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Effects of *Streptococcus thermophilus* on volatile sulfur compounds produced by *Porphyromonas gingivalis*

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ABSTRACT

Halitosis as oral malodour is an unpleasant odour caused by volatile sulfur compounds (VSCs). VSCs are produced primarily by anaerobic bacteria that abundantly produce proteinase as trypsin-like enzyme. General therapies, such as mouthwash and plaque control, do not provide a continuous effect on oral halitosis. *Streptococcus thermophilus* is a probiotic bacterium that is beneficial for human health. The aim of this study was to evaluate the effect of *S. thermophilus* on *Porphyromonas gingivalis*-producing VSCs and to analyze the inhibitory mechanism of halitosis. *P. gingivalis* was cultured with or without *S. thermophilus*, and the emission of VSCs from the spent culture medium was measured by gas chromatography. In order to analyze the inhibitory effect, the antibacterial activity of *S. thermophilus* against *P. gingivalis* was assessed. After the spent culture medium or whole bacterial of *S. thermophilus* was mixed with the spent culture medium of *P. gingivalis*, VSCs were again measured by gas chromatograph. When *S. thermophilus* and *P. gingivalis* were co-cultivated, VSCs were present at a lower level than those of single-cultured *P. gingivalis*. *S. thermophilus* inhibited growth of *P. gingivalis*, and the whole bacteria and the spent culture medium of *S. thermophilus* reduced emission of VSCs gas. *S. thermophilus* may reduce oral malodour by inhibition of *P. gingivalis* growth and neutralizing VSCs with their metabolites or themselves.

1. Introduction

Halitosis is known as bad breath, oral malodour or fetor oris. Temporary halitosis is caused by consumption of foods or drinks. Persistent malodour is mainly due to metabolites of oral microorganisms.[1] Persistent malodour occurs mainly by production of volatile sulfur compounds (VSCs) from putrefaction of proteinaceous substrates.[2,3] In the oral cavity, putrefaction of proteins is mainly due to anaerobic gram-negative bacteria, such as *Porphyromonas gingivalis*, *Tannrella forsythia* and *Treponema denticola*.[2,4,5] These three bacteria as periodontopathogens have a characteristic of benzoyl-D,L-arginine-naphthylamide (BANA)-positive bacteria by secretion of trypsin-like enzyme[6], by which the periodontopathogens can produce large amount of hydrogen sulfide (H_2S), methyl mercaptan (CH_3SH) and dimethyl sulfide ($(CH_3)_2S$) from methionine and cysteine in serum protein.[7] Until now, halitosis has typically been treated with mechanical therapy like dental floss and mouth rinse with chemical agents for reduction of VSC.[1,3] Recently, improvement of halitosis using *Streptococcus salivarius* K12 has been explored.[8,9]

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Streptococcus thermophilus is a probiotic, gram-positive and facultative anaerobe. It is used in preparation of homemade yogurt.[10] *S. thermophilus* is beneficial on gastrointestinal health by producing its exopolysaccharide or bacteriocin.[11,12] *S. thermophilus* showed antibacterial activity against oral Streptococci such as *S. mutans*, *S. oralis* and *S. sobrinus*.[13] Furthermore, *S. thermophilus* adheres to tooth-like surfaces such as hydroxyapatite with casein-containing dairy products, and comparatively inhibits the attachment of *S. mutans* and *S. sobrinus*. Thus, *S. thermophilus* has been considered to prevent dental caries.[14] However, the effect of *S. thermophilus* on provocation of oral malodour by periodontopathogens has not been investigated.

The purpose of this study was to evaluate the effect of *S. thermophilus* on emission of *P. gingivalis*-producing VSCs and to analyze the inhibitory effect on reduction of VSCs emission.

2. Materials and methods

2.1. Bacterial strain and cultivation

P. gingivalis ATCC 33277 was used for generation of halitosis-related VSCs and cultivated in Brain Heart Infusion broth (BHI; BD bioscience, San Jose, CA, USA) supplemented with hemin (1 μg/ml) and vitamin K (0.2 μg/ml) at 37 °C anaerobically. *S. thermophilus* strain HY2, HY3 HY9012 were donated from Yakult (Korea yakult Com, Gyeonggi, Korea) and cultured with BHI broth at 37 °C in anaerobic chamber (H_2 5%, CO_2 10% and N_2 85%).

2.2. Measurement of volatile sulfur compounds

Since halitosis is mainly caused by VSCs such as hydrogen sulfide, methyl mercaptan and dimethyl sulfide, the level of the compounds was measured from the spent culture medium of *P. gingivalis* after treating or non-treating with various cell densities of *S. thermophilus*. *P. gingivalis* was cultivated in the presence or absence of *S. thermophilus* at 37 °C for 36 h. Each bacterial suspension (1 ml) was transferred to a new 50 ml conical tube and then vortexed for 30 s followed by measurement of VSCs with Oral Chroma™ gas chromatograph (FIS Inc., Itami, Hyogo, Japan). In order to investigate the inhibitory mechanism of VSCs, each *P. gingivalis* and *S. thermophilus* were cultivated and then the spent culture medium and whole bacteria were separated by centrifugation at 7000 × *g* for 10 min at 4 °C. The supernatants (1 ml) were transferred into fresh 15 ml conical tubes. The spent culture medium of *P. gingivalis* was mixed with various volume of the spent culture medium or whole bacteria of *S. thermophilus* in a 50 ml conical tube and incubated at room temperature for 5 min. VSCs were collected in 5 ml of gas above the mixed solution using 10 ml syringe and measured the level by gas chromatograph.

2.3. Antibacterial activity of S. thermophilus against P. gingivalis

Since *S. thermophilus* produces a bacteriocin, the antibacterial activity of *S. thermophiles* against *P. gingivalis* was evaluated. Susceptibility assay was performed according to the methods of Clinical Laboratory Standard Institute (CLSI). Briefly, 180 μl of fresh BHI broth including hemin (1 μg/ml) and vitamin K (0.2 μg/ml) was dispensed in each well of a 96-well polystyrene plate (SPL Lifescience, Gyeonggi, Korea), and then 180 μl of the spent culture medium of two species was added to the first row of the plate. Two-fold serial dilutions were made using a multi-channel micropipette. *P. gingivalis* was counted by Petroff–Hasser bacteria counter (Hausser Scientific, Horsham, PA, USA) and then diluted to 3×10^6 cells/ml with BHI broth including hemin (1 μg/ml) and vitamin K (0.2 μg/ml). The bacterial suspensions (20 μl; 6×10^4 cells) were inoculated in each well. The plates were incubated at 37 °C in anaerobic chamber for 36 h and the optical density was measured at 660 nm using an ELISA reader.

2.4. Co-cultivation of P. gingivalis and S. thermophilus

Bacterial co-cultivation was carried out according to the method described by Lee and Baek [15] *P. gingivalis* and *S. thermophilus* were co-cultured using Millicell cell culture insert (Millipore, Billerica, MA, USA). BHI broth was mixed with hemin and vitamin K and then dispensed in two new tubes. *P. gingivalis* and *S. thermophilus* were inoculated into each tube. After hanging Millicell cell culture insert in a well of 12-well plate, *P. gingivalis* and *S. thermophilus* were inoculated in the apical and basolateral side, respectively. Contamination of each bacterium in the separating chamber was investigated by observation using a microscope, and *P. gingivalis* colonies were counted after plating on BHI agar plate.

2.5. Statistical analysis

Statistically significant differences were analyzed by Mann–Whitney *U*-test using SPSS ver. 10 (SPSS Inc., Chicago, IL). *P*-values <0.05 were considered statistically significant.

3. Results

3.1. Effect of S. thermophilus on the VSCs-producing P. gingivalis

P. gingivalis produces halitosis-associated VSCs using proteolytic enzyme. The effect of *S. thermophilus* on VSC production of *P. gingivalis* was investigated. When *P. gingivalis* was co-cultured with *S. thermophilus*, the level of hydrogen sulfur, methyl sulfide and dimethyl sulfide were reduced in the presence of *S. thermophilus* (Fig. 1). Especially, methyl mercaptan was decreased by 90% in the present of *S. thermophilus* (Table 1).

3.2. Antibacterial activity of S. thermophilus against P. gingivalis

To investigate the correlation of VSCs reduction and growth inhibition of *P. gingivalis*, antibacterial activity of *S. thermophilus* against *P. gingivalis* was examined according to CLSI. The spent culture medium of *S. thermophilus* were prepared and tested the antibacterial activity against *P. gingivalis*. The spent culture medium of *S. thermophilus* strain HY2, HY3 and HY9012