J Clin Periodontol 2006; 33: 226–232 doi: 10.1111/j.1600-051X.2006.00893.x

Journal of Clinical Periodontology

Inhibitory effect of *Weissella cibaria* isolates on the production of volatile sulphur compounds

Mi-Sun Kang[1], Byung-Gook Kim[2], Jin Chung[3], Hyun-Chul Lee[1] and Jong-Suk Oh[1]

[1]Department of Microbiology and Immunology, School of Medicine, Chonnam National University, Gwangju, Korea; [2]Dental Science Research Institute, Chonnam National University, Gwangju, Korea; [3]Department of Microbiology, College of Dentistry, Pusan National University, Busan, Korea

Kang M-S, Kim B-G, Chung J, Lee H-C, Oh J-S. Inhibitory effect of Weissella cibaria *isolates on the production of volatile sulphur compounds. J Clin Periodontol 2006; 33: 226–232. doi: 10.1111/j.1600-051X.2006.00893.x.* Blackwell Munksgaard, 2006.

Abstract

Aims: The objective of this study was to characterize the inhibitory effects of *Weissella cibaria* isolates on volatile sulphur compounds (VSC) production both in vitro and in vivo.

Material and Methods: We isolated and identified three hydrogen peroxide-generating lactobacilli from children's saliva, and assessed their inhibitory effects on VSC production and *Fusobacterium nucleatum* proliferation. Clinical studies were conducted with 46 subjects in order to measure the VSC of their mouth air.

Results: These lactobacilli were identified as *W. cibaria*. These isolates inhibited the production of VSC by *F. nucleatum* ($p < 0.05$). The concentration of *F. nucleatum* was decreased by 5-log cycles as a result of exposure to the *W. cibaria* strains ($p < 0.05$), whereas the catalase-treated *W. cibaria* cultures exerted no evident inhibitory effects on *F. nucleatum* replication. In the clinical studies, gargling with one isolate resulted in a significant reduction in the levels of H_2S and CH_3SH by approximately 48.2% ($p < 0.01$) and 59.4% ($p < 0.05$), respectively.

Conclusions: These results indicate that *W. cibaria* isolates possess the ability to inhibit VSC production under both in vitro and in vivo conditions, demonstrating that they bear the potential for development into novel probiotics for use in the oral cavity.

Key words: *Fusobacterium nucleatum*; hydrogen peroxide; volatile sulphur compounds; *Weissella cibaria*

Accepted for publication 14 October 2005

Oral malodour, also known as halitosis, is a widespread problem, which is believed to affect a large proportion of the population. It is estimated that more than 50% of the population in North America and about 25% of the population in Japan have halitosis (Tessier & Kulkarni 1991, Miyazaki et al. 1995, Bosy 1997). Volatile sulphur compounds (VSC), which include hydrogen sulphide (H_2S) and methyl mercaptan (CH_3SH), both of which comprise about 90% of the VSC contents in breath, have generally been used as indicators for halitosis (Tonzetich 1971). These compounds are believed to be produced by Gram-negative anaerobic bacteria, including *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Treponema denticola* (Persson et al. 1990, De Boever & Loesche 1995). Cysteine and methionine are the sulphur-harbouring amino acids known to function as substrates for these bacteria, and they can be catabolysed into VSC, in particular, H_2S and CH_3SH.

Several studies have demonstrated that, among the many known oral bacteria, *F. nucleatum* produces copious VSC (Pianotti et al. 1986, Claesson et al. 1990, Persson et al. 1990). *F. nucleatum* has been isolated in cases of skin ulcers, peritonsillar abscesses, septic arthritis, and endocarditis, and is also naturally present as a microfloral component in the mouths of healthy or humans with diseases. This microorganism is not only predominant in terms of its numbers in the oral cavity, but also possesses the capacity to form aggregates with other bacteria, and to function as a bridge between the primary and secondary settlers on the surfaces of the teeth (Bolstad et al. 1996).

Many authors have reported that antimicrobial compounds, including metal salts and chlorhexidine, can reduce or inhibit halitosis via interaction with sulphur in the VSC precursors, as well as via the inhibition of VSC-producing bacteria (Yaegaki & Suetaka 1989, Young et al. 2001, Roldan et al. 2004, Fine et al. 2005). However, some of these agents exhibit only temporary effects, or are associated with undesirable side effects when used in the oral cavity for long periods. Lactic acid bacteria are known to be normal flora in the mouth, intestine, and vagina (Ahrne et al. 1998). They are considered to perform an opportunistic function in the development of dental caries via the generation of lactic acid (Babaahmady

et al. 1998). However, some lactic acid bacteria have been shown to protect the gastrointestinal systems of humans and animals from a variety of pathogenic infections (Gill et al. 2001), and these organisms have been extensively studied for their possible utility in probiotics. Recently, Burton et al. (2005) have reported that probiotic bacterial strains, which were originally sourced from the indigenous oral microbiotas of healthy humans, might have potential applications as adjuncts for both the prevention and treatment of halitosis.

Weissella is a lactic acid bacterial species, and was formerly included in the genus *Lactobacillus*. *Weissella cibaria* is a Gram-positive, non-spore forming, non-motile, heterofermentative, and catalase-negative bacillus, which is generally isolated in fermented foods, and also exists in humans (Bjorkroth et al. 2002). *W. cibaria* has never, as yet, been associated with any evidence of pathogenicity.

The objective of this study, then, was to determine the inhibitory effects, if any, generated by *W. cibaria* isolates on the production of VSC by *F. nucleatum*. Additional clinical studies were also conducted in order to determine the inhibitory effects of solutions containing *W. cibaria* on morning bad breath in a series of healthy adults.

Material and Methods

Isolation of hydrogen peroxide-generating lactobacilli

The oral examinations were conducted with 460 kindergarten children of ages between 4 and 7 years, all of whom lived in Gwangju, South Korea. We obtained saliva samples from children who had little supragingival plaque and no oral diseases including dental caries. These samples were then serially diluted with sterile 0.9% NaCl solution and spread onto MRS (Difco, Detroit, MI, USA) or Rogosa (Difco) agar plates. After 48 h of incubation at 37°C, the colonies were selected and subcultured on fresh MRS agar plates. The lactobacilli were successively transferred to MRS agar plates several times until pure colonies were obtained. Each of the lactobacilli was then tested with regard to its ability to generate hydrogen peroxide using a modified version of the method described by Eschenbach et al. (1989). Each of the lactobacilli was plated onto MRS agar, which contained 0.25 mg/ml of TMB (3,3′,5,5′-tetramethylbenzidine, Sigma, St Louis, MO, USA) and 0.01 mg/ml of peroxidase (Sigma), and was incubated at 37°C for 48 h, after which the isolates were exposed to the ambient air. The horseradish peroxidase in the medium induced the oxidation of TMB in the presence of hydrogen peroxide, hydrogen peroxide-generating bacteria, resulting in the formation of blue colonies. Because the peroxidase was unstable, these plates were used within 3 days of their preparation.

The levels of hydrogen peroxide in the spent culture supernatants of 140 lactobacilli isolates were determined spectrophometrically by the *o*-dianisidine horseradish peroxidase method (Tomas et al. 2004). Three strains that generated substantial levels of hydrogen peroxide, were designated as CMU, CMS2, and CMS3, and were identified via a standard biochemical test, using the API CH50 system (Biomerieux, Marcy, l'Etoile, France), and also by 16S rDNA sequence analysis.

Bacterial strains and culture conditions

Four VSC-producing anaerobic bacteria were examined, including *F. nucleatum* ATCC 10953, *P. gingivalis* A7A1-28, *T. denticola* ATCC 35405, and *Prevotella loescheii* ATCC 15930. Each species was from a laboratory stock culture. *F. nucleatum*, *P. gingivalis,* and *P. loescheii* were grown in BHI broth (Difco) that had been enriched with 0.5% yeast extract, 5 μg/ml haemin (Sigma), and 1 μ/ml menadione (Sigma), at 37°C for 24 h under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2). *T. denticola* was grown in GM-1 broth (Blakemore & Canale-Parola 1976) at 37°C for 72 h under anaerobic conditions. The isolates were maintained in MRS broth. All of the strains were maintained as 20% glycerol stock at −80°C. Before use in each experiment, the strains were propagated twice in the appropriate broth.

Coaggregation assays

Three lactobacilli isolates were tested with regard to their capacity to coaggregate with anaerobic bacteria including *F. nucleatum*, *P. gingivalis*, *T. denticola*, and *P. loescheii*. These isolates were grown in MRS broth at 37°C for 18 h under anaerobic conditions. The anaerobic bacteria were cultured in the appropriate broth for 24–72 h. Coaggregation experiments were conducted via the spectrophotometric assay (Handley et al. 1987) with some modifications. The cells were harvested by centrifugation at 4000 × *g* for 15 min., washed three times with coaggregation buffer (Cisar et al. 1979), and re-suspended in coaggregation buffer at a concentration of approximately 10^9 cells/ml. An equal volume (1 ml) of bacterial cells of a coaggregation pair was mixed together by vortexing for 10 s, and incubated at 37°C with gentle agitation at 110 rpm. The tubes were then removed after 30 min, and were allowed to stand for 3 min in order to precipitate the aggregates. The supernatant fluid (0.5 ml) was removed carefully, and its optical density was measured at 660 nm with a spectrophotometer (U2000, Hitachi, Nissei, Japan). The percentage of coaggregation was calculated using the equations of Handley et al. (1987).

In vitro studies — VSC analysis of incubated bacterial culture

We investigated the capacities of the isolates to inhibit VSC production of *F. nucleatum*. After coaggregation, 1 ml of the coaggregation buffer was discarded, and 2 ml of the test medium (growth medium (pH 7.0) containing 0.1% cysteine, 0.2% $FeSO_4$, and 0.1 M MES (2-morpholinoethanesulphonic acid)) was carefully added in a sterile tube that was sealed with a silicon plug without breaking the aggregates in order to detect H_2S production (Padron & Dockstader 1972, Langendijk et al. 1999). The test tubes were then incubated at 37°C for 24 h under anaerobic conditions. H_2S production was assessed by determining the degree of appearance of insoluble black iron sulphide (FeS) precipitates in the test tubes. In order to determine the effects exerted by the hydrogen peroxide that was generated by the isolates; a test medium containing 1000 U/ml of catalase (Sigma) was used. In addition, a sample of the vapour above the culture was removed using a gas-tight syringe, and was analysed via portable gas chromatograph (Oral Chroma CHM-1, Abilit, Osaka, Japan). In order to determine the effects exerted by all of these factors on the proliferation of *F. nucleatum*, the cultures were serially diluted and plated onto CVE agar (erythromycin-crystal violet agar) for *F. nucleatum* (Walker et al. 1979), and on MRS agar for the lactobacilli isolates. The experimental

values were statistically analysed using the Mann–Whitney test.

Clinical studies — VSC analysis of mouth air

Forty-six healthy subjects (28 males and 18 females) between the ages of 20 and 30, all of whom were dental school students at the Chonnam National University in Gwangju, South Korea, and had volunteered to participate in the study, were enrolled. All of the test subjects were instructed to refrain from smoking, drinking, and eating, after they had brushed their teeth in the evening, until the next morning when the VSC analyses were conducted. The VSC analyses were conducted before and after gargling of the test solution containing isolate CMU. The test solution was prepared by the centrifugation, washing, and suspension of the culture (10^9 CFU/ml) in sterile distilled water. Sterile distilled water was used as negative control. Commercial lactic acid bacteria including *Lactobacillus casei* and *Weissella confusa* were isolated from yogurts in a market and used as a placebo rinse. Like the test solution, placebo was prepared by centrifugation, washing, and suspension of the culture (10^9 CFU/ml) with sterile distilled water.

On the first day, the VSC analyses were conducted in the morning, before the subjects had brushed their teeth (7:00–8:00 hours). After the analysis, the subjects gargled twice a day with 15 ml of the test solution for 2 min. In brief, gargling was carried out first in the afternoon (between 14:00 and 15:00 hours), and then again in the evening (between 19:00 and 20:00 hours), at least 30 min after the subjects had last brushed their teeth. The next morning, the VSC analyses were conducted, and the volunteers were questioned with regard to whether they had experienced any adverse oral symptoms, and then the oral soft tissue examinations were performed. VSC analyses with negative control or placebo were also tested as described above.

A portable gas chromatograph (Oral Chroma) was used to assess the level of VSC in mouth air. Before each analysis, the subjects were instructed to keep their mouth closed and to breathe through their noses for a period of 30 s. A 1 ml disposable syringe was inserted into the centre of each subject's oral cavity through the lips and teeth, while the mouth was kept closed. Subsequent to the aspiration of 1 ml of oral air with a syringe, 0.5 ml of the air was injected into the gas chromatograph. The measurement was repeated three times for each subject. Descriptive studies were conducted using SPSS version 12.0, and the significance of the differences was assessed via a paired *t*-test.

Results

Identification of hydrogen peroxide-generating lactobacilli

The almost complete 16S rDNA sequences (1451, 1483, and 1483 nucleotide (nt)) were determined, and sequence searches of GenBank and ribosomal database project libraries showed that all of the selected strains had a high level of 16S rDNA similarity (100%) to the reference strain of the species, *W. cibaria* LMG 17699^T (accession no. AJ295989), and were hence designated as *W. cibaria* CMU, CMS2, and CMS3. The levels of hydrogen peroxide produced by these strains are shown in Table 1. These strains were the highest producers, and for this reason they were selected for the current study.

Coaggregation of the *W. cibaria* isolates

The coaggregation assay showed that *F. nucleatum* coaggregated most strongly with the three *W. cibaria* strains, followed by *T. denticola*, *P. loescheii*, and *P. gingivalis* (Table 2). As all three of the *W. cibaria* strains were determined to coaggregate most efficiently with *F. nucleatum*, we decided to use *F. nucleatum* in further study.

Inhibitory effects on the VSC production of *F. nucleatum*

All three of the *W. cibaria* strains were shown to inhibit the production of H_2S by *F. nucleatum*, as is shown in Figs 1b–d, respectively, with no black pigment being observed in the culture supernatants, whereas black pigments were observed in the cultures that had been treated with 1000 U/ml catalase (Figs 1e–g). Therefore, it appears that the inhibitory effects exerted by the *W. cibaria* strains were abolished as a result of the addition of catalase.

The control assay containing only *F. nucleatum* had a very strong odour and produced a very high concentration of VSC above the maximum detection level (342.2 ng/10 ml of H_2S; 616.1 ng/10 ml of CH_3SH). *W. cibaria* CMU and *W. cibaria* CMS2 completely (99.9%) inhibited the production of H_2S, and reduced CH_3SH production

Table 1. Levels of hydrogen peroxide produced by three isolates

Strains	Hydrogen peroxide (mM)
Weissella cibaria CMU	3.91
W. cibaira CMS2	2.98
W. cibaria CMS3	1.62

Fig. 1. Inhibition of hydrogen sulphide production by *Fusobacterium nucleatum* ATCC 10953, afforded by *Weissella cibaria* isolates. (A) *F. nucleatum* only; (B–D) *W. cibaria* CMU, CMS2, and CMS3 mixed with *F. nucelatum*, respectively; E, F, and G, *W. cibaria* CMU, CMS2, and CMS3 mixed with *F. nucleatum* after catalase treatment, respectively. Black pigmentation was a marker for H_2S production.

Table 2. Coaggregation reactions between the *Weissella cibaria* isolates and the volatile sulphur compounds (VSC)-producing anaerobic bacteria

Strains	Coaggregation (%)		
	CMU	CMS2	CMS3
Fusobacterium nucleatum ATCC 10953	88.9 ± 2.7	89.3 ± 2.7	84.9 ± 3.1
Porphyromonas gingivalis A7A1-28	33.3 ± 1.3	28.3 ± 1.3	31.4 ± 1.3
Treponema denticola ATCC 35405	68.9 ± 2.9	73.1 ± 0.9	61.6 ± 3.2
Prevotella loescheii ATCC 15930	66.0 ± 3.0	66.5 ± 5.4	31.7 ± 2.9

Values are expressed as the means ± SD from three separate experiments.

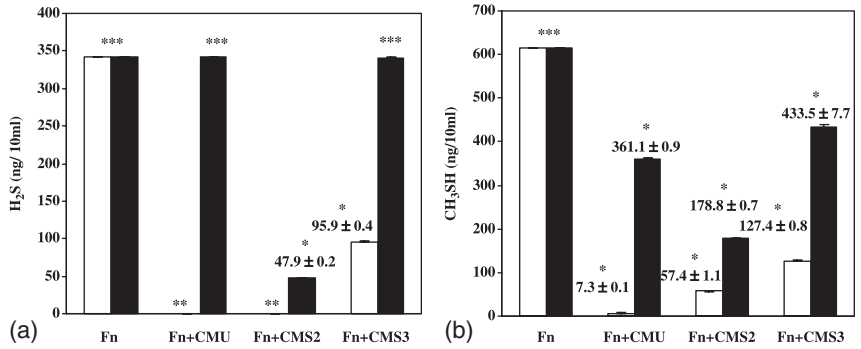

Fig. 2. Inhibitory effects of *W. cibaria* isolates on the production of H_2S (a) and CH_3SH (b) by *F. nucleatum* ATCC 10953 before (□) and after (■) catalase treatment. Fn represents *F. nucleatum*. *Significance of differences was defined as $p<0.05$; **W. cibaria* CMU and CMS2 completely inhibited the production of H_2S by *F. nucleatum* (0.0 ± 0.0 ng/10 ml), $p<0.05$; ***Values are volatile sulphur compounds concentrations obtained above the maximum detection level.

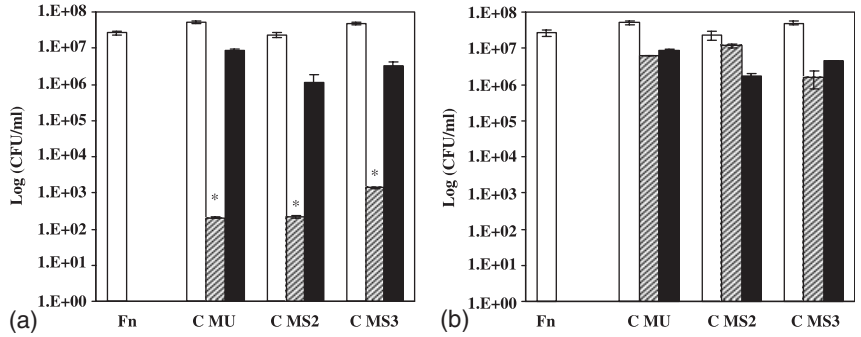

Fig. 3. Inhibitory effects of *W. cibaria* isolates on the proliferation of *F. nucleatum* ATCC 10953 both before (a) and after (b) catalase treatment. □, Control group; ▨, *F. nucleatum* in mixed culture; ■, *W. cibaria* in mixed culture. *Significance of differences was defined as $p<0.05$.

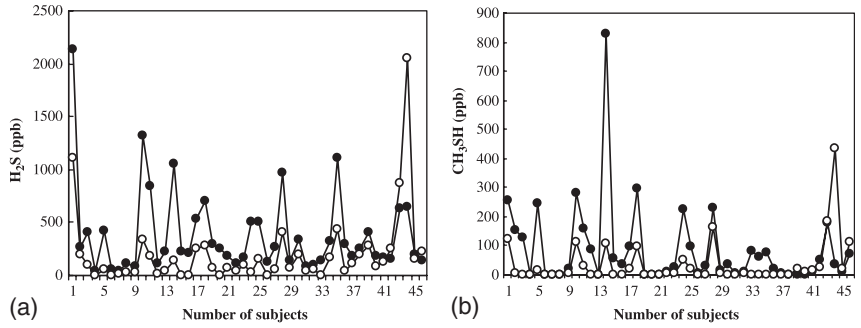

Fig. 4. Distribution of H_2S (a) and CH_3SH (b) measured in each of the subjects before (●) and after (○) gargling with the test solution.

by approximately 99% (7.3 ± 0.1 ng/10 ml) and 91% (57.4 ± 1.1 ng/10 ml), respectively ($p<0.05$). *W. cibaria* CMS3 also reduced the production of H_2S and CH_3SH, by approximately 72% and 79%, respectively ($p<0.05$). *W. cibaria* CMU and CMS3, which were treated with catalase were not noted to exert any inhibitory effects on H_2S production by *F. nucleatum,* but *W. cibaria* CMS2 still evidenced strong inhibition (about 86%) of H_2S production ($p<0.05$) even after catalase treatment. However, all three of the *W. cibaria* strains treated with catalase reduced CH_3SH production by 30%–70% ($p<0.05$) (Fig. 2).

The concentrations of *F. nucleatum* decreased by 5-log cycles ($2.1 \times 10^2 \pm 1.2 \times 10^1$ CFU/ml, $p<0.05$) in the groups in which *W. cibaria* isolates had been added, as compared with the control group ($2.6 \times 10^7 \pm 2.7 \times 10^5$ CFU/ml), whereas the catalase-treated *W. cibaria* cultures were determined to exert no apparent inhibitory effects on the replication of *F. nucleatum* (Fig. 3). These results demonstrated that the hydrogen peroxide generating *W. cibaria* isolates decreased *F. nucleatum*-mediated VSC production by inhibiting the replication of *F. nucleatum*.

Inhibitory effects on the VSC production of mouth air

Figure 4 showed the distribution of VSC concentrations (p.p.b.) measured in each of the subjects, both before and after they had gargled with the test solutions containing *W. cibaria* CMU. On an average, this strain was found to effect a reduction in the concentrations of H_2S and CH_3SH by about 48.2% and 59.4%, respectively (Table 3). The statistical analysis showed that gargling with *W. cibaria* CMU significantly reduced the production of both H_2S ($p<0.01$) and CH_3SH ($p<0.05$). However, when sterile distilled water or commercial lactic acid bacteria were used as a control rinse, there were no statistically significant reductions in VSC concentrations.

Discussion

The *Weissella* genus was recently separated from the *Lactobacillus* genus, as a result of recent developments in DNA technology (Stiles & Holzapfel 1997). *W. cibaria*, which was initially described by Bjorkroth et al. (2002), has been isolated from a variety of sources, ranging from fermented foods to human faeces. However, thus far, this species has not been reported in human saliva.

Among the variety of known oral anaerobic bacteria, *F. nucleatum* has been known to possess a powerful ability to coaggregate with other oral bacteria, and to contribute substantially to VCS production in the oral cavity (Claesson et al. 1990, Bolstad et al. 1996). This coaggregation ability may

Table 3. VSC concentrations (ppb) before and after the treatments and percentage of reduction before and after for each treatment

Treatments	Measurement	VSC (ppb)		Reduction (%)
		before	after	
Distilled water[†]	H_2S	326.4 ± 32.2	288.5 ± 36.4	11.6
($n = 46$)	CH_3SH	79.3 ± 16.7	81.0 ± 14.2	–
Lactobacillus casei[‡]	H_2S	316.2 ± 70.6	312.0 ± 84.2	1.3
($n = 10$)	CH_3SH	81.6 ± 39.6	87.2 ± 36.4	–
Weissella confusa[‡]	H_2S	385.8 ± 50.9	449.2 ± 61.2	–
($n = 10$)	CH_3SH	80.7 ± 37.4	82.0 ± 36.2	–
W. cibaria CMU[§]	H_2S	383.1 ± 59.3	198.3 ± 51.8	48.2*
($n = 46$)	CH_3SH	85.5 ± 20.9	34.7 ± 11.3	59.4**

Values are expressed as means ± SE.
*,**Significances of differences were defined as $p < 0.01$ and $p < 0.05$, respectively.
[†]Negative control.
[‡]Commercial lactic acid bacteria were used as a placebo rinse.
[§]The test solution.

constitute an important factor in the establishment and maintenance of a healthy oral floral system because of the production of a microenvironment around *F. nucleatum*, in which the inhibiting substances generated by the coaggregated bacteria would be affected. The ability of the *W. cibaria* isolates to coaggregate was found to be at its highest in reactions with *F. nucleatum* among all of the tested anaerobic bacterial strains (Table 2). Therefore, we expected that this bacterial strain, because of its potent ability to coaggregate with *W. cibaria* isolates, might generate definite results. Therefore, we selected *F. nucleatum* for use in further studies regarding the inhibition of isolates on VSC production and proliferation.

Three *W. cibaria* strains were determined to exhibit profound inhibitory abilities with regard to VSC production. The concentrations of *F. nucleatum* were also reduced by 5-log cycles as a result of the activity of all three *W. cibaria* isolates, as compared with the control group (Fig. 3). Therefore, we were compelled to conclude that the substances generated from the *W. cibaria* isolates exerted reducing effects on VSC production by inhibiting *F. nucleatum* proliferation.

Hydrogen peroxide is one of the principal antibacterial materials, which are associated with lactic acid bacteria (Hiller et al. 1993) along with organic acids and bacteriocins. Hydrogen peroxide has been shown to facilitate the maintenance of a stable ecological system, and also protects against invading pathogens (Reid et al. 1990). The oral anaerobic bacteria exhibit a higher degree of susceptibility to hydrogen peroxide than do other genera of aerobic or facultative anaerobic bacteria (Leke et al. 1999, Ohwada et al. 1999). Hydrogen peroxide has also been shown to significantly reduce concentrations of sulphur gas in vivo (Suarez et al. 2000). In the present study, we found that *W. cibaria* isolates generated a substantial quantity of hydrogen peroxide, which was sufficient to inhibit the proliferation of *F. nucleatum*. Moreover, as the final pH of the mixed cultures that contained each of the *W. cibaria* isolates was found to be quite high (above pH 5.5) (data not shown), we were able to dismiss the possibility that organic acid was responsible for the observed inhibition of *F. nucleatum* proliferation. The inhibitory effects on VSC production and *F. nucleatum* proliferation were observed to be attenuated by treatment of the *W. cibaria* isolates with catalase. Therefore, we concluded that the hydrogen peroxide generated by the *W. cibaria* isolates resulted in the attenuation of VSC production by inhibiting *F. nucleatum* proliferation. Via its coaggregation with *W. cibaria, F. nucleatum* might be killed by the hydrogen peroxide generated by *W. cibaria*.

Oral care procedures reducing anaerobic bacteria could prevent halitosis as well as other oral diseases. Many attempts have been made to inhibit the production of VSC with disinfectants or metal salts. Chlorhexidine and zinc chloride-containing mouthwashes have been shown to be rather effective in the reduction of oral malodour (Yaegaki & Suetaka 1989, Carvalho et al. 2004, Roldan et al. 2004). However, these agents tend to be diluted by the saliva and swallowed into the gullet together with saliva, and thus their effects in the oral cavity are rather short-lived (Rosenberg 2002). It has been reported that probiotic bacteria originally derived from the indigenous oral microbiota of healthy humans might potentially be applied as adjuncts for the prevention and treatment of halitosis (Burton et al. 2005). Bacteria isolated from human oral microflora would be able to proliferate in the oral cavities of other humans. Further, lactic acid bacteria such as *Lactobacillus acidophilus* and *Lactobacillus casei* have been determined to inhibit the in vitro proliferation of anaerobic bacteria via the production of a strong acid (Babaahmady et al. 1998). However, in the oral cavity of a healthy person, this strong acid would then normally be neutralised by the buffering function of saliva. Furthermore, as this strong acid can induce the development of dental caries, the continuous administration of these bacteria may give rise to oral hygiene problems (Babaahmady et al. 1998). The *W. cibaria* strains used in the current study had been isolated from the saliva of a group of healthy children, and none of the members of this genus have, thus far, been associated with any pathogenicity. In the present study, the *W. cibaria* isolates resulted in a higher ecological pH than that which would normally be observed in conjunction with lactobacilli. These beneficial properties and effects observed in conjunction with these isolates were encouraging with regard to the utility of this species within the context of probiotic use.

Oral malodour is caused by many factors including VSC. The mechanism of VSC production is complicated and involved many factors including various pathogenic bacteria. To verify our in vitro results, we attempted a clinical study with healthy adults to determine the effects exerted by *W. cibaria* CMU on the breath malodour. In this clinical trial, the results showed that the *W. cibaria* isolate significantly reduced VSC level in the mouth. *F. nucleatum* acts as a bridge-organism that facilitates the colonization of other bacteria by coaggregation, and contributes to VCS production in the oral cavity. In this study, we hypothesised that *W. cibaria* strains with the ability to coaggregate with *F. nucleatum* might have an advantage over non-coaggregating organisms which were more easily removed from the oral environment by salivary flow,

and focussed on the ability of *W. cibaria* strains to coaggregate with *F. nucleatum*. Also, *W. cibaria* isolates efficiently adhered to mouth epithelial cells (data not shown). Furthermore, when one of these strains was applied in the oral cavity, it effectively reduced VSC concentrations. Even if *W. cibaria* isolates effectively reduced VSC level in vivo, the effects of this strain on other potential Gram-negative anaerobes such as *P. gingivalis* and *P. intermedia* were not determined in this study, which limited the activity of this strain on clinical oral halitosis, we could suggest *W. cibaria* isolates as potential probiotic candidates against halitosis. These isolates might also constitute attractive alternatives to chemicals in terms of the inhibition of VSC production. However, the subjects of our in vivo study were healthy dental school students, and the morning breath was estimated. Therefore, long-term studies would be necessary for other groups of people such as the elderly, smoking group or patients having halitosis.

In conclusion, the present study indicated that *W. cibaria* strains isolated from human saliva were capable of inhibiting the production of VSC, both under in vitro and in vivo conditions.

Acknowledgements

This work was supported by grant No. R01-2002-000-00173-0 from the Basic Research Program of the Korea Science & Engineering Foundation.

References

Ahrne, S., Nobaek, S., Jeppsson, B., Adlerberth, I., Wold, A. E. & Molin, G. (1998) The normal *Lactobacillu*s flora of healthy human rectal and oral mucosa. *Journal of Applied Microbiology* **85**, 88–94.

Babaahmady, K. G., Challacombe, S. J., Marsh, P. D. & Newman, H. N. (1998) Ecological study of *Streptococcus mutans, Streptococcus sobrinus* and *Lactobacillus* spp. at sub-sites from approximal dental plaque from children. *Caries Research* **32**, 51–58.

Bjorkroth, K. J., Schillinger, U., Geisen, R., Weiss, N., Hoste, B., Holzapfel, W. H., Korkeala, H. J. & Vandamme, P. (2002) Taxonomic study of *Weissella confusa* and description of *Weissella cibaria* sp. nov., detected in food and clinical samples. *International Journal of Systematic and Evolutionary Microbiology* **52**, 141–148.

Blakemore, R. P. & Canale-Parola, E. (1976) Arginine catabolism by *Treponema denticola*. *Journal of Bacteriology* **128**, 616–622.

Bolstad, A. I., Jensen, H. B. & Bakken, V. (1996) Taxonomy, biology, and periodontal aspects of *Fusobacterium nucleatum*. *Clinical Microbiology Reviews* **9**, 55–71.

Bosy, A. (1997) Oral malodour: philosophical and practical aspects. *Journal of the Canadian Dental Association* **63**, 196–201.

Burton, J. P., Chilcott, C. N. & Tagg, J. R. (2005) The rationale and potential for the reduction of oral malodour using *Streptococcus salivarius* probiotics. *Oral Diseases* **11**, 29–31.

Carvalho, M. D., Tabchoury, C. M., Cury, J. A., Toledo, S. & Nogueira-Filho, G. R. (2004) Impact of mouthrinses on morning bad breath in healthy subjects. *Journal of Clinical Periodontology* **31**, 85–90.

Cisar, J. O., Kolenbrander, P. E. & McIntire, F. C. (1979) Specificity of coaggregation reactions between human oral streptococci and strains of *Actinomyces viscosus* or *Actinomyces naeslundii*. *Infection and Immunity* **24**, 742–752.

Claesson, R., Edlund, M. B., Persson, S. & Carlsson, J. (1990) Production of volatile sulfur compounds by various *Fusobacterium* species. *Oral Microbiology and Immunology* **5**, 137–142.

De Boever, E. H. & Loesche, W. J. (1995) Assessing the contribution of anaerobic microflora of the tongue to oral malodor. *Journal of the American Dental Association* **126**, 1384–1393.

Eschenbach, D. A., Davick, P. R., Williams, B. L., Klebanoff, S. J., Young-Smith, K., Critchlow, C. M. & Holmes, K. K. (1989) Prevalence of hydrogen peroxide-producing *Lactobacillus* species in normal women and women with bacterial vaginosis. *Journal of Clinical Microbiology* **27**, 251–256.

Fine, D. H., Furgang, D., Sinatra, K., Charles, C., McGuire, A. & Kumar, L. D. (2005) In vivo antimicrobial effectiveness of an essential oil-containing mouth rinse 12 h after a single use and 14 days' use. *Journal of Clinical Periodontology* **32**, 335–340.

Fuller, R. (1991) Probiotics in human medicine. *Gut* **32**, 439–442.

Gill, H. S., Shu, Q., Lin, H., Rutherfurd, K. J. & Cross, M. L. (2001) Protection against translocating *Salmonella typhimurium* infection in mice by feeding the immuno-enhancing probiotic *Lactobacillus rhamnosus* strain HN001. *Medical Microbiology and Immunology* **190**, 97–104.

Handley, P. S., Harty, D. W., Wyatt, J. E., Brown, C. R., Doran, J. P. & Gibbs, A. C. (1987) A comparison of the adhesion, coaggregation and cell-surface hydrophobicity properties of fibrillar and fimbriate strains of *Streptococcus salivarius*. *Journal of General Microbiolgy* **133**, 3207–3217.

Hiller, S. L., Krohn, M. A., Rabe, L. K., Klebanoff, S. J. & Eschenbach, D. A. (1993) The normal vaginal flora H_2O_2-producing lactobacilli, and bacterial vaginosis in pregnant women. *Clinical Infectious Diseases* **16**, S273–S281.

Langendijk, P. S., Hagemann, J. & van der Hoeven, J. S. (1999) Sulfate-reducing bacteria in periodontal pockets and in healthy oral sites. *Journal of Clinical Periodontology* **26**, 596–599.

Leke, N., Grenier, D., Goldner, M. & Mayrand, D. (1999) Effects of hydrogen peroxide on growth and selected properties of *Porphyromonas gingivalis*. *FEMS Microbiology Letters* **174**, 347–353.

Miyazaki, H., Sakao, S., Katoh, Y. & Takehara, T. (1995) Oral malodor in the general population of Japan. In: Rosenberg, M. (ed). *Bad Breath: Research Perspectives*, pp. 119–136. Tel Aviv: Ramot Publishing.

Ohwada, T., Shirakawa, Y., Kusumoto, M., Masuda, H. & Sato, T. (1999) Susceptibility to hydrogen peroxide and catalase activity of root nodule bacteria. *Bioscience, Biotechnology and Biochemistry* **63**, 457–462.

Padron, A. P. & Dockstader, W. B. (1972) Selective medium for hydrogen sulfide production by salmonellae. *Applied Microbiology* **23**, 1107–1112.

Persson, S., Edlund, M. B., Claesson, R. & Carlsson, J. (1990) The formation of hydrogen sulfide and methyl mercaptan by oral bacteria. *Oral Microbiology and Immunology* **5**, 195–201.

Pianotti, R., Lachette, S. & Dills, S. (1986) Desulfuration of cysteine and methionine by *Fusobacterium nucleatum*. *Journal of Dental Research* **65**, 913–917.

Reid, G., Bruce, A. W., McGroarty, J. A., Cheng, K. J. & Costerton, J. W. (1990) A review of bacterial interference in medical and veterinary applications. *Clinical Microbiology Reviews* **3**, 335–344.

Roldan, S., Herrera, D., Santa-Cruz, I., O'Connor, A., Gonzalez, I. & Sanz, M. (2004) Comparative effects of different chlorhexidine mouth-rinse formulations on volatile sulphur compounds and salivary bacterial counts. *Journal of Clinical Periodontology* **31**, 1128–1134.

Rosenberg, M. (2002) The science of bad breath. *Scientific American* **286**, 72–79.

Senpuku, H., Tada, A., Yamaga, T., Hanada, N. & Miyazaki, H. (2004) Relationship between volatile sulphide compounds concentration and oral bacteria species detection in the elderly. *International Dental Journal* **54**, 149–153.

Stiles, M. E. & Holzapfel, W. H. (1997) Lactic acid bacteria of foods and their current taxonomy. *International Journal of Food Microbiology* **36**, 1–29.

Suarez, F. L., Furne, J. K., Springfield, J. & Levitt, M. D. (2000) Morning breath odor: influence of treatments on sulfur gases. *Journal of Dental Research* **79**, 1773–1777.

Tessier, J. F. & Kulkarni, G. V. (1991) Bad breath: etiology, diagnosis and treatment. *Oral Health* **81**, 19–22.

Tomas, M. S., Claudia Otero, M., Ocana, V. & Elena Nader-Macias, M. (2004) Production of antimicrobial substances by lactic acid bacteria I: determination of hydrogen peroxide. *Methods in Molecular Biology* **268**, 347–353.

Tonzetich, J. (1971) Direct gas chromatographic analysis of sulphur compounds in mouth air in man. *Archives of Oral Biology* **16**, 587–597.

Walker, C., Ratliff, D., Muller, D., Mandell, R. & Socransky, S. S. (1979) Medium for selective isolation of *Fusobacterium nucleatum* from human periodontal pockets. *Journal of Clinical Microbiology* **10**, 844–849.

Yaegaki, K. & Suetaka, T. (1989) The effect of zinc chloride mouthwash on the production of oral malodor, the degradations of salivary cellular elements, and proteins. *Journal of Dental Health* **39**, 377–386.

Young, A., Jonski, G., Rölla, G. & Waler, S. M. (2001) Effects of metal salts on the oral production of volatile sulfur-containing compounds (VSC). *Journal of Clinical Periodontology* **28**, 776–781.

Address:
Jong-Suk Oh
Department of Microbiology
School of Medicine
Chonnam National University
5 Hak-Dong, Dong-Gu, Gwangju 501-746
Korea
E-mail: joh@chonnam.ac.kr

Clinical Relevance

It has been known that oral malodour is associated with VSC produced by various oral pathogenic bacteria. In the present study, we evaluated the inhibitory effects of *W. cibaria* isolates on the VSC production both in vitro and in vivo. Our results showed that *W. cibaria* isolates successfully inhibited the production of VSC by *F. nucleatum* and also reduced VSC concentration in the clinical study.