Clin Oral Invest (2011) 15:485–493
DOI 10.1007/s00784-010-0422-x

ORIGINAL ARTICLE

Effects of a composition containing lactoferrin and lactoperoxidase on oral malodor and salivary bacteria: a randomized, double-blind, crossover, placebo-controlled clinical trial

Kouichirou Shin · Ken Yaegaki · Takatoshi Murata · Hisataka Ii · Tomoko Tanaka · Izumi Aoyama · Koji Yamauchi · Tomohiro Toida · Keiji Iwatsuki

Received: 27 January 2010 / Accepted: 26 April 2010 / Published online: 29 May 2010

Abstract We report a clinical trial of the effects of test tablets containing bovine lactoferrin and lactoperoxidase on oral malodor and salivary bacteria. Fifteen subjects with volatile sulfur compounds (VSCs) in mouth air above the olfactory threshold (H_2S >1.5 or CH_3SH >0.5 ng/10 ml) as detected by gas chromatography were enrolled in the trial. Either a test or a placebo tablet was ingested twice at 1-h intervals in two crossover phases. Mouth air was monitored for VSC levels at the baseline before ingestion of a tablet, 10 min after the first ingestion, 1 h (just before the second ingestion), and 2 h after the first ingestion. Whole saliva was analyzed at the baseline and at 2 h for bacterial numbers. At 10 min, the level of CH_3SH was significantly lower in the test group (median [interquartile range]=0.28 [0.00–0.68]ng/10 ml) compared to that in the placebo group (0.73 [0.47–1.00]ng/10 ml; P=0.011). The median concentration of CH_3SH in the test group was below the olfactory threshold after 10 min until 2 h, whereas the level in the placebo group was above the threshold during the experimental period. No difference in the numbers of salivary bacteria was detected by culturing or quantitative PCR, but terminal restriction fragment length polymorphism detected one fragment with a significantly lower copy number at 2 h in the test group (mean ± standard error, 4.89±0.11 $\log_{10}$ copies/10 μl) compared to that in the placebo group (5.38±0.15 $\log_{10}$ copies/10 μl; P=0.033). These results indicate a suppressive effect of the test composition on oral malodor and suggest an influence on oral bacteria.

Keywords Lactoferrin · Lactoperoxidase · Oral malodor · Salivary bacteria · Clinical trial

K. Shin · K. Yamauchi · T. Toida · K. Iwatsuki
Food Science & Technology Institute,
Morinaga Milk Industry Co., Ltd.,
5-1-83 Higashihara,
Zama, 228-8583 Kanagawa, Japan

K. Yaegaki (✉) · T. Murata · H. Ii · T. Tanaka · I. Aoyama
Department of Oral Health, Nippon Dental University,
1-9-20 Fujimi, Chiyoda-ku,
Tokyo 102-8159, Japan
e-mail: yaegaki-k@tky.ndu.ac.jp

Introduction

Oral malodor affects up to one half of the general population and has become of great concern to many people in the past few decades [1, 2]. The putrefactive action of microorganisms producing volatile sulfur compounds (VSCs) against proteinaceous components in tongue coating, saliva, and gingival crevicular fluid is generally agreed to be responsible for oral malodor [2–4]. Tongue cleaning is very effective in reducing oral malodor, and antimicrobial products in the form of mouthwashes or dentifrices have also been reported to be effective [2, 4–6]. However, antimicrobial compounds like chlorhexidine cause some side effects [2, 7]. Zinc mouthwash, which is one of the most effective products, causes an uncomfortable bitter taste [2, 8].

Biological procedures for reducing oral malodor may involve fewer side effects and are environmentally safe. Lactoferrin (LF), a member of the transferrin family and a component of milk, saliva, tears, and secondary neutrophil granules, exhibits antimicrobial activity [9]. LF has a wide range of biological functions other than antimicrobial

activities, such as demonstrating immuno-modulatory effects and regulating both cell proliferation and iron uptake [9, 10]. Lactoperoxidase (LPO), a member of the mammalian heme peroxidase family, is a component of milk, saliva, and other exocrine secretions [11]. In these secretions, LPO catalyzes the hydrogen peroxide-dependent oxidation of thiocyanate (SCN^-) to hypothiocyanite ($OSCN^-$), which is a potent antimicrobial agent against bacteria, fungi, and viruses [11]. LF and LPO, both known to be antimicrobial components in saliva, have been reported to inhibit the metabolism and growth of oral pathogens [12–14]. The inhibitory effects of LF on biofilm formation by periodontopathic bacteria have also been reported [15]. Oral administration of LF has been shown to reduce the number of periodontal pathogens in subgingival plaque [16]. Recently, a composition containing LPO, glucose oxidase (GO), glucose, and citrate buffer salts has been developed [17]. This composition showed bactericidal activity against oral bacteria in vitro in the presence of saliva or SCN^-. Tablets that slowly dissolve in saliva seem to be a possible candidate for an oral-hygiene product to introduce antimicrobial agents into the oral cavity effectively and to retain these agents transiently on the tongue surface to reduce oral malodor.

Therefore, combining LF and the above composition is of interest in assessing clinical efficacy in oral hygiene, especially against oral malodor mainly produced by oral pathogens [18]. The aim of this randomized, double-blind, crossover, placebo-controlled clinical study was to assess the short-term effects of this composition containing LF and LPO on oral malodor and salivary bacteria.

Materials and methods

Composition of the test tablet

The compositions of the test and placebo tablets are presented in Table 1. LF purified from bovine milk (Morinaga Milk Industry, Tokyo, Japan), LPO purified from bovine milk (Biopole, Gembloux, Belgium), and GO originating from *Penicillium chrysogenum* (Sumizyme PGO, Shin-Nihon Chemical, Aichi, Japan) were used. The tablets (Morinaga Milk Industry) were round in shape with a 12-mm diameter and 6-mm thickness.

Subjects

Fifteen healthy volunteers aged 26–54 years (mean age 30.3 years; 11 male, four female) were recruited. The volunteers had not received antibiotic therapy in the preceding 2 weeks, had no untreated carious lesions or periodontitis, and were found in screening examinations to have VSCs in mouth air above the olfactory threshold [hydrogen sulfide (H_2S) >1.5 ng or methylmercaptan (CH_3SH) >0.5 ng/10 ml air] using gas chromatography [19]. Cooperation with the trial was achieved by careful explanation of the procedures involved and clarification of the overall aims of the study. Written informed consent was obtained from all volunteers. The research protocol was reviewed and approved by the research ethics committee of Nippon Dental University.

Table 1 Compositions of the test and placebo tablets

Component	Amount per tablet (mg)	
	Test	Placebo
LF	100.0	–
LPO	1.8	–
GO	24.0	–
Glucose	27.0	–
Trisodium citrate dihydrate	31.2	–
Citric acid	14.1	–
Erythritol	270.0	–
Xylitol	67.5	–
Maltitol	323.9	754.8
Cornstarch	–	100.0
Coloring materials	–	4.7
Flavor	2.7	2.7
Menthol	1.8	1.8
Sucrose fatty acid ester	18.0	18.0
Glycerol fatty acid ester	18.0	18.0

The total weight of each tablet was 900 mg

Study design

The present study was a randomized, double-blind, crossover, placebo-controlled clinical trial. The subjects were randomly assigned to one of two groups using a series of randomized numbers. In the first crossover phase, the subjects in one group (n=8) ingested the test tablets and the subjects in the other group (n=7) ingested the placebo tablets. There was a 1-week washout period between the two crossover phases. In the second crossover phase, each subject ingested the alternative tablets to the first phase.

Oral malodor assessment

On the day of assessment, the subjects were asked to abstain from eating, drinking, and using oral-hygiene practices from midnight until the end of the experiment. The subjects ingested a test or placebo tablet twice in the morning at a 1-h interval. Foods, especially such a tiny tablet, could be expected to be used frequently because of

their convenience, so the current clinical trial was designed taking into consideration the usual procedure of ingesting a food tablet several times a day. Each tablet was sucked for 10 min and then chewed and swallowed if still remaining.

Van del Velde et al. [20] have reported that only VSCs among the 700 compounds in mouth air are significantly correlated with the strength of oral malodor, as Tonzetich suggested [19]. VSCs in two mouth-air samples were analyzed using a GC8A gas chromatograph equipped with a flame photometric detector (Shimadzu, Kyoto, Japan), as described previously [21], at the baseline before ingestion of a tablet, 10 min after the first ingestion, 1 h (just before the second ingestion), and 2 h after the first ingestion. The second ingestion occurred immediately following the mouth-air analysis at 1 h. To sample the mouth air, 20 cm of polytetrafluoroethylene sampling tube (3.3 mm outside diameter) connected to the inlet of a six-port valve with the 10-ml sample loop of the gas chromatograph was inserted into the center of the oral cavity through the lips and teeth, and the lips remained closed around it for 1 min. Fifteen milliliters of mouth air was aspirated with a gas-tight syringe connected to the outlet of the valve. The concentrations of H_2S and CH_3SH, the main components of oral-malodorous sulfur compounds, were determined in each sample. Total VSC concentration was obtained as the sum of the H_2S and CH_3SH concentrations. The average concentrations of H_2S, CH_3SH, and total VSCs at each time points were calculated and expressed as nanograms/10 ml as previously reported [21].

Salivary bacteria assessment

Unstimulated whole saliva samples of approximately 2 ml were collected at the baseline (before starting the oral malodor assessment) and at 2 h (after finishing the oral malodor assessment). Each saliva sample was vortexed and a 0.5-ml portion stored at −20°C for later use in quantitative PCR. The remaining saliva sample was sampled with a culture swab (BD, Tokyo, Japan) for the determination of the number of lactobacilli, total streptococci, and mutans streptococci (*Streptococcus mutans* and *Streptococcus sobrinus*), using selective plates of Rogosa SL agar (BD), Mitis–Salivarius agar (BD), or improved Mitis–Salivarius agar as previously described [22]. The number of bacteria was expressed as $\log_{10}$ colony-forming units (CFU)/swab.

Quantitative PCR

DNA was extracted from each saliva sample, and then quantitative PCR was performed using oligonucleotide primers and probes targeting the 16S rRNA of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, or total bacteria as previously described [23]. The number of bacteria (the target DNA sequences) was expressed as $\log_{10}$ copies/10 μl saliva.

Terminal restriction fragment length polymorphism analysis

Terminal restriction fragment length polymorphism (T-RFLP) analysis was performed on the DNA extracted from the saliva sample [24]. Fragments of the bacterial 16S rRNA gene were amplified by PCR with the extracted DNA in the presence of 6-FAM labeled universal forward primer, D88 (5′-GAGAGTTTGATYMTGGCTCAG-3′), and unlabeled universal reverse primer, E94 (5′-GAAG GAGGTGWTCCARCCGCA-3′). The PCR product was digested with *HaeIII* (Promega, Tokyo, Japan) and analyzed using the ABI 3130 Genetic Analyzer (Applied Biosystems, Tokyo, Japan) with GeneScan 1200 LIZ (Applied Biosystems) as the internal size standard. The electropherograms were analyzed using GeneScan software (version 3.7, Applied Biosystems), and the fragment sizes in base and peak areas were estimated using the local Southern method. The copy number of each terminal-restriction fragment (T-RF) was calculated from the number of total bacteria obtained by quantitative PCR and the percentage of peak area for each fragment compared to total peak area. The number of bacteria (T-RFs) was expressed as $\log_{10}$ copies/10 μl saliva. Phylogenic analysis of the T-RFs generated with the above-mentioned primer pairs and restriction-enzyme digestion was performed on the basis of base size, with the help of the TRFMA database containing information on the nucleotide sequences of approximately 650 species of oral bacteria and their T-RFs [25].

Statistical analysis

Data from the experiments with different tablets were separately calculated and expressed as median [lower quartile–upper quartile] or means ± standard error for each group (n=15). Normal distribution was not found in the concentrations of VSCs at baseline than their medians for each group. Thus, the intra-group differences in the concentrations of VSC during the experimental period were analyzed using the Friedman test. The differences in the concentrations of VSCs and number of bacteria between baseline and each time point in each group were analyzed using the Wilcoxon signed rank test. The differences in the concentrations of VSCs and number of bacteria between the test and placebo groups at each time point were analyzed using the Wilcoxon test. The detection limit of the quantitative PCR or T-RFLP result was 1.00 or 4.75 $\log_{10}$ copies/10 μl for the statistical analysis. Values of $P<0.05$ were accepted as significant. Statistical analysis was performed using the software program JMP (version 5,

SAS Institute Japan, Tokyo, Japan) or KaleidaGraph (version 3.6, Hulinks, Tokyo, Japan).

Results

Concentration of H_2S in the mouth air

Changes in the concentration of H_2S in the mouth air are shown in Fig. 1a. Significant intra-group differences over the duration of the experimental period were detected in both groups. Compared to the baseline, the concentration of H_2S was significantly lower in both groups at 10 min, 1 h, and 2 h. The median concentration of H_2S in the test group was below the olfactory threshold level (1.5 ng/10 ml) at 2 h, whereas the concentrations in the placebo group were over the threshold level during the entire experimental period.

Concentration of CH_3SH in the mouth air

Changes in the concentration of CH_3SH in the mouth air are shown in Fig. 1b. Significant intra-group differences over the duration of the experimental period were detected in both groups. Compared to the baseline, the concentration of CH_3SH was significantly lower in both groups at 10 min, 1 h, and 2 h. The concentration of CH_3SH in the test group (0.28 [0.00–0.68]ng/10 ml) was significantly lower at 10 min compared to that in the placebo group (0.73 [0.47–1.00]ng/10 ml; P=0.011). The median concentration of CH_3SH in the test group was below the olfactory threshold level (0.5 ng/10 ml) after 10 min until 2 h, whereas the concentration in the placebo group was above the threshold level during the entire experimental period.

Concentration of total VSCs in the mouth air

Changes in the concentration of total VSCs in the mouth air are shown in Fig. 1c. Significant intra-group differences in the total VSC concentration over the duration of the experimental period were detected in both groups. Compared to the concentrations of VSCs at the baseline, the concentration was significantly lower in both groups at 10 min, 1 h, and 2 h. The concentration of total VSCs in the test group (1.85 [0.79–2.75]ng/10 ml) was significantly lower at 10 min compared to that in the placebo group (2.70 [1.93–6.37]ng/10 ml; P=0.049).

Number of salivary bacteria

The number of salivary bacteria determined by culturing and quantitative PCR is shown in Table 2. No significant difference was detected in the number of bacteria for each of the species between baseline and 2 h in each group or between the two groups at each time point.

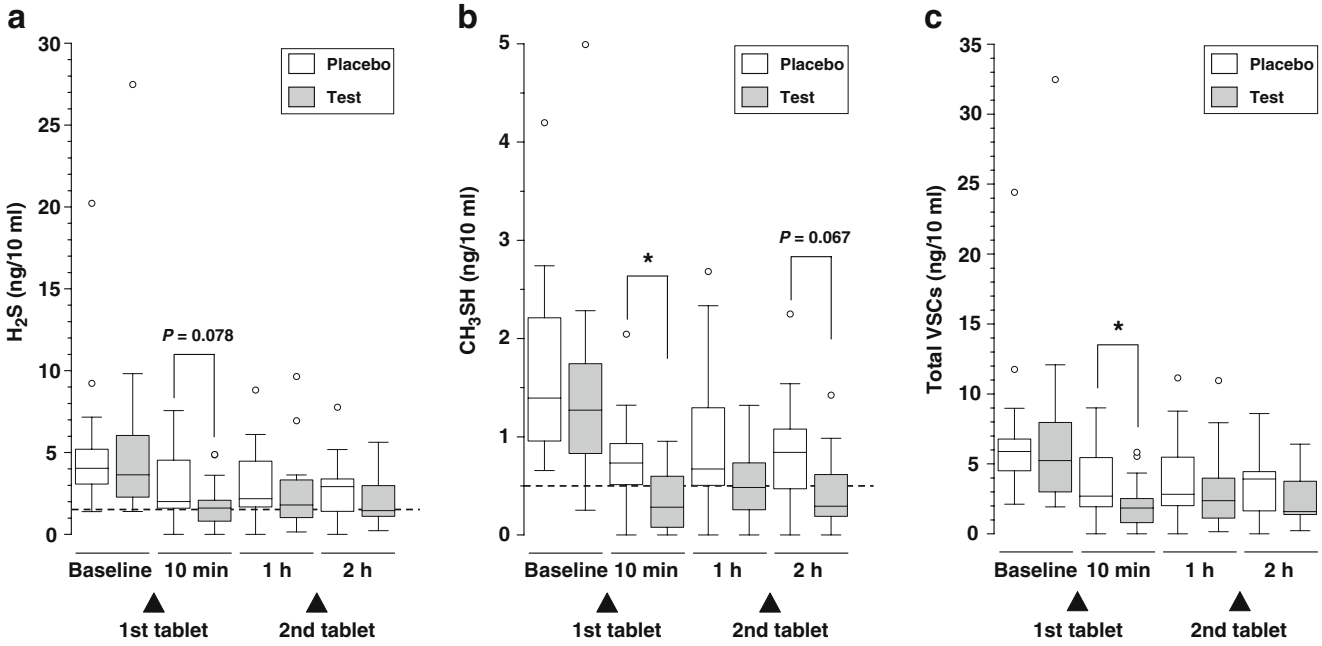

Fig. 1 The effects of ingesting test tablets on the concentrations of VSCs in mouth air. **a** H_2S, **b** CH_3SH, **c** total VSCs. Data are expressed as sample minimum (*lower edge of whisker*), lower quartile (*lower edge of box*), median (*traverse line in box*), upper quartile (*upper edge of box*), sample maximum (*lower edge of whisker*), and outlier (*circles*) in box-and-whisker plots. The olfactory thresholds of H_2S (1.5 ng/10 ml) and CH_3SH (0.5 ng/10 ml) are shown in *broken lines*. The time points of tablet ingestion are indicated by *closed triangles*. *P<0.05 between the test and placebo groups

Table 2 Salivary bacteria analyzed by culturing and quantitative PCR

Bacteria	Group	Baseline	2 h
Culturing		Number of bacteria (log_{10} CFU/swab)	
Lactobacilli	Placebo	2.76±0.06	2.74±0.04
	Test	2.77±0.05	2.75±0.04
Total streptococci	Placebo	5.75±0.22	5.53±0.20
	Test	5.79±0.25	5.78±0.21
Mutans streptococci	Placebo	2.97±0.15	2.84±0.09
	Test	2.86±0.09	2.84±0.09
Quantitative PCR		Number of bacteria (log_{10} CFU/10 μl)	
A. actinomycetemcomitans	Placebo	1.11±0.08	1.16±0.10
	Test	1.10±0.07	1.16±0.12
P. gingivalis	Placebo	1.49±0.27	1.57±0.31
	Test	1.47±0.25	1.52±0.28
P. intermedia	Placebo	1.65±0.28	1.61±0.25
	Test	1.72±0.30	1.64±0.27
F. nucleatum	Placebo	5.13±0.28	5.06±0.27
	Test	4.96±0.35	4.92±0.28
Total bacteria	Placebo	7.46±0.18	7.31±0.15
	Test	7.38±0.22	7.25±0.17

Data are expressed as mean ± standard error. The detection limit of the quantitative PCR was 1.00 log_{10} copies/10 μl for the statistical analysis

T-RFLP analysis detected an average of 39 T-RFs in the saliva samples. Table 3 summarizes the 15 T-RFs; a significant difference was detected between baseline and 2 h for each group, and between the two groups at each time point. The test and placebo groups showed 11 (162, 203, 249, 257, 294, 306, 320, 323, 329, 574, and 915 bases) and four (262, 302, 913, and 915 bases) fragments, respectively, with significantly reduced copy numbers at 2 h compared to those at the baseline. These fragments were not assigned to specific bacterial species. The T-RF 265 bases in length had a significantly lower copy number at 2 h in the test group (4.89±0.11 log_{10} copies/10 μl) compared to that of the placebo group (5.38±0.15 log_{10} copies/10 μl; P=0.033). This fragment was assigned to bacterial species belonging to *Prevotella*, *Porphyromonas*, *Streptococcus*, *Treponema*, *Eubacterium*, *Clostridiales*, *Bacteroidales*, and *Desulfomicrobium* [24, 25].

Discussion

LF and LPO are known to be constituents of both mammalian milk and saliva [9–11]. These components in saliva and gingival crevicular fluid may contribute to the oral health status [16, 17, 26–29]. However, this is the first study of the clinical efficacy against oral malodor of a composition containing LF and LPO. Using foods for controlling oral malodor provides many benefits besides fewer side effects. Although oral-hygiene products always require a special place for their use, e.g., a bathroom or washbasin, foods such as a tablet can easily be used anywhere and conveniently taken without unnecessary disposal after ingestion.

The results of the current clinical trial indicate short-term suppressive effects of the test composition on oral malodor. We designed this study employing 15 subjects because the suppressive effects of other foods or oral hygiene products on oral malodor were successfully demonstrated with a similar number of subjects [21]. The subjects were randomly assigned to one of two groups in each crossover phase; moreover, no statistical difference in their VSC levels at baseline was observed between the test and placebo groups. In controlling oral malodor, the test tablet may be more effective than previously examined food products on the market, including mint tablets, parsley-seed oil capsules, and sugarless chewing gum [21]. These results demonstrate that the test tablet might be a new measure for suppression of oral malodor in addition to the currently existing treatment measures such as tongue brushing, mouth rinsing, etc. [1, 2, 4].

In the present study, the extent of oral malodor reduction by the tablets was also evaluated by comparing the concentration of VSCs in the mouth air of the subjects to the olfactory threshold of VSCs [19]. The olfactory thresholds of CH_3SH and H_2S are above 0.5 and 1.5 ng/10 ml mouth air, respectively. The median concentration of CH_3SH in the test group was below the objectionable concentration (olfactory threshold) of CH_3SH after 10 min until 2 h, whereas the concentration in the placebo group was above the objectionable concentration during the entire experimental period. The median concentration of H_2S in the test group was below the olfactory threshold at 2 h,

Table 3 Salivary bacteria analyzed by T-RFLP

Fragment size (bases)	Group	Number of fragments (log_{10} copies/10 μl)		TRFMA database assignments
		Baseline	2 h	
162	Placebo	5.30±0.20	5.06±0.16	Not identified
	Test	5.40±0.16	5.16±0.13*	
203	Placebo	6.50±0.15	6.32±0.09	*Myxococcus coralloides*
	Test	6.44±0.20	6.25±0.16*	*Desulfobulbus* sp. oral clone CH031
				Corallococcus exiguous
				Corallococcus sp. SDU-2
249	Placebo	5.16±0.16	5.08±0.13	Not identified
	Test	5.36±0.17	5.12±0.14*	
257	Placebo	5.10±0.11	4.97±0.10	*Bifidobacterium* sp. oral strain A32ED
	Test	5.15±0.12	4.96±0.11*	*Leptotrichia* sp. oral clone EI022
262	Placebo	6.29±0.25	6.02±0.22*	*Flavobacterium*-like sp. oral clone AZ105
	Test	6.32±0.28	6.14±0.22	*Bifidobacterium* sp. oral strain H6-M4
				Bacteroides cf. *forsythus* oral clone BU063
				Prevotella sp. oral clone DO045
				TM7 phylum sp. oral clone FR058
265	Placebo	5.08±0.08	5.38±0.15	*Prevotella* spp. (10 phylotypes)
	Test	5.03±0.14	4.89±0.11**	*Porphyromonas*-like sp. oral clone DA065
				Streptococcus mitis
				Treponema spp. (5 phylotypes)
				Prevotella loescheii
				Eubacterium sp. oral clone BU014
				Clostridiales bacterium oral clone P4PA_66 P1
				Bacteroidales oral clone MCE7_20
				Bacteroides-like sp. oral clone X083
				Prevotella dentalis
				Desulfomicrobium orale
				Streptococcus sp. oral clone FP064
294	Placebo	5.31±0.20	5.29±0.19	*Oscillatoria corallinae*
	Test	5.45±0.18	5.27±0.15*	*Sphingomonas* sp. oral clone AW030
				Spiroplasma litorale
302	Placebo	5.00±0.09	4.87±0.08*	*Firmicutes* spp. (2 phylotypes)
	Test	4.98±0.08	4.96±0.09	*Eubacterium saburreum*
				Eubacterium saburreum-like sp. oral clone CK004
				Peptostreptococcus spp. (2 phylotypes)
				Eubacterium spp. (4 phylotypes)
				Human oral bacterium C73
				Lachnospiraceae oral clone MCE9_104
				Streptococcus mitis
306	Placebo	6.54±0.20	6.29±0.19	*Streptococcus mitis*
	Test	6.52±0.23	6.12±0.25*	*Streptococcus* spp. (8 phylotypes)
				Streptococcus oralis
				Streptococcus cristatus
				Streptococcus sanguinis
320	Placebo	5.37±0.17	5.32±0.13	*Streptococcus gordonii*
	Test	5.48±0.15	5.18±0.15*	*Streptococcus intermedius*
				Streptococcus constellatus
				Streptococcus mutans

Table 3 (continued)

Fragment size (bases)	Group	Number of fragments (log_{10} copies/10 µl)		TRFMA database assignments
		Baseline	2 h	
				Firmicutes oral clone CH017
				Filifactor alocis
				Clostridiales spp. (2 phylotypes)
323	Placebo	5.36±0.18	5.20±0.15	Not identified
	Test	5.51±0.13	5.26±0.12*	
329	Placebo	5.31±0.21	5.19±0.15	*Flexibacter litoralis*
	Test	5.33±0.18	5.14±0.14*	
574	Placebo	5.63±0.22	5.50±0.15	*Bacteroidales* oral clone MCE7_120
	Test	5.50±0.18	5.21±0.15*	*Porphyromonas*-like sp. oral clone DA064
913	Placebo	5.28±0.16	5.06±0.12*	*Tannerella forsythensis*
	Test	5.12±0.14	5.00±0.11	
915	Placebo	5.16±0.13	4.88±0.09*	*Tannerella forsythensis*
	Test	5.08±0.10	4.81±0.05*	*Bacteroidetes* sp. oral clone FX069
				Bacteroides-like sp. oral clone AU126
				Porphyromonas sp. oral clone HF001

T-RFs with significant intra- or inter-group differences are listed. Data are expressed as mean log_{10} copies/10 µl ± standard error. The detection limit was 4.75 log_{10} copies/10 µl for the statistical analysis.

*$P<0.05$ between baseline and 2 h; **$P<0.05$ between the test and placebo groups

whereas the concentration in the placebo group was above the threshold level during the entire experimental period. In previous reports, the H_2S concentration of 1.5 ng/10 ml was still at a detectable but not objectionable level, and that of 2.5 ng/10 ml was at a slightly objectionable level [18, 19, 30]. Our results indicate that the median concentrations of H_2S in the test group after 10 min until 2 h were not objectionable, whereas the concentration in the placebo group increased to the objectionable concentration at 2 h. The actual numbers of subjects who showed the lower concentration than the olfactory threshold in the test and placebo groups were 2 and 0 at baseline, 10 and 4 at 10 min, 8 and 4 at 1 h, and 8 and 5 at 2 h, respectively in CH_3SH concentration, and 1 and 1 at baseline, 7 and 3 at 10 min, 7 and 3 at 1 h, and 8 and 4 at 2 h, respectively in H_2S, although any statistical difference between the test and placebo groups was not found in both CH_3SH and H_2S by χ^2 test.

The suppressive effect of the test tablet on the concentration of VSCs in the mouth air was more clearly observed in the levels of CH_3SH compared to the levels of H_2S. The odor of CH_3SH has been found to be more objectionable with a lower threshold of objectionability compared to H_2S [19]. Anaerobic periodontopathic bacteria have been implicated in the production of VSCs, especially CH_3SH, in the oral cavity [31–33]. LF and LPO have an inhibitory effect on several species of periodontopathic bacteria in vitro [14, 15, 17, 34]. The suppressive effect of LF on the number of periodontopathic bacteria in the subgingival plaque was also demonstrated [16]. The in vitro experiments based on the method previously described by Shin et al. [17] showed that the test tablet reduced the number of oral bacteria such as *A. actinomycetemcomitans* by more than 4 log units after incubation for 5 min in the presence of SCN^- at a physiological concentration in saliva, whereas the placebo tablet exhibited no bactericidal activity (data not shown). It is likely that the ingestion of the test tablet suppressed metabolic activity or the living number of periodontopathic bacteria residing in the oral microbial community (microflora). LF and LPO have also been shown to inhibit acid production and the uptake of amino acids or other nutrients [12, 13, 35]. In the metabolic pathway that produces VSCs, the uptake of amino acids or peptides is an essential step in utilizing sulfur-containing substrates [2, 3, 36, 37]. LF and LPO inhibit the activity of protease, which is a key enzyme in the degradation of high molecular weight proteins into low molecular weight peptides that provide substrates for VSC production [38, 39].

T-RFLP has been applied to the analysis of microflora profiles in oral and extra-oral specimens from humans [24, 40–43]. T-RFLP is a rapid and effective molecular method for analyzing the profile of microflora in oral specimens, as information on the constituents of oral microflora has been accumulated by sequencing the clone library of bacterial 16S rRNA [44–46] and a useful database has been established from the genetic information [25]. We improved the T-RFLP profiling reported by Takeshita et al. [24], i.e., we used the new internal size standard GeneScan 1200 LIZ to improve the accuracies of the standard peaks. The base

size of each T-RF was estimated and searched for in the TRFMA database, which contains information of the theoretical T-RFs of oral bacteria using the same primer sets and restriction enzymes as those used in the current study. Because the total bacterial numbers obtained by quantitative PCR analysis were diverse among saliva samples, the copy numbers of T-RFs estimated from the percentage area of the fragments and the copy numbers of total bacteria obtained from the analysis of 16S rRNA were compared. In the T-RFLP analysis of the present study, the 265-base T-RF had a significantly lower copy number at 2 h in the test group compared to the placebo group. This fragment was assigned to bacterial species belonging to *Prevotella*, *Porphyromonas*, and *Treponema*, which demonstrate the ability to produce VSCs both in vitro and in vivo [31–33, 36, 37]. These results suggest that the ingestion of a test tablet influenced the number of oral bacteria, especially VSC-producing microorganisms, although neither culturing nor quantitative PCR results could demonstrate the effects of the test tablet on the number of oral pathogenic bacteria in saliva.

In this study, the placebo tablet showed some suppression of VSC production, although to an extent less than that of the test tablet. Since fatty acid esters of sucrose and glycerol, which were added as lubricants to the tablets, are known to have the properties of detergents with bacteriostatic activity [47, 48], the suppressive effect of the placebo tablet on oral malodor may be due to the actions of these components. Trisodium citrate dihydrate and citric acid were added as buffer salts to the test tablet to make the solvent a slightly acidic condition, which itself does not influence the number of living bacteria in vitro but provide LPO with optimal condition for showing its antibacterial activity [17]. There were also differences in the contents of sugar and sugar alcohols between the test and placebo tablets. Glucose was added in the test tablet as a substrate for GO. The test tablet contains erythritol, xylitol, and maltitol at lower percentage of total sugar alcohols compared to the placebo tablet, which contains maltitol only. These sugar alcohols are not utilized by mutans streptococci and classified as non-cariogenic [49]. Furthermore, sugar alcohols, such as xylitol and maltitol, added to chewing gum were demonstrated not to affect the production of VSCs in vivo [21]

In conclusion, the results of this clinical study indicate short-term effects on oral malodor when ingesting a test tablet containing LF and LPO. As antimicrobial effects of the tablet were found in vivo, further clinical studies for a longer experimental period of several months should be conducted to demonstrate the long-term effects of the test tablet.

Conflict of interest statement Four of the authors, K. Shin, K. Yamauchi, T. Toida, and K. Iwatsuki, are employees of Morinaga Milk Industry Co., Ltd. All other authors declare that they have no conflict of interest related to this study.

Sources of funding This study was supported by research grants from Morinaga Milk Industry Co., Ltd.

References

1. Yaegaki K, Coil JM (2000) Examination, classification, and treatment of halitosis; clinical perspectives. J Can Dent Assoc 66:257–261
2. Loesche W, Kazor C (2002) Microbiology and treatment of halitosis. Periodontology 2000 28:256–279
3. Yaegaki K, Sanada K (1992) Biochemical and clinical factors influencing oral malodor in periodontal patients. J Periodontol 63:783–789
4. Scully C, Greenman J (2008) Halitosis (breath odor). Periodontology 2000 48:66–75
5. Roldan S, Winkel EG, Herrera D, Sanz M, Van Winkelhoff AJ (2003) The effects of a new mouthrinse containing chlorhexidine, cetylpyridinium chloride and zinc lactate on the microflora of oral halitosis patients: a dual-centre, double blind placebo-controlled study. J Clin Periodontol 30:427–434
6. Farrell S, Baker RA, Somogyi-Mann M, Witt JJ, Gerlach RW (2006) Oral malodor reduction by a combination of chemotherapeutical and mechanical treatments. Clin Oral Invest 10:157–163
7. Tilliss TS, Stach DJ, Cross-Poline GN (1992) Use of toothpicks for chlorhexidine staining. J Clin Periodontol 19:398–400
8. Young A, Jonski G, Rölla G (2003) Inhibition of orally produced volatile sulfur compounds by zinc, chlorhexidine or cetylpyridinium chloride—effect of concentration. Eur J Oral Sci 111:400–404
9. Lönnerdal B, Iyer S (1995) Lactoferrin: molecular structure and biological function. Annu Rev Nutr 15:93–110
10. Wakabayashi H, Yamauchi K, Takase M (2006) Lactoferrin research, technology and applications. Int Dairy J 16:1241–1251
11. Thomas EL, Bozeman PM, Learn DB (1991) Lactoperoxidase: structure and catalytic properties. In: Everse J, Everse KE, Grisham MB (eds) Peroxidases in chemistry and biology, vol 1. CRC, Boca Raton, pp 123–142
12. Arnold RR, Russell JE, Champion WJ, Brewer M, Gauthier JJ (1982) Bactericidal activity of human lactoferrin: differentiation from the stasis of iron deprivation. Infect Immun 35:792–799
13. Thomas EL, Milligan TW, Joyner RE, Jefferson MM (1994) Antibacterial activity of hydrogen peroxide and the lactoperoxidase–hydrogen peroxide–thiocyanate system against oral streptococci. Infect Immun 62:529–535
14. Ihalin R, Loimaranta V, Lenander-Lumikari M, Tenovuo J (2001) The sensitivity of *Porphyromonas gingivalis* and *Fusobacterium nucleatum* to different (pseudo)halide–peroxidase combinations compared with mutans streptococci. J Med Microbiol 50:42–48
15. Wakabayashi H, Yamauchi K, Kobayashi T, Yaeshima T, Iwatsuki K, Yoshie H (2009) Inhibitory effects of lactoferrin on growth and biofilm formation of *Porphyromonas gingivalis* and *Prevotella intermedia*. Antimicrob Agents Chemother 53:3308–3316
16. Kondo I, Kobayashi T, Wakabayashi H, Yamauchi K, Iwatsuki K, Yoshie H (2008) Effects of oral administration of bovine lactoferrin on periodontitis patients. Jpn J Conserv Dent 51:281–291 (in Japanese)
17. Shin K, Horigome A, Wakabayashi H, Yamauchi K, Yaeshima T, Iwatsuki K (2008) In vitro and in vivo effects of a composition containing lactoperoxidase on oral bacteria and breath odor. J Breath Res 2:017014 (5 pp)
18. Awano S, Koshimune S, Kurihara E, Gohara K, Sakai A, Soh I, Hamasaki T, Ansai T, Takehara T (2004) The assessment of methyl mercaptan, an important clinical marker for the diagnosis of oral malodor. J Dent 32:555–559

19. Tonzetich J, Ng SK (1976) Reduction of malodor by oral cleansing procedures. Oral Surg 42:172–181
20. Van den Velde S, van Steenberghe D, Van Hee P, Quirynen M (2009) Detection of odorous compounds in breath. J Dent Res 88:285–289
21. Lodhia P, Yaegaki K, Khakbaznejad A, Imai T, Sato T, Tanaka T, Murata T, Kamoda T (2008) Effect of green tea on volatile sulfur compounds in mouth air. J Nutr Sci Vitaminol 54:89–94
22. Tamaki Y, Nomura Y, Takeuchi H, Ida H, Arakawa H, Tsurumoto A, Kumagai T, Hanada N (2006) Study of the clinical usefulness of a dental drug system for selective reduction of mutans streptococci using a case series. J Oral Sci 48:111–116
23. Tadokoro K, Yamaguchi T, Kawamura K, Shimizu H, Egashira T, Minabe M, Yoshino T, Oguchi H (2010) Rapid quantification of periodontitis-related bacteria using a novel modification of Invader PLUS technologies. Microbiol Res 165:43–49
24. Takeshita T, Nakano Y, Yamashita Y (2007) Improved accuracy in terminal restriction fragment length polymorphism phylogenetic analysis using a novel internal size standard definition. Oral Microbiol Immumol 22:419–428
25. Nakano Y, Takeshita T, Yamashita Y (2006) TRFMA: a web-based tool for terminal restriction fragment length polymorphism analysis based on molecular weight. Bioinformatics 22:1788–1789
26. Salvolini E, Martarelli D, Di Giorgio R, Mazzanti L, Procaccini M, Curatola G (2000) Age-related modifications in human unstimulated whole saliva: a biochemical study. Aging Clin Exp Res 12:445–448
27. Jentsch H, Sievert Y, Göcke R (2004) Lactoferrin and other markers from gingival crevicular fluid and saliva before and after periodontal treatment. J Clin Periodontol 31:511–514
28. Tenovuo J (2002) Clinical applications of antimicrobial host proteins lactoperoxidase, lysozyme and lactoferrin in xerostomia: efficacy and safety. Oral Dis 8:23–29
29. Nagy K, Urban E, Fazekas O, Thurzo L, Nagy E (2007) Controlled study of lactoperoxidase gel on oral flora and saliva in irradiated patients with oral cancer. J Craniofac Surg 18:1157–1164
30. Oho T, Yoshida Y, Shimazaki Y, Yamashita Y, Koga T (2001) Characteristics of patients complaining of halitosis and the usefulness of gas chromatography for diagnosing halitosis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 91:531–534
31. De Boever EH, Loesche WJ (1995) Assessing the contribution of anaerobic microflora of the tongue to oral malodor. J Am Dent Assoc 126:1384–1393
32. Tanaka M, Yamamoto Y, Kuboniwa M, Nonaka A, Nishida N, Maeda K, Kataoka K, Nagata H, Shizukuishi S (2004) Contribution of periodontal pathogens on tongue dorsa analyzed with real-time PCR to oral malodor. Microbes Infect 6:1078–1083
33. Riggio MP, Lennon A, Rolph HJ, Hodge PJ, Donaldson A, Maxwell AJ, Bagg J (2008) Molecular identification of bacteria on the tongue dorsum of subjects with and without halitosis. Oral Dis 14:251–258
34. Aguilera O, Andrés MT, Heath J, Fierro JF, Douglas CW (1998) Evaluation of the antimicrobial effect of lactoferrin on *Porphyromonas gingivalis*, *Prevotella intermedia* and *Prevotella nigrescens*. FEMS Immunol Med Microbiol 21:29–36
35. Hamon CB, Klebanoff SJ (1973) A peroxidase-mediated, *Streptococcus mitis*-dependent antimicrobial system in saliva. J Exp Med 137:438–450
36. Persson S, Edlund MB, Claesson R, Carlsson J (1990) The formation of hydrogen sulfide and methyl mercaptan by oral bacteria. Oral Microbiol Immunol 5:195–201
37. Yoshimura M, Nakano Y, Yamashita Y, Oho T, Saito T, Koga T (2000) Formation of methyl mercaptan from L-methionine by *Porphyromonas gingivalis*. Infect Immun 68:6912–6916
38. Sano E, Miyauchi R, Takakura N, Yamauchi K, Murata E, Le QT, Katsunuma N (2005) Cysteine protease inhibitors in various milk preparations and its importance as a food. Food Res Int 38:427–433
39. Curtis MA, Aduse-Opoku J, Rangarajan M (2001) Cysteine proteases of *Porphyromonas gingivalis*. Crit Rev Oral Biol Med 12:192–216
40. Hayashi H, Sakamoto M, Benno Y (2002) Fecal microbial diversity in a strict vegetarian as determined by molecular analysis and cultivation. Microbiol Immunol 46:819–831
41. Sakamoto M, Takeuchi Y, Umeda M, Ishikawa I, Benno Y (2003) Application of terminal RFLP analysis to characterize oral bacterial flora in saliva of healthy subjects and patients with periodontitis. J Med Microbiol 52:79–89
42. Hommez GM, Verhelst R, Claeys G, Vaneechoutte M, De Moor RJ (2004) Investigation of the effect of the coronal restoration quality on the composition of the root canal microflora in teeth with apical periodontitis by means of T-RFLP analysis. Int Endod J 37:819–827
43. Rogers GB, Carroll MP, Serisier DJ, Hockey PM, Jones G, Bruce KD (2004) Characterization of bacterial community diversity in cystic fibrosis lung infections by use of 16S ribosomal DNA terminal restriction fragment length polymorphism profiling. J Clin Microbiol 42:5176–5183
44. Kroes I, Lepp PW, Relman DA (1999) Bacterial diversity within the human subgingival crevice. Proc Natl Acad Sci U S A 96:14547–14552
45. Kazor CE, Mitchell PM, Lee AM, Stokes LN, Loesche WJ, Dewhirst FE, Paster BJ (2003) Diversity of bacterial populations on the tongue dorsa of patients with halitosis and healthy patients. J Clin Microbiol 41:558–563
46. Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE (2005) Defining the normal bacterial flora of the oral cavity. J Clin Microbiol 43:5721–5732
47. Thomas LV, Davies EA, Delves-Broughton J, Wimpenny JW (1998) Synergist effect of sucrose fatty acid esters on nisin inhibition of Gram-positive bacteria. J Appl Microbiol 85:1013–1022
48. Brissette JL, Cabacungan EA, Pieringer RA (1986) Studies on the antibacterial activity of dodecylglycerol. J Biol Chem 261:6338–6345
49. van Loveren C (2004) Sugar alcohols: what is the evidence for caries-preventive and caries-therapeutic effects ? Caries Res 38:286–293