Research Article

Inhibitory Effect of *Enterococcus faecium* WB2000 on Volatile Sulfur Compound Production by *Porphyromonas gingivalis*

Nao Suzuki,1 Takuya Higuchi,2 Masato Nakajima,2 Akie Fujimoto,2 Hiromitsu Morita,2 Masahiro Yoneda,2 Takashi Hanioka,1 and Takao Hirofuji2

1Department of Preventive and Public Health Dentistry, Fukuoka Dental College, 2-15-1 Tamura, Sawara-ku, Fukuoka 814-0193, Japan
2Department of General Dentistry, Fukuoka Dental College, 2-15-1 Tamura, Sawara-ku, Fukuoka 814-0193, Japan

Correspondence should be addressed to Nao Suzuki; naojsz@college.fdcnet.ac.jp

Received 17 August 2016; Accepted 18 September 2016

Academic Editor: Yoshitaka Hara

Copyright © 2016 Nao Suzuki et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Volatile sulfur compounds (VSCs) produced by oral anaerobes are the major compounds responsible for oral malodor. *Enterococcus faecium* WB2000 is recognized as an antiplaque probiotic bacterium. In this study, the effect of *E. faecium* WB2000 on VSC production by *Porphyromonas gingivalis* was evaluated, and the mechanism of inhibition of oral malodor was investigated. *P. gingivalis* ATCC 33277 was cultured in the presence of four lactic acid bacteria, including *E. faecium* WB2000. Subsequently, *P. gingivalis* ATCC 33277, W50, W83, and two clinical isolates were cultured in the presence or absence of *E. faecium* WB2000, and the emission of VSCs from spent culture medium was measured by gas chromatography. The number of *P. gingivalis* ATCC 33277 in mixed culture with *E. faecium* WB2000 decreased at 6 h, and the rate of decrease was higher than that in mixed cultures with the other lactic acid bacteria. The numbers of five *P. gingivalis* strains decreased at similar rates in mixed culture with *E. faecium* WB2000. The concentration of methyl mercaptan was lower in spent culture medium from *P. gingivalis* and *E. faecium* WB2000 cultures compared with that from *P. gingivalis* alone. Therefore, *E. faecium* WB2000 may reduce oral malodor by inhibiting the growth of *P. gingivalis* and neutralizing methyl mercaptan.

1. Introduction

Oral malodor is caused mainly by the metabolism of sulfur amino acids by anaerobic bacteria inhabiting the oral cavity [1]. The main compounds responsible for oral malodor are volatile sulfur compounds (VSCs), such as hydrogen sulfide (H$_2$S), methyl mercaptan (CH$_3$SH), and dimethyl sulfide; these compounds are produced by some periodontopathic bacteria. Indeed, *Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*, and *Prevotella intermedia* generate considerable amounts of H$_2$S and CH$_3$SH [2].

Probiotic bacteria, defined as live microorganisms that benefit the health of the host when administered in adequate amounts (FAO/WHO 2001), are thought to play a role in the maintenance of oral health [3]. Enterococci are facultatively anaerobic, Gram-positive cocci that form a part of the normal flora of the gastrointestinal tract of animals and humans. They are also frequently found in fermented food, such as cheese and meat [4]. *Enterococcus faecium* and *Enterococcus faecalis* are the most clinically relevant members of the genus *Enterococcus*. Traditionally, they are regarded as low-grade pathogens but have emerged as important causes of nosocomial infections [5]. Clinical use of *E. faecium* and *E. faecalis* during food fermentation and as probiotics requires a careful safety evaluation [6].

*E. faecium* strains have been reported to inhibit biofilm formation by cariogenic bacteria *in vitro* [7, 8]. In addition, a previous double-blind randomized trial in which the subjects cleaned their teeth using a dentifrice containing *E. faecium* WB2000 or placebo for 4 weeks revealed improvements in salivary flow, salivary buffering capacity, and plaque accumulation [9]. This study aimed to investigate the effect of *E. faecium* WB2000 on VSC production by *P. gingivalis* and the mechanism of inhibition of oral malodor by *E. faecium* WB2000.
2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions. The bacterial strains used in the study are listed in Table 1. Enterococcus faecium WB2000, previously classified as Streptococcus faecalis [10], was provided by Wakamoto Pharmaceutical. The selective medium for P. gingivalis consisted of Brucella agar (Becton Dickinson, Le Pont de Claix, France) supplemented with 5% horse blood, hemin (5 μg/mL), vitamin K (1 μg/mL), and gentamicin (50 μg/mL). Lactic acid bacteria were cultivated on BL agar (Nissui, Tokyo, Japan). Bacterial strains were cultivated at 37°C anaerobically for 40 h and suspended in sterile physiological saline to an optical density at 560 nm (OD₅₆₀) of 1.0 for P. gingivalis and an OD₅₆₀ of 0.02 for lactic acid bacteria.

2.2. Cocultivation of P. gingivalis and Lactic Acid Bacteria. Bacterial cocultivation was carried out using 100 μL of P. gingivalis suspension, 100 μL of lactic acid bacterial suspension, and 10 mL fresh GAM broth (Nissui, Tokyo, Japan) supplemented with 0.7% glucose, hemin (5 μg/mL), and vitamin K (1 μg/mL) at 37°C anaerobically. Viable bacterial counts in the culture medium were determined at 6, 12, 24, and 48 h on the appropriate agar medium at 37°C anaerobically for 48 h.

2.3. Measurement of Volatile Sulfur Compounds. The VSC concentration in spent medium from P. gingivalis cultured in the presence or absence of E. faecium WB2000 was measured after 24 and 48 h. Aliquots (0.2 mL) of spent culture medium were added to 5 mL conical tubes, which were sealed and incubated at room temperature for 5 min. Then, 0.5 mL of the gas phase was collected and measured by gas chromatography (model GC2014, Shimadzu Works, Kyoto, Japan).

3. Results

3.1. Effect of Lactic Acid Bacteria on the Growth of P. gingivalis ATCC 33277. The number of viable P. gingivalis ATCC 33277 decreased to less than the detection limit after 6 h in mixed culture with E. faecium WB2000 (Figure 1(a)). In mixed cultures with the other three lactic acid bacteria (S. salivarius JCM 5707, L. salivarius CIP 103140, and L. reuteri JCM 1112), the number of viable P. gingivalis ATCC 33277 decreased to less than the detection limit at 12 h. In mixed culture with P. gingivalis ATCC 33277, E. faecium WB2000 grew more...
3.2. Effect of E. faecium WB2000 on the Growth of Various P. gingivalis Strains. The effect of E. faecium WB2000 on the growth of five P. gingivalis strains (ATCC 33277, ATCC 53978, ATCC BAA-308, 2-1, and 7-1) was evaluated. The numbers of all P. gingivalis strains decreased to less than the detection limit at 24 and 48 h in mixed cultures with E. faecium WB2000 (Figure 2). In contrast, the number of E. faecium WB2000 reached a plateau at 24 h.

3.3. Effect of E. faecium WB2000 on VSC Production by P. gingivalis Strains. The concentrations of VSCs in spent culture medium were measured by gas chromatography (Table 2). The levels of H₂S produced by P. gingivalis strains were lower than those of CH₃SH. The concentrations of H₂S in mixed culture media were higher than that in medium in which only P. gingivalis was cultured, with the exception of medium from mixed cultures of two P. gingivalis clinical isolates (2-1 and 7-1) and E. faecium WB2000 after 48 h. In contrast, the CH₃SH concentration in mixed culture medium was lower than that in medium from culture of P. gingivalis alone, with the exception of medium from mixed cultures of two P. gingivalis strains (W50 and 2-1) and E. faecium WB2000 after 24 h. The levels of CH₃SH in spent medium from P. gingivalis cultured for 48 h in the presence or absence of E. faecium WB2000 are shown in Figure 3. Although CH₃SH production by P. gingivalis was strain dependent, the CH₃SH concentration was markedly lower in spent medium from mixed cultures of all P. gingivalis strains and E. faecium WB2000 than in medium from culture of the latter microorganism only.

4. Discussion

The hypothetical mechanisms of probiotic action in the oral cavity are (1) involvement in binding of oral microorganisms to proteins, (2) effects on plaque formation and its complex ecosystem by competing and interfering with interbacterial attachment, (3) involvement in the metabolism of substrates, and (4) production of compounds that inhibit oral bacteria [11]. E. faecium has been reported to inhibit biofilm formation by cariogenic bacteria [7, 8]. Kumada et al. [8] reported
Table 2: The hydrogen sulfide and methyl mercaptan concentrations in spent culture medium (ng/mL).

| Pg strain   | Culture medium       | Hydrogen sulfide | Methyl mercaptan |
|-------------|----------------------|------------------|------------------|
|             |                      | 24 h | 48 h | 24 h | 48 h |
| ATCC 33277 | Pg                   | 0.45 | 0.86 | 11.45 | 87.34 |
|            | Pg + Ef WB2000       | 0.94 | 1.49 | 2.38 | 1.53 |
| ATCC 53978 (W50) | Pg       | 0.87 | 2.20 | 3.40 | 507.27 |
|            | Pg + Ef WB2000       | 1.44 | 2.91 | 4.36 | 2.92 |
| ATCC BAA-308 (W83) | Pg       | 0.68 | 2.46 | 62.53 | 558.22 |
|            | Pg + Ef WB2000       | 1.75 | 2.83 | 6.61 | 4.32 |
| 2-1        | Pg                   | 0.43 | 2.18 | 1.70 | 164.44 |
|            | Pg + Ef WB2000       | 1.11 | 2.03 | 3.26 | 2.81 |
| 7-1        | Pg                   | 0.86 | 9.24 | 58.18 | 799.95 |
|            | Pg + Ef WB2000       | 2.06 | 2.44 | 6.03 | 4.05 |

Pg: Porphyromonas gingivalis; Ef: Enterococcus faecium.

Figure 3: The levels of CH$_3$SH in spent medium from P. gingivalis cultured for 48 h in the presence or absence of E. faecium WB2000 (ng/mL). Grey bars: single culture; white bars: dual culture with E. faecium WB2000.

a protein that inhibited biofilm formation by streptococci. In addition, our previous study suggested that E. faecium inhibits the growth of some mutans streptococci [7]. In this study, E. faecium WB2000 inhibited the growth of, as well as reduced CH$_3$SH production by, P. gingivalis. A study on L. salivarius TI 2711 reported that a low pH (≤6.0) and the presence of lactic acid (40–50 mmol/L) induced P. gingivalis death [12]. The pH of E. faecium WB2000 culture medium was 4.4 after 24 h of incubation (data not shown). The growth rate of E. faecium WB2000 was higher than that of the other lactic acid bacteria, suggesting that this organism rapidly inhibited the growth of P. gingivalis.

P. gingivalis strains produced CH$_3$SH and a low level of H$_2$S, as reported previously [13]. E. faecium WB2000 suppressed CH$_3$SH production by P. gingivalis but did not inhibit production of H$_2$S in the current study. Some probiotic bacteria produce VSCs in the presence of cysteine or methionine [14]. The GAM broth used in the current study contains cysteine, and thus E. faecium WB2000 might have produced H$_2$S. Streptococcus thermophilus inhibited the growth and H$_2$S, CH$_3$SH, and CH$_3$SCH$_3$ production of P. gingivalis [13]. To determine whether E. faecium WB2000 specifically inhibits production of CH$_3$SH, different media and culture conditions should be used. Furthermore, future studies are needed to evaluate other P. gingivalis strains producing high levels of H$_2$S.

In addition to E. faecium and S. thermophilus, other organisms have been reported to inhibit oral malodor. Streptococcus salivarius K12 suppressed the growth of Solobacterium moorei, which produces H$_2$S and is involved in halitosis [15, 16]. Lactobacillus salivarius WB21 and L. reuteri have been reported to inhibit oral malodor in clinical trials [17–19]. L. salivarius WB21 reduced the number of Fusobacterium nucleatum and ubiquitous bacteria in saliva [19].

E. faecium WB2000 has been used in traditional Japanese medicine (Strong Wakamoto®) to treat gastrointestinal discomfort, and its effect on dry eye was reported recently [20]. Moreover, the effect of dentifrice containing E. faecium WB2000 on plaque control has been investigated [9]. The findings of this study suggest that E. faecium WB2000 may reduce oral malodor by inhibiting the growth of P. gingivalis and neutralizing CH$_3$SH. The effect of E. faecium WB2000 on oral malodor will be investigated in a future clinical trial involving the use of dentifrice.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

This study was supported in part by a Grant-in-Aid for Young Scientists (no. 16K20707) and by Grants-in-Aid for Scientific Research (nos. 26463203 and 26463175) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

[1] C. Scully, S. Porter, and J. Greenman, "What to do about halitosis," British Medical Journal, vol. 308, no. 6923, pp. 217–218, 1994.
[2] S. Persson, M. B. Edlund, R. Claesson, and J. Carlsson, “The formation of hydrogen sulfide and methyl mercaptan by oral bacteria,” Oral Microbiology and Immunology, vol. 5, no. 4, pp. 195–201, 1990.
[3] I. Stamatova and J. H. Meurman, “Probiotics: health benefits in the mouth,” American Journal of Dentistry, vol. 22, no. 6, pp. 329–338, 2009.
[4] G. Pesavento, C. Calonico, B. Ducci, A. Magnanini, and A. Lo Nostro, “Prevalence and antibiotic resistance of Enterococcus spp. isolated from retail cheese, ready-to-eat salads, ham, and raw meat,” Food Microbiology, vol. 41, pp. 1–7, 2014.
[5] A. M. Guzman Prieto, W. van Schaik, M. R. Rogers et al., “Global emergence and dissemination of Enterococci as nosocomial pathogens: attack of the clones?” Frontiers in Microbiology, vol. 7, article 788, 2016.
[6] T. J. Eaton and M. J. Gasson, “Molecular screening of Enterococcus virulence determinants and potential for genetic exchange between food and medical isolates,” Applied and Environmental Microbiology, vol. 67, no. 4, pp. 1628–1635, 2001.
[7] N. Suzuki, M. Yoneda, Y. Hatano, T. Iwamoto, Y. Masuo, and T. Hirofuji, “Enterococcus faecium WB2000 inhibits biofilm formation by oral cariogenic streptococci,” International Journal of Dentistry, vol. 2011, Article ID 834151, 5 pages, 2011.
[8] M. Kumada, M. Motegi, R. Nakao et al., “Inhibiting effects of Enterococcus faecium non-biofilm strain on Streptococcus mutans biofilm formation,” Journal of Microbiology, Immunology and Infection, vol. 42, no. 3, pp. 188–196, 2009.
[9] Y. Hatano, N. Suzuki, M. Yoneda, and T. Hirofuji, “Clinical study on the improvement effect of a lactic acid bacterium-containing dentifrice (Avantbise) on oral hygiene,” The Japanese Journal of Conservative Dentistry, vol. 55, pp. 219–225, 2012 (Japanese).
[10] K. H. Schleifer and R. Kilpper-Bälz, “Transfer of Streptococcus faecalis and Streptococcus faecium to the genus Enterococcus nom. rev. as Enterococcus faecalis comb. nov. and Enterococcus faecium comb. nov.” International Journal of Systematic Bacteriology, vol. 34, no. 1, pp. 31–34, 1984.
[11] J. H. Meurman, “Probiotics: do they have a role in oral medicine and dentistry?” European Journal of Oral Sciences, vol. 113, no. 3, pp. 188–196, 2005.
[12] T. Matsuoka, M. Nakanishi, Y. Aiba, and Y. Koga, “Mechanism of Porphyromonas gingivalis killing by Lactobacillus salivarius T1 2711,” Journal of the Japanese Society of Periodontology, vol. 46, no. 2, pp. 118–126, 2004 (Japanese).
[13] S.-H. Lee and D.-H. Baek, “Effects of Streptococcus thermophilus on volatile sulfur compounds produced by Porphyromonas gingivalis,” Archives of Oral Biology, vol. 59, no. 11, pp. 1205–1210, 2014.
[14] R. Sreekumar, Z. Al-Attabi, H. C. Deeth, and M. S. Turner, “Volatile sulfur compounds produced by probiotic bacteria in the presence of cysteine or methionine,” Letters in Applied Microbiology, vol. 48, no. 6, pp. 777–782, 2009.
[15] A. S. Stephen, D. P. Naughton, R. L. Pizziy, D. J. Bradshaw, and G. R. Burnett, “In vitro growth characteristics and volatile sulfur compound production of Solobacterium moorei,” Anaerobe, vol. 26, pp. 53–57, 2014.
[16] L. Masdea, E. M. Kulik, I. Hauser-Gerspach, A. M. Ramseier, A. Filippi, and T. Waltimo, “Antimicrobial activity of Streptococcus salivarius K12 on bacteria involved in oral malodour,” Archives of Oral Biology, vol. 57, no. 8, pp. 1041–1047, 2012.
[17] T. Iwamoto, N. Suzuki, K. Tanabe, T. Takeshita, and T. Hirofuji, “Effects of probiotic Lactobacillus salivarius WB21 on halitosis and oral health: an open-label pilot trial,” Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontontology, vol. 110, no. 2, pp. 201–208, 2010.
[18] M. K. Keller, A. Bardow, T. Jensdottir, J. Lykkeaa, and S. Twetman, “Effect of chewing gums containing the probiotic bacterium Lactobacillus reuteri on oral malodour,” Acta Odontologica Scandinavica, vol. 70, no. 3, pp. 246–250, 2012.
[19] N. Suzuki, M. Yoneda, K. Tanabe et al., “Lactobacillus salivarius WB21-containing tablets for the treatment of oral malodor: a double-blind, randomized, placebo-controlled crossover trial,” Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology, vol. 117, no. 4, pp. 462–470, 2014.
[20] M. Kawashima, S. Nakamura, Y. Izuta, S. Inoue, and K. Tsushima, “Dietary supplementation with a combination of lactoferrin, fish oil, and Enterococcus faecium WB2000 for treating dry eye: a rat model and human clinical study,” The Ocular Surface, vol. 14, no. 2, pp. 255–263, 2016.