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Original Article

Preventive effects of *Ligilactobacillus salivarius* WB21 on halitosis and periodontitis

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Purpose: Halitosis is generally defined as an unpleasant odor and generated by periodontitis related bacteria. *Ligilactobacillus salivarius* WB21 is a probiotics isolated from the human intestine. The aim of present study was to investigate antimicrobial activity of *L. salivarius* WB21 against periodontopathogens using susceptibility assay and co-cultivation system and inhibitory effects the WB21 strain on emission of volatile sulfur compounds (VSCs). **Materials and Methods:** The antimicrobial activity was performed using susceptibility assay and co-cultivating condition with cell culture insert. The levels of VSCs were measured by a gas chromatograph. **Results:** The spent culture medium of WB21 strain significantly inhibited the growth of Porphyromonas gingivalis, Tannerella forsythia, and Treponema denticola at concentration of 12.5% or higher ($P < 0.05$). Also, the growth of the periodontitis related bacteria was significantly inhibited in co-cultivating with *L. salivarius* WB21 in condition of 1:5 ratio or higher ($P < 0.05$). In co-cultivation conditions with *L. salivarius* WB21, the levels of VSCs produced by periodontitis related bacteria were significantly decreased in mixed conditions of 1 : 1 ratio or higher ($P < 0.05$). **Conclusion:** *L. salivarius* WB21 may be a probiotics candidate to prevent periodontitis and halitosis. **(J Dent Rehabil Appl Sci 2025;41(4):297-303)**

Key words: probiotics; *L. salivarius* WB21; halitosis; periodontitis; preventive effect

Introduction

Halitosis is generally defined as an unpleasant or offensive odor which is emitted from oral cavity.[1] Also, halitosis is caused from either a local or systemic origin and emanates from mouth. Various studies have shown that approximately 90% of halitosis cases originate in the oral cavity, not the digestive tract.[2] Halitosis originated by the mouth is mainly caused by metabolites which are volatile sulfur compounds (VSCs) from putrefaction of proteinaceous substrates.[3] Furthermore, these VSCs were produced by the action of anaerobic Gram-negative bacteria known as periodontitis related bacteria (*Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*) in the oral cavity.[4,5] These bacteria, are called red-complex, produce trypsin-like enzymes, by which peptide containing methionine was broken down.[6,7] The VSCs produced by the bacteria are hydrogen sulfide (H_2S), methyl mercaptan (CH_3SH), and dimethyl sulfide (CH_3SCH_3). The concentration of these VSCs is used as an indicator of halitosis.[8]

As interest in oral health using probiotics has grown recently, studies on the effects of probiotics have been also increased.[9] Oral diseases occur when the balance of bacteria in oral cavity is broken, increasing the proportion of pathogenic bacteria and causing the diseases such as dental caries and

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periodontitis.[10] The occurrence of these oral diseases is similar to the occurrence of intestinal diseases, thus oral health can be maintained by balanced microbiome with beneficial bacteria.[11,12] Additionally, research is being conducted to maintain a balanced microbiome using probiotics based on the concept.[13] *Ligilactobacillus salivarius* WB21, is a lactic acid bacterium, is known to be potentially effective probiotic against *Helicobacter pylori*.[14] Generally, *L. salivarius* produces antimicrobial peptide as bacteriocin called salivaricin and has efficacy against pathogens.[14-16] *L. salivarius* WB21 has antimicrobial activity against *P. gingivalis* and *Streptococcus mutans*.[15] However, studies on the antimicrobial activity and anti-halitogenic effects of *L. salivarius* WB21 on *T. denticola* and *T. forsythia* excluding *P. gingivalis*.

The aim of present study was to investigate antimicrobial activity of *L. salivarius* WB21 against periodontopathogens using susceptibility assay and co-cultivation system and inhibitory effects WB21 strain on emission of VSCs gaseous.

Materials and Methods

Ligilactobacillus salivarius WB21 (Deposit institution: National Institute of Technology and Evaluation, Japan, Deposit No.: FERM BP-7792) supplied by Ju Yeong NS Co., Ltd. (Seoul, Republic of Korea), was cultivated using MRS broth (BD biosciences, Franklin Lakes, USA) at 37°C, aerobically. *Porphyromonas gingivalis* ATCC 33277 was using brain heart infusion (BHI) (BD biosciences) supplemented with hemin (1 μg/ml) and vitamin K (0.2 μg/ml). *Tannerella forsythia* ATCC 43037 was cultivated using modified NOS medium,[17] and *Treponema denticola* ATCC 35405 was cultivated TYGVS medium.[18] Periodontitis related bacteria were managed at 37°C in anaerobic condition (5% H_2, 10% CO_2, and 85% N_2).

The susceptibility assay was carried out according to the methods of Clinical Laboratory Standard Institute (CLSI). The susceptibility of periodontitis related bacteria was used spent conditioned media (SCM) derived from *L. salivarius* after filtrating 0.22 μm of a polyvinylidene fluoride (PVDF) filter (Millipore, Billerica, USA). The appropriate medium for each bacterium was dispensed into 96-well plate (SPL life Sciences, Pocheon, Korea). The prepared SCM was added into the first well containing the fresh medium and conducted 2-fold serial dilution to the 11th well. After adjusting bacterial concentration at 0.5 McFarland, the bacteria were inoculated into the prepared 96-well plate. The plates were incubated at 37°C in anaerobic condition for 36 h, and the optical density was measured at 660 nm of wavelength with microplate reader (BioTek, Winooski, USA).

L. salivarius WB21 and periodontitis-related bacteria were co-cultivated using 12-well cell culture insert (SPL life Sciences). The suspension of pathogens and *L. salivarius* WB21 were inoculated inside and outside of the insert according to the manufacturer's volume, respectively. The plate was incubated at 37°C in anaerobic condition for 36 h. The bacterial growth was measured with optical density using microplate reader and observed with microscope. Also, Contamination of each bacterium in separating chamber was checked by observation of microscope.

Since halitosis is mainly caused by volatile sulfur compounds (VSCs) produced by periodontitis related bacteria, the level of the VSCs was measured from the suspension of co-cultivated bacteria. The bacterial suspensions (0.5 ml) were transferred to a new 50 ml tube, and the tube was then rotated on a rotator for 1 min to allow sufficient volatilization of VSCs from the suspension. The VSCs were collected in the tube using 1 ml syringe (BD biosciences) followed by measurement of VSCs with Oral Chroma^{TM} gas chromatograph (FIS inc., Itami, Japan).

The data was examined distribution using Kolmogorov-Smirnov test and analyzed by Kruskal Wallis test. As a post-hoc test, Mann-Whitney U test was performed using IBM SPSS statistics Ver. 22 (IBM, Armonk, USA). *P*-values less than 0.05 were considered statistically significant.

Results

When the antimicrobial activity of the SCM from *L. salivarius* WB21 was performed, the SCM inhibited the growth of *P. gingivalis*, *T. denticola*, and *T. forsythia* (Fig. 1). The growth of *P. gingivalis* was significantly

decreased at the concentration above 6.25% the SCM ($P < 0.05$). Also, the growth of *T. denticola* and *T. forsythia* was significantly reduced at the concentration above 12.5% the SCM ($P < 0.05$).

To investigate the direct effect when applied oral cavity, the effect in a co-existence environment was examined. When the effect of the WB21 strain was observed using co-cultivation system, it was confirmed using a microscope that there was no contamination between *L. salivarius* WB21 and periodontitis-related bacteria (Fig. 2). Also, the levels of *P. gingivalis*, *T. denticola* and *T. forsythia* were significantly reduced in the inoculating condition with the WB21 ratio of 1 : 5 or more ($P < 0.05$) (Fig. 3).

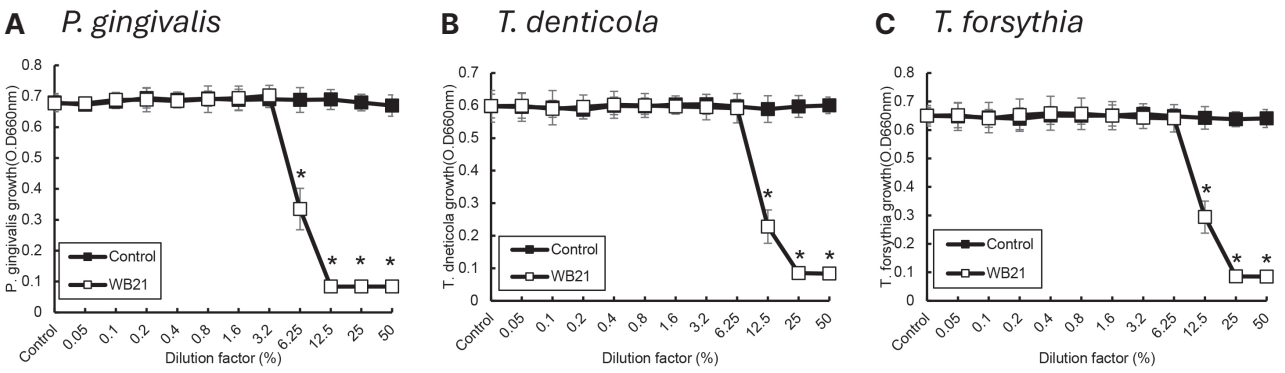

Fig. 1. Antimicrobial activity of the SCM of *L. salivarius* WB21 against Periodontopathogens. The SCM of *L. salivarius* WB21 was collected. After preparing various concentrations of the SCM in 96-well plate, *P. gingivalis* (A), *T. denticola* (B), and *T. forsythia* (C) were inoculated into the prepared 96-well plate. The plates were incubated, and the bacterial growth was measured by spectrophotometer. Asterix (*) indicates significant difference between control group and sample group ($P < 0.05$).

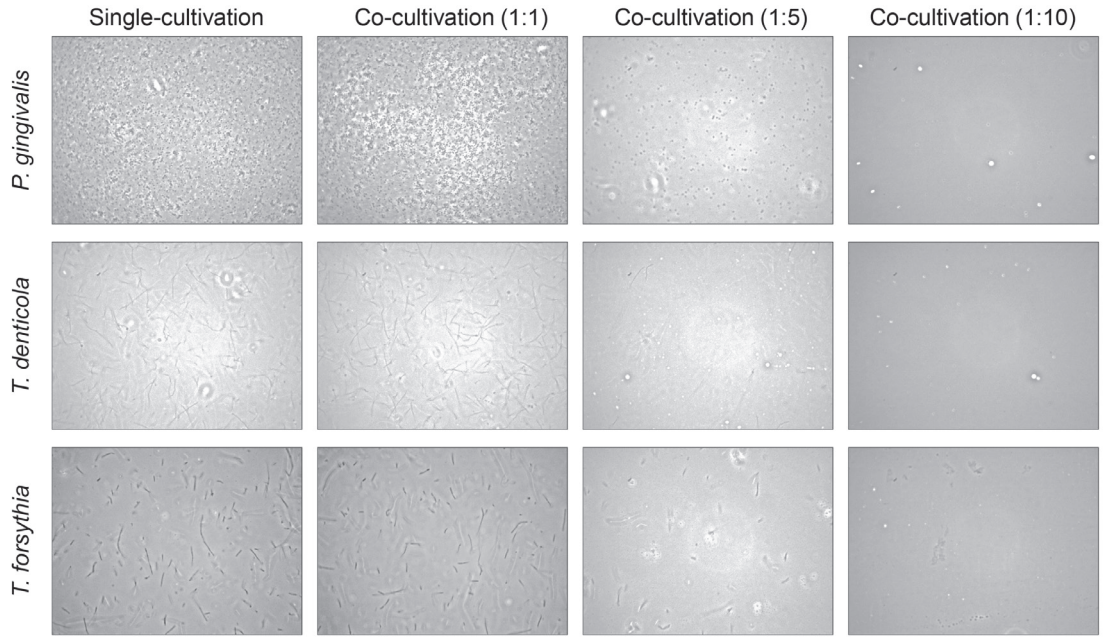

Fig. 2. The growth of periodontopathogens in co-cultivation with *L. salivarius* WB21. Periodontopathogens were cultured with or without *L. salivarius* WB21 in co-cultivating system at concentration of 1 : 1, 1 : 5, and 1 : 10 ratio. After incubating, the bacterial suspensions were observed by phase contrast microscope and get the image.

L. salivarius WB21 and periodontopathogens were co-cultivated and then collected VSCs gas. Total VSCs were measured using a gas chromatograph. Total VSCs produced by *P. gingivalis* were significantly decreased in co-cultivating with WB21 ratio of 1 : 1 or higher ($P < 0.05$) (Fig. 4A). Methyl mercaptan (CH_3SH) produced by *P. gingivalis* were also reduced significantly in co-cultivating with WB21 ratio of 1 : 1 ($P < 0.05$). In case of *T. denticola* and *T. forsythia*, when the WB21 strain was co-cultured in the inoculating condition of 1 : 1 ratio or higher, not only total VSCs but also Hydrogen sulfur (H_2S) and methyl mercaptan (CH_3SH) showed to be significantly reduced (Fig. 4B and 4C).

Discussion

Probiotics were used for the purpose of maintaining health by improving environment of the intestinal microbiome, but recently, the concept of functionality has been added, the use of probiotics is to maintain the health of specific organs such as

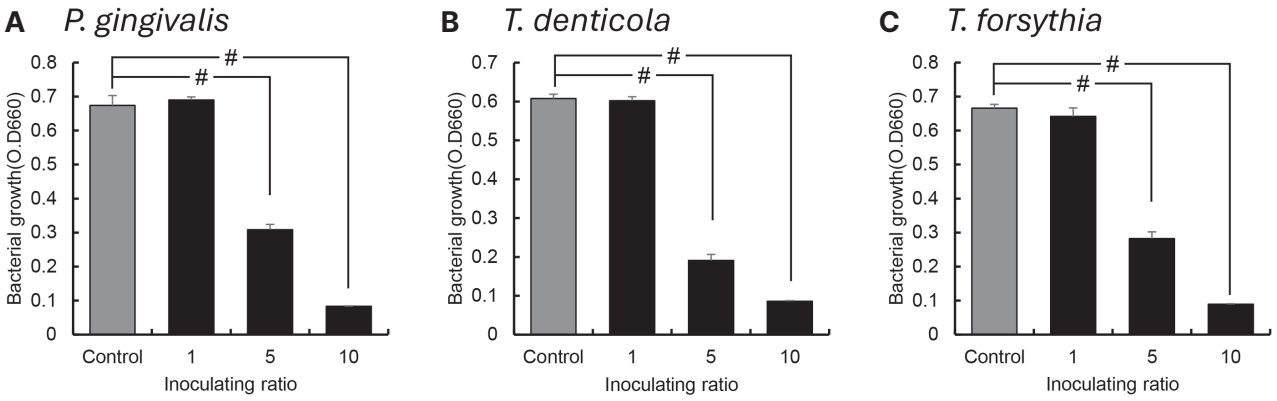

Fig. 3. Inhibitory effects of *L. salivarius* WB21 on growth of periodontopathogens. Periodontopathogens were cultured with or without *L. salivarius* WB21 in co-cultivating system at concentration of 1 : 1, 1 : 5, and 1 : 10 ratio. After incubating, the growth of *P. gingivalis* (A), *T. denticola* (B), and *T. forsythia* (C) was measured by spectrophotometer. Sharp (#) indicates significant difference between control group and sample group ($P < 0.01$).

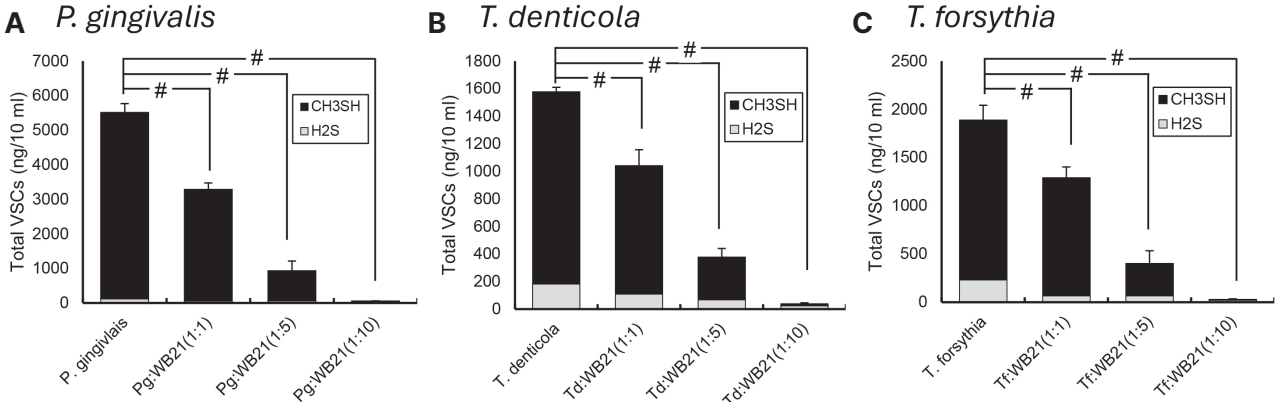

Fig. 4. Inhibitory effects of *L. salivarius* WB21 on production of VSCs from periodontopathogens. Periodontopathogens were cultured with or without *L. salivarius* WB21 in co-cultivating system at concentration of 1 : 1, 1 : 5, and 1 : 10 ratio. After incubating, the suspensions of *P. gingivalis* (A), *T. denticola* (B), and *T. forsythia* (C) were transferred into 50 ml conical tube, and the tubes were rotated for 1 min. the VSCs gaseous was harvested above on the suspension and measured by gas chromatograph. Sharp (#) indicates significant difference between control group and sample group ($P < 0.01$).

liver, skin, genital organ, and oral cavity, and probiotics that act on specific organs of the body have been discovered.[19,20] *L. salivarius* WB21 was also studied on the effects of each organ, and this study investigated the effect of WB21 on halitosis and the induction of periodontitis.

First, to investigate whether *L. salivarius* WB21 produces bacteriocins like existing strains, the antibacterial activity against periodontal bacteria was examined using the spent culture medium from WB21 strain. The SCM of WB21 strain has antibacterial activity against *P. gingivalis*, *T. forsythia*, and *T. denticola* as red-complex bacteria. Although quantitative analysis of antibacterial activity using the MIC test was performed,[21] this experiment is insufficient to confirm whether there is an antibacterial effect against periodontitis related bacteria when they coexist in the oral cavity. So, next, the growth of red-complex bacteria was investigated using co-cultivating condition with *L. salivarius* WB21. When the WB21 strain was co-cultivated with periodontopathogens at a 5-fold increase, the growth of *P. gingivalis*, *T. denticola*, and *T. forsythia* showed to be reduced. This result indicates that the WB21 strain may inhibit the growth of periodontitis related bacteria when applied to the oral cavity, and it is expected to have preventive effects on periodontitis.

Red-complex bacteria are associated with halitosis as well as periodontitis. These bacteria secret trypsin-like enzymes, which produce VSCs.[22,23] Therefore, after co-cultivating *L. salivarius* WB21 and red-complex bacteria, VSCs contained in the suspension of *P. gingivalis*, *T. forsythia*, and *T. denticola* were measured. Although there were differences in the degree of occurrence among periodontitis related bacteria, it was checked that VSCs were generated in the suspension of the single-cultured bacteria. The level of VSCs gaseous appeared to decrease in the suspension cultured with WB21 strain at concentration of 1 : 1 ratio. When co-cultivated with *L. salivarius* WB21 at concentration of 1 : 5 ratio, the growth of periodontitis pathogens was reduced. Which may have led to a decrease in VSCs levels. However, when co-cultivating with *L. salivarius* WB21 at concentration of 1 : 1 ratio, the growth of periodontitis pathogens was not reduced. These results indicate that WB21 inhibits the production of enzymes that generate VSCs in periodontitis pathogens.

Conclusion

L. salivarius WB21 showed antimicrobial activity against periodontitis related pathogens such as *P. gingivalis*, *T. forsythia*, and *T. denticola*. Furthermore, *L. salivarius* WB21 inhibited the production of VSCs by periodontitis related pathogens. Basis of the results, *L. salivarius* WB21 may be a probiotic candidate to prevent periodontitis and halitosis. To support the results obtained through in vitro experiments, future clinical trials will be conducted, and more definitive conclusions about the probiotic effects can be obtained through clinical trials.

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Ligilactobacillus salivarius WB21의 구취 및 치주염에 대한 예방효과

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목적: 구취는 일반적으로 치주염 관련 세균에 의해 발생하는 불쾌한 냄새로 정의된다. *Ligilactobacillus salivarius* WB21은 사람 장에서 분리된 프로바이오틱스이다. 본 연구의 목적은 감수성 분석 및 공동 배양 시스템을 사용하여 치주 병원균에 대한 *L. salivarius* WB21의 항균 활성과 휘발성 황 화합물(VSC) 배출에 대한 WB21 균주의 억제 효과를 조사하는 것이다.

연구 재료 및 방법: 항균 활성은 감수성 분석 및 세균 배양액과 혼합 배양 시스템을 이용한 혼합조건을 사용하여 측정하였다. VSC의 수준은 가스 크로마토그래피로 측정하였다.

결과: *L. salivarius* WB21 균주의 배양되었던 배지 12.5% 농도 이상에서 *Porphyromonas gingivalis*, *Tannerella forsythia* 및 *Treponema denticola*의 성장을 유의하게 억제하였다($P < 0.05$). 또한, *L. salivarius* WB21과 혼합 배양 시, 치주염관련 세균과 WB21종을 1 : 5 비율 이상의 환경에서 치주염 관련 세균의 성장이 유의하게 억제되었다($P < 0.05$). *L. salivarius* WB21과 혼합 배양 시, 치주염 관련세균과 WB21종을 1 : 1 비율 이상의 환경에서 치주염 관련 세균이 생성하는 VSC 수치가 유의하게 감소하였다($P < 0.05$).

결론: *L. salivarius* WB21은 치주염과 구취를 예방하는 프로바이오틱스 후보 물질이 될 수 있다고 사료된다.

(구강회복응용과학지 2025;41(4):297-303)

주요어: 프로바이오틱스; *Ligilactobacillus salivarius* WB21; 구취; 치주염; 예방효과

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