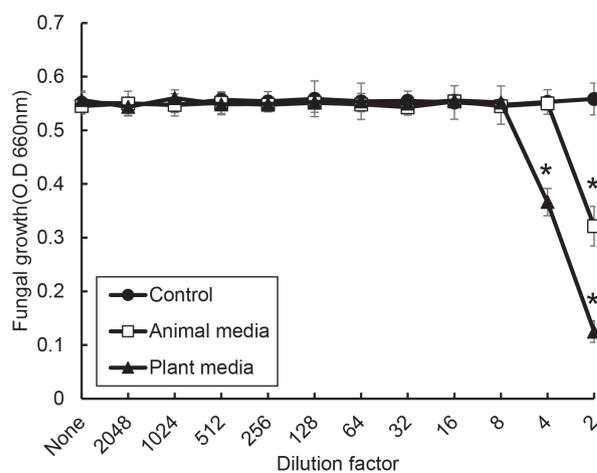


Fig. 4. Antifungal activity of *S. salivarius* K12 against *C. albicans*. The spent culture medium was collected from *S. salivarius* K12 cultured with medium containing animal and plant protein. *C. albicans* was incubated in the presence or absence of the SCM of *S. salivarius* K12. Fungal growth was measured by a spectrophotometer. The experiments were carried out 3 times in duplicate, and data are expressed as mean ± standard deviation. * indicates statistically significant differences compared control group.

Discussion

S. salivarius K12 is a probiotic bacterium and isolated from the saliva of healthy child.¹⁴ This probiotic produces salivarin A2 and salivarin B that effectively inhibits the growth of *S. pyogenes* as a pharyngitis related bacterium.^{15,16} Furthermore, *S. salivarius* K12 has the antimicrobial activity against oral bacteria and fungus and can colonize in oral cavity.^{13,17} Due to these characteristics, *S. salivarius* K12 is considered to be more suitable for prevention and treatment of bacteria-related oral disease than other probiotics. However, there is still not enough information about the habit of *S. salivarius* K12. Therefore, in this study, the growth and antimicrobial activity of *S. salivarius* K12 according to the culture condition were investigated.

S. salivarius K12 showed faster growth in medium containing plant protein than animal proteins. Probiotics require nutrients and environments for colonization and growth like pathogenic bacteria because of protozoa. So, substances that help the function and growth of probiotics are called prebiotics, and oligosaccharide is representative. In this study, although it has not been examined exactly what component, it was observed that a protein derived from soybean, a representative plant protein, helps the growth of *S. salivarius* K12. This indicates that soy protein may be a prebiotic for *S. salivarius* K12. In experiment of pH levels, *S. salivarius* K12 was observed to grow very slowly in pH 5.5 as an acidic condition. Most bacteria produce lactic acid through carbohydrate metabolism and create the acidic environment. Even in a pH 5.5 environment, if *S. salivarius* K12 is active in carbohydrate metabolism, it can induce dental caries, but it is considered that *S. salivarius* K12 may slowly metabolize because the growth is slow in this environment. Therefore, this information supports that *S. salivarius* K12 is safe to apply for the prevention of oral diseases, and that substances with a pH buffer function may be included to increase the preventive effect of *S. salivarius* K12.

In order to investigate whether the two media affects only the growth of *S. salivarius* K12 or affects the antimicrobial and the antifungal activity of *S.*

salivarius k12, the susceptibility of *S. mutans* and *P. gingivalis* was evaluated using the SCM of *S. salivarius* K12 cultured in medium containing animal or plant protein. *S. mutans* is a Gram-positive microbe and closely related with dental caries,⁷ and *P. gingivalis* is a Gram-negative anaerobe and a periodontopathogen.³ Both bacteria showed more susceptibility for the SCM from medium containing plant protein than animal protein. Furthermore, *C. albicans* appeared more susceptibility for the SCM from medium containing plant protein compared to animal protein

Conclusion

The present study shown that the necessity of the condition to maximize the function of *S. salivarius* K12 as a probiotic. Eventually, in order to apply *S. salivarius* K12 to bacterial oral disease, co-substances may be needed for *S. salivarius* K12 to colonize in the oral cavity and exhibit stronger the antimicrobial activity.

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Streptococcus salivarius K12의 배양조건에 따른 항균활성의 특징

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목적: 본 연구의 목적은 *Streptococcus salivarius* K12의 성장과 항균활성에 대한 배양조건의 영향을 알아보는 것이다.

연구 재료 및 방법: *S. salivarius* K12는 동물 또는 식물 단백질을 함유한 배지 또는 중성 및 산성 조건의 배지에서 배양되었다. *S. salivarius* K12의 성장은 2시간마다 분광광도계로 측정하였다. *S. salivarius* K12의 *Streptococcus mutans*, *Porphyromonas gingivalis* 및 *Candida albicans*에 대한 항균 또는 항진균 활성을 배양액을 이용한 감수성 분석으로 조사하였다.

결과: *S. salivarius* K12의 성장은 식물 단백질과 중성 pH 조건에서 더 빠른 성장을 보였다. *S. salivarius* K12의 항균 및 항진균 활성은 동물성 단백질보다 식물성 단백질을 함유한 배지에서 더 강하게 나타났다.

결론: *S. salivarius* K12를 세균성 구강질환에 적용하기 위해서는 *S. salivarius* K12가 구강 내에서 군집화하여 항균 활성을 향상시키기 위한 보조물질이 필요할 수 있다.

(구강회복응용과학지 2021;37(4):244-50)

주요어: *S. salivarius* K12; 프로바이오틱스; 프리바이오틱스; 항균 활성

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