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Weissella cibaria CMU suppresses *mgl* gene expression and enzyme activity associated with bad breath

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The oral care probiotic strain *Weissella cibaria* CMU (oraCMU) inhibits volatile sulphur compounds associated with halitosis, presumably by inhibiting the growth of associated oral pathogens. In the present study, we investigated whether oraCMU inhibits the production of these compounds by suppressing the expression of *mgl*. This gene encodes L-methionine-α-deamino-γ-mercaptomethane-lyase (METase) and is involved in the production of methyl mercaptan (CH_3SH) by *Porphyromonas gingivalis*. Therefore, we specifically investigated the effects of oraCMU on the growth, CH_3SH production, METase activity, and *mgl* expression of *P. gingivalis*. The minimum inhibitory concentrations of cell-free supernatant and secreted proteins from oraCMU were 125 mg/mL and 800 μg/mL, respectively. At sub-minimum inhibitory concentration levels, these metabolites inhibited CH_3SH production, but they also reduced *P. gingivalis* viability. Only heat-killed oraCMU decreased CH_3SH production without affecting P. gingivalis viability. Heat-killed oraCMU also inhibited METase activity toward L-methionine and *mgl* mRNA expression ($p < 0.05$). In summary, we demonstrated the inhibition of volatile sulphur compounds via the antimicrobial action of oraCMU and, for the first time, the inhibition of such compounds by heat-killed oraCMU, which occurred at the molecular level.

Keywords: Probiotics, Halitosis, *Porphyromonas gingivalis*, Methyl mercaptan, L-methionine-α-deamino-γ-mercaptomethanelyase

Introduction

Bad breath (halitosis) is an unpleasant odour from the mouth, of which volatile sulphur compounds (VSCs) are the main constituents [1]. Oral VSCs originate from the use of sulphur-containing amino acids by oral Gram-negative anaerobic bacteria, such as *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Prevotella intermedia*, and *Treponema denticola* [2,3]. Specifically, cysteine and methionine are sulphur-harbouring amino acids known to act as substrates for these bacteria, and which can be catabolized to produce hydrogen sulphide (H_2S) and methyl mercaptan (CH_3SH) [4]. Reportedly, ~90% of the VSC content in the human breath is composed of H_2S and CH_3SH [5].

L-methionine-α-deamino-γ-mercaptomethane-lyase (METase) catalyses the α, γ-elimination of L-methionine to

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produce CH_3SH, α-ketobutyrate, and ammonia, while L-cysteine desulfhydrase produces H_2S, pyruvate, and ammonia from L-cysteine [4]. *P. gingivalis* is known to produce large amounts of CH_3SH through METase, which is encoded by the *mgl* gene [6]. METase can be used as a target to reduce VSCs, however, this enzyme is not found in mammals and appears to be an intracellular enzyme, i.e., crude bacterial extracts obtained by removing cell membranes exhibited lyase activities [4].

Recently, oral-derived probiotics have been studied as a substitute to currently available antimicrobial chemicals for the management of bad breath [7,8]. Probiotic strains, such as *Streptococcus salivarius* K12 [9], *Lactobacillus salivarius* WB21 [10], and *Lactobacillus reuteri* (DSM 17938 and ATCC PTA 5289) [11] have been shown to suppress halitosis. In particular, *Weissella cibaria* strain CMU is a lactic acid bacterium (LAB) isolated from the saliva of healthy Korean children that is now actively commercialized as an oral care probiotic in Korea [12,13]. *W. cibaria* is a short rod-shaped, heterofermentative Gram-positive LAB [14] and is also widely distributed in kimchi [15]. These strains reportedly act by inhibiting the growth of halitosis-causing bacteria through antimicrobial action, however; to our knowledge, halitosis inhibition has not been investigated at the molecular level.

Although the ingestion of commercial oral probiotics presumably suppresses the metabolic activity or abundance of bacteria in the oral microflora, the mechanism of action involved in reducing the VSCs has not yet been elucidated in detail. Therefore, the aims of the present study were to 1) investigate the in vitro effects of *W. cibaria* CMU on the METase activity of *P. gingivalis* involved in the production of VSCs and 2) determine whether *W. cibaria* CMU could reduce CH_3SH production at sub-minimum inhibitory concentration (sub-MIC) levels by suppressing the expression of *mgl* in *P. gingivalis*.

Materials and Methods

1. Bacterial strains and growth conditions

W. cibaria CMU (oraCMU; OraPharm Inc, Seoul, Korea), which has oral care probiotic properties [13], was used to clarify the mechanism of VSC inhibition *in vitro*. oraCMU was grown aerobically in MRS broth (Difco Laboratories, Detroit, MI, USA) at 37℃ for 16 hours. To prepare the cell-free supernatant (CFS) of oraCMU, the cells were removed by centrifugation (4000 × *g*, 20 minutes, 4℃) and then filter-sterilized (0.45 μm pore size; Millipore, Burlington, MA, USA). To prepare the secreted proteins (SPs), the total SPs in the stationary phase of oraCMU growth were precipitated as previously described [16], with some modifications. Sodium deoxycholate (Sigma Aldrich, St. Louis, MO, USA) was added to the CFS at a final concentration of 0.2% (v/v), mixed, and incubated on ice for 30 minutes. Thereafter, chilled trichloroacetic acid (TCA; Sigma Aldrich) was added at a final concentration of 6% (v/v), vortexed for 30 seconds, and allowed to precipitate overnight at 4℃. The proteins were recovered by centrifugation (9300 × *g*, 10 minutes, 4 ℃). The resultant pellet was washed twice with 2 mL of chilled acetone (Sigma Aldrich), harvested by centrifugation (15000 × *g*, 10 minutes, 4℃), dried at 25℃, and finally re-solubilized in 1 mL of 0.02 M Tris (pH 8.8). To prepare the heat killed (HK)-oraCMU, the bacteria were harvested, washed twice, and resuspended with phosphate-buffered saline (PBS). The bacteria were then exposed to heat (100℃) for 10 minutes. *P. gingivalis* KCTC 5352 was purchased from the Korean Culture Collection for Type Cultures (KCTC, Daejeon, Korea). *P. gingivalis* was grown anaerobically (AnaeroPack-Anaero; Mitsubishi Gas Chemical Co., Tokyo, Japan) in tryptic soy broth (Kisan Bio Co., Ltd., Seoul, Korea) supplemented with 5 μg/mL hemin (Kisan Bio) and 0.5 μg/mL menadione (Kisan Bio) at 37℃ for 48 hours.

2. Bacterial susceptibility assay

The antibacterial activities of the CFS and SP of oraCMU were determined using a broth microdilution method, as previously described [17]. The MIC was defined as the lowest dilution of tested substances at which no growth was detected. Growth of *P. gingivalis* in the presence of sub-MICs of oraCMU was determined at 600 nm with a microplate reader (VersaMax; Molecular devices, San Jose, CA, USA) after anaerobic incubation at 37℃ for 48 hours. The antibacterial activity of oraCMU was also determined via phase contrast microscopic analysis (DCS 6002; Dr. PREVENT, Seoul, Korea).

3. Methyl mercaptan production assays

CH_3SH production assays were conducted according to previously reported methods [18] to determine the effects of sub-MICs of CFS or SP of oraCMU on the production of CH_3SH. Specifically, *P. gingivalis* was cultured in 96-well microtiter plates treated with sub-MICs of CFS or SP of oraCMU. The effect of HK-oraCMU on CH_3SH production was also investigated. *P. gingivalis* and HK-oraCMU were adjusted to OD_{600} = 0.05

(–5 × 10^7 CFU/mL) and 0.5 (–5 × 10^8 CFU/mL) using growth medium, respectively. Equal amounts of *P. gingivalis* and HK-oraCMU were inoculated into 96-well plates. After 48 hours of anaerobic incubation, 10 μL of L-methionine (0.6%, w/v) and 10 μL of 5,5'-dithiobis (2-nitrobenzoic acid) (0.06%, w/v) were added to each well and the plates were further cultured for 12 hours. The production of CH_3SH was measured with a microplate reader at 430 nm.

4. XTT reduction assay

To determine if the reduced CH_3SH production at sub-MIC levels of oraCMU was due to growth suppression, the viability of *P. gingivalis* was assessed using an 2, 3-bis [2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) cell proliferation assay kit (iNtRON, Seongnam, Korea) [19]. *P. gingivalis* was grown in the presence of sub-MIC levels of CFS, SP, and HK-oraCMU in a 96-well plate using protocols similar to those described for the MIC assay. After 48 hours incubation at 37℃, 50 μL of the XTT solution (0.2 mg/mL) was added to each well, and the plate was further incubated at 37℃ for 1 hour. The viability of *P. gingivalis* was measured at 490 nm.

5. L-methionine-α-deamino-γ-mercaptomethane-lyase assay

To determine the effect of HK-oraCMU on the METase of *P. gingivalis*, METase activity was measured as described previously [6]. Simply, a crude bacterial extract from *P. gingivalis* was prepared using the NoviPure Microbial Protein Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The protein contents in the extract were measured using a DC protein assay kit (Bio-Rad, Hercules, CA, USA) and bovine serum albumin as the standard. The *P. gingivalis* extract (with a protein concentration of 150 μg/mL) was incubated with 1 mL of the HK-oraCMU (OD_{600} = 0.5) at 37℃ for 10 minutes. The substrate solution of L-methionine (40 μL, Sigma Aldrich) was added to 360 μL of the reaction mixture so that the final concentration of substrate was 10 mM. After incubation for 90 minutes at 37℃, the reaction was stopped by mixing with 200 μL of 4.5 % (w/v) TCA. The crude bacterial extract solution was centrifuged (12,000 × *g*, 5 minutes, 4℃) and 100 μL of the supernatant was mixed with 100 μL of 0.05 % (w/v) 3-methyl-2-benzothiazolone hydrazine hydrochloride (MBTH; Sigma Aldrich) in 1 M sodium acetate (pH 5.2). The amount of α-ketobutyrate was measured at 340 nm with a microplate reader after incubation for 30 minutes at 50℃. PBS-treated crude bacterial extract of *P. gingivalis* was simultaneously used as the control.

6. Analysis of *mgl* expression by quantitative real-time polymerase chain reaction (qRT-PCR)

P. gingivalis grown in the presence of HK-oraCMU was collected by centrifugation to investigate whether *mgl* gene expression of *P. gingivalis* was inhibited by the HK-oraCMU. Total RNA was isolated using the easy-spin total RNA extraction kit (iNtRON) according to the manufacturer's instructions. Relative quantification of gene expression by real-time PCR was performed using the Rotor-Gene Q system (QIAGEN). cDNA synthesis and real-time PCR reactions were conducted using a QuantiFast SYBR green RT-PCR kit (QIAGEN) to quantify *mgl* mRNA expression. *P. gingivalis*-specific 16S rRNA expression was used as the internal control. The following primer sequences were used: *mgl* (5'-TCGTGCTTATGAGCGATGTC-3'; 5'-GGAAGTCACCCTCGTGGATA-3') [20] and *P. gingivalis*-specific 16S rRNA (5'-TGTAGATGACTGATGGTGAAAACC-3'; 5'-TTTAGAGATTCGCATCCGGT-3') [21]. The $2^{-\Delta\Delta CT}$ method was used to compare the relative expression levels of the *mgl* gene. Results were expressed as fold-change values relative to the control samples.

7. Statistical analysis

Experiments were conducted in triplicate and the resultant mean values were analysed further. Statistical analyses were performed using IBM SPSS software (version 21.0; IBM Co., Armonk, NY, USA). A Mann-Whitney U test was used to determine any statistically significant differences among treatments ($p < 0.05$).

Results

1. Determination of antibacterial activity of oraCMU against *P. gingivalis*

As shown in Fig. 1, the CFS (MIC = 125 mg/mL) and SP (MIC = 800 μg/mL) of oraCMU inhibited the growth of *P. gingivalis* ($p < 0.05$). Phase contrast microscopy revealed that the *P. gingivalis* cells were destroyed by oraCMU and thus their numbers were markedly reduced (Fig. 2). In addition, the cell morphology of *P. gingivalis* in the oraCMU-treated group was

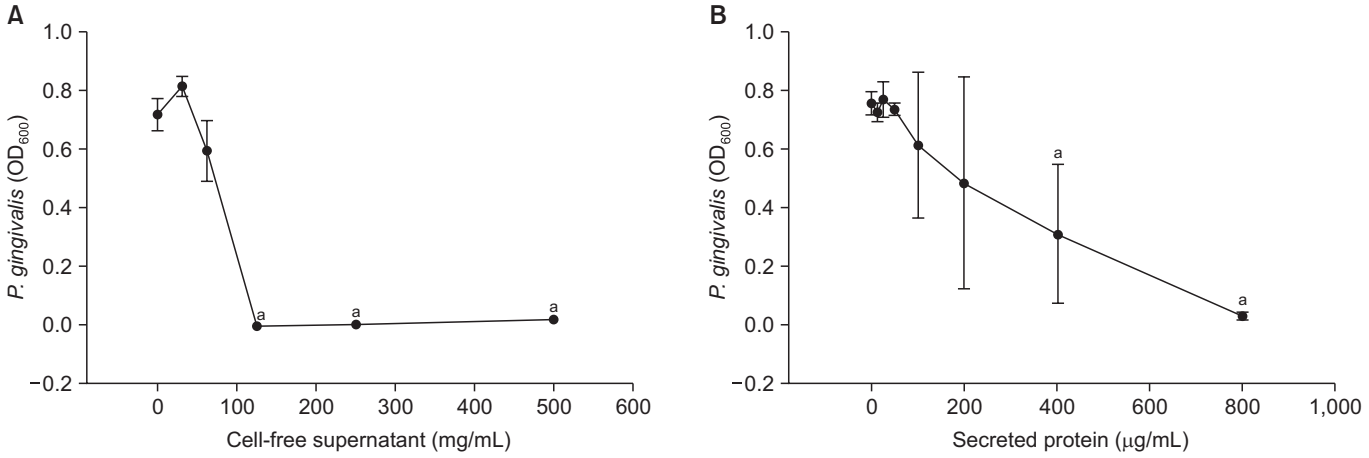

Fig. 1. Determination of the minimum inhibitory concentration of the cell-free supernatant and secreted protein of oraCMU on *Porphyromonas gingivalis*. Serial doses of cell-free supernatant (A) or secreted protein (B) were added to *P. gingivalis* cultures in 96-well plates and incubated under anaerobic conditions for 24 hours. The growth of *P. gingivalis* was measured by optical density (600 nm). Values represent means ± standard deviations of three independent experiments.
[a]$p < 0.05$.

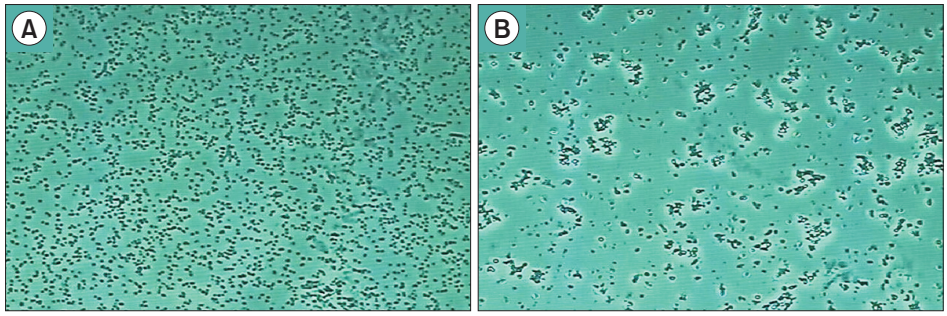

Fig. 2. Phase-contrast microscopic analysis of antimicrobial activity in the presence of MIC of secreted protein of oraCMU. (A) Untreated *Porphyromonas gingivalis* control. (B) *P. gingivalis* with secreted protein at 800 μg/mL. Magnification: × 4000.

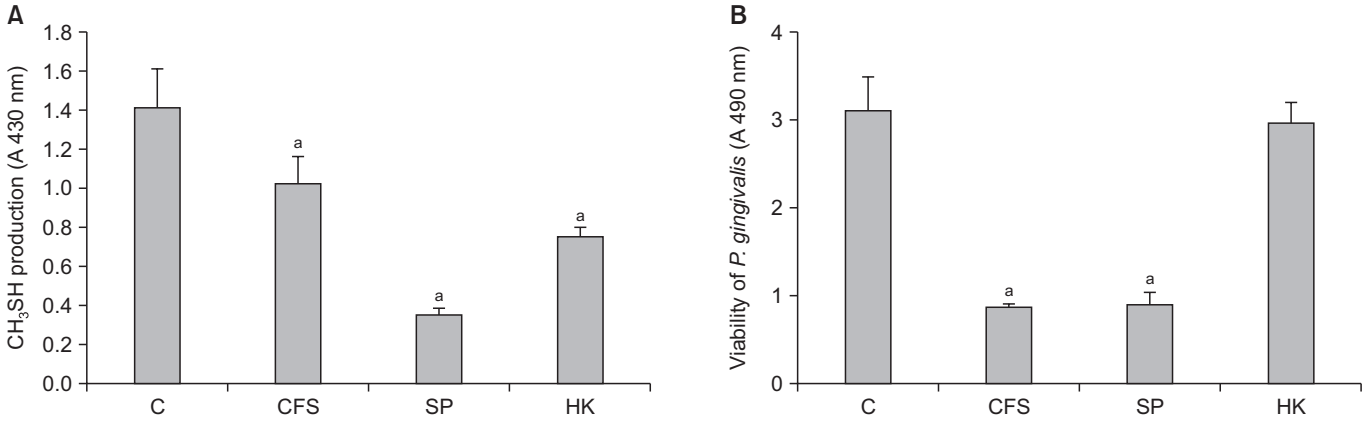

Fig. 3. Effects of cell-free supernatant (CFS), secreted protein (SP), and heat killed (HK)-oraCMU on the methyl mercaptan (CH_3SH) production by *Porphyromonas gingivalis*. (A) *In vitro* methyl mercaptan production in the presence of sub-minimum inhibitory concentration (MIC) (CFS, 62.5 mg/mL; SP, 250 μg/mL). (B) Viability of *P. gingivalis* in the presence of sub-MIC determined by an 2, 3-bis [2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) reduction assay. Values represent means ± standard deviations of three independent experiments.
C, non-treated control.
[a]$p < 0.05$.

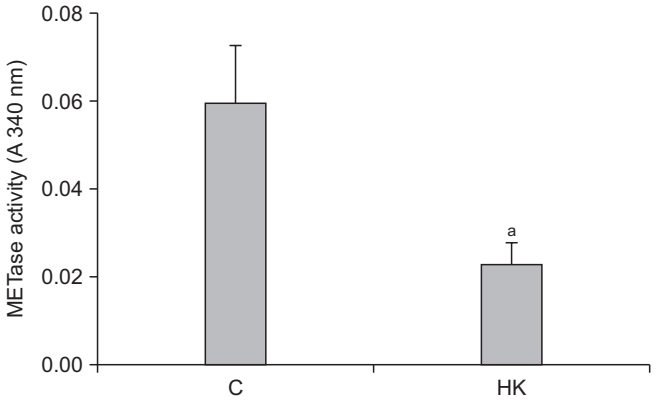

Fig. 4. Effect of heat-killed (HK)-oraCMU on L-methionine-α-deamino-γ-mercaptomethanelyase (METase) activity in *Porphyromonas gingivalis*. Bars and error bars indicate the means ± standard deviations of three independent experiments.
C, non-treated control.
[a]$p < 0.05$.

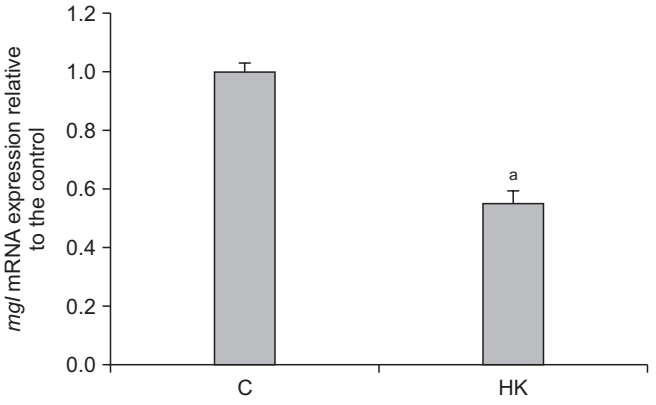

Fig. 5. Effect of heat-killed (HK)-oraCMU on the mRNA expression of *mgl* in *Porphyromonas gingivalis*. Bars and error bars indicate the mean ± standard deviation of three independent experiments.
C, non-treated control.
[a]$p < 0.05$.

not uniform in the form of short rods, but showed abnormal morphological changes and the cells appeared to rupture and gather together.

2. Effects of oraCMU on the production of methyl mercaptan

The production of CH_3SH by *P. gingivalis* decreased as the CFS or SP concentrations of oraCMU increased (data not shown). The CH_3SH production of *P. gingivalis* was significantly inhibited by the CFS and SP of oraCMU at sub-MIC levels of 62.5 mg/mL and 250 μg/mL, respectively ($p < 0.05$). The XTT reduction assay revealed that the viability of *P. gingivalis* was greatly reduced at these sub-MIC levels compared to the untreated control. On the other hand, HK-oraCMU inhibited the production of CH_3SH by *P. gingivalis* without affecting the viability of *P. gingivalis* (Fig. 3). Therefore, HK-oraCMU was selected and used in further experiments.

3. Effects of HK-oraCMU on METase activity

Crude bacterial extracts of *P. gingivalis* were used to determine whether HK-oraCMU affected the METase involved in the production of CH_3SH in *P. gingivalis*. The crude bacterial extract of *P. gingivalis* exposed to HK-oraCMU for 10 minutes showed significantly lower METase activity toward L-methionine ($p < 0.05$) than that of the control (i.e., incubated with PBS only) (Fig. 4).

4. Effect of HK-oraCMU on *mgl* expression

The inhibitory effect of HK-oraCMU on *mgl* expression is shown in Fig. 5. The mRNA expression levels of *mgl* were normalized by amplification of the 16S rRNA of *P. gingivalis* as an internal control. The HK-oraCMU significantly inhibited the *mgl* expression in *P. gingivalis* by 45% compared to the untreated control ($p < 0.05$).

Discussion

Bad breath is known to occur mainly in the oral cavity and is caused by bacteria that form a biofilm on the tongue [3]. Our previous studies revealed at least 3 different mechanisms, involving organic acids, hydrogen peroxide, and secretory proteins, by which oraCMU suppresses the proliferation of malodour-inducing bacteria [17]. oraCMU has been demonstrated to inhibit halitosis in human clinical studies [12] as well as animal studies using Beagle dogs [22,23]. In addition, previous studies confirmed that oraCMU has a colonization ability in the oral cavity [24]. Therefore, oraCMU is likely to affect halitosis-causing bacteria in tongue coatings. However, until now, halitosis suppression by oraCMU had not been studied at the molecular level. The present study thus aimed to identify the mechanism of VSC reduction of oraCMU at the molecular level.

P. gingivalis, a major periodontal pathogen that is associated with chronic periodontitis, is known to possess METase that can break down L-methionine to produce large amounts of CH_3SH [4,6]. In the present study, oraCMU exhibited bacteri-

cidal and METase-inactivating effects on *P. gingivalis*. METase activity decreased in the presence of HK-oraCMU without antimicrobial activity. These results suggested that the inactivation of METase was not likely to be directly correlated with the viability of *P. gingivalis*. Our previous study reported that HK-oraCMU could inhibit the expression of the pro-inflammatory cytokine genes induced by *F. nucleatum* to a similar extent to live bacteria [25]. Yu et al. [26] also reported that treatment with HK-*W. cibaria* JW15 decreased nitric oxide production and suppressed the expression of pro-inflammatory cytokines in lipopolysaccharide -induced RAW 264.7 cells.

Various *W. cibaria* strains have been shown to exert beneficial traits, such as immunomodulatory effects [27], anti-inflammatory effects [28], and anticancer effects [29], as well as to produce dextran, which is used as commercial prebiotics [30]. In addition, *W. cibaria* has been shown to be safe for consumption and is thus a registered food ingredient in the Korean Food and Drug Administration [31]. Recently, products containing *W. cibaria* have been marketed for the prevention of bad breath. The inactivation of METase by oral care probiotics such as oraCMU may represent safe and effective approaches for the prevention of oral malodour. Since the removal of L-methionine by METase is an important process in the production of VSC, the inactivation of bacterial METase is thought to be a more fundamental approach than masking or deodorising to inhibit oral malodour for extended periods of time.

In the present study, HK-oraCMU inhibited the METase activities of the crude bacterial extract of *P. gingivalis*. The concentration of HK-oraCMU (~5 × 10^8 CFU/mL) used in the current study was within the range of concentrations of live bacteria used in previous studies using Beagle dogs. These concentrations inhibited the production of CH_3SH in Beagle dogs [22,23]. In addition, we demonstrated that *P. gingivalis* METase activity on L-methionine was significantly lower than that of the untreated control when reacted with HK-oraCMU for 10 minutes. Compared to other delivery products, such as capsules and powders, we recommend that tablets are the most appropriate product type to enable a sufficient amount of time for the inactivation of METase of *P. gingivalis*. In addition, commercialized tablets made from oraCMU should be designed to dissolve slowly in the mouth to extend the duration and active period in the mouth.

The METase inactivation by oraCMU may be one of the possible mechanisms of action to reduce VSCs. METase is encoded by the gene *mgl*, which is reportedly responsible for the production of CH_3SH as well as for the virulence of *P. gingivalis* [6]. Since CH_3SH is known to be more correlated with oral malodour intensity than H_2S [5], we only assessed the *mgl* gene in the present study. Specifically, we investigated the inhibitory effect of oraCMU on the CH_3SH production of *P. gingivalis*.

The antimicrobial effect of oraCMU against *P. gingivalis* is consistent with the more general action of reducing VSC in a previous study [13]. Because the presence of specific periodontal bacteria on the tongue is known to be highly correlated with the strength of malodour, a reduction in these microbes can improve halitosis. In addition to the antimicrobial mechanisms, we also demonstrated a more specific mechanism for CH_3SH inhibition by HK-oraCMU at the genetic level, i.e., the suppression of *mgl* expression at sub-MIC levels without affecting cell viability of *P. gingivalis*. Although the suppression of the transcriptional levels of the *mgl* gene of oral anaerobic bacteria by some substances has been reported [20,32,33], this is the first study to demonstrate the specific effect of oral care probiotics on halitosis suppression at the genetic level.

In addition, we showed that not only the live but also the HK-oraCMU suppressed the odour component, and that the mechanism of CH_3SH suppression occurred as a result of the inhibition of the *mgl* gene of *P. gingivalis*. The METase activity of *P. gingivalis* was confirmed to be susceptible to HK-oraCMU. For industrial product development, we recommend that not only live but also dead bacteria be used during the distribution period since both are able to suppress halitosis, and thus the burden of stability guarantee for the functioning of probiotics would presumably be lower.

In conclusion, the present study indicated that the CFS of oraCMU suppressed the production of VSCs not only through its bactericidal effects on *P. gingivalis*, but also via its inactivating effects on bacterial METase. In addition, this study demonstrated that HK-oraCMU inhibited CH_3SH production by inhibiting METase activity as well as inhibiting the expression of the *mgl* gene of *P. gingivalis*. The results of this study are expected to further our understanding of the mechanism of action of oraCMU in the oral cavity.

Acknowledgements

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Conflicts of interest

No potential conflict of interest relevant to this article was reported.

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