Study on the Effect of Lactoferrin and Lactoperoxidase on Oral Health and Its Mechanisms

（ラクトフェリンおよびラクトパーオキシダーゼの

口腔衛生に対する効果とその作用機序に関する研究）

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Contents

Abbreviations

BOP	Bleeding on probing
CH_3SH	Methyl mercaptan
DTT	Dithiothreitol
FCS	Fetal calf serum
FIC	Fractional inhibitory concentration
GO	Glucose oxidase
H_2S	Hydrogen sulfide
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HOSCN	Hypothiocyanous acid
IC50	50% inhibitory concentration
LF	Lactoferrin
LPO	Lactoperoxidase
MBTH	3-methyl-2-benzothiazolone hydrazine hydrochloride
SCN^-	Thiocyanate
TSA	Trypticase soy agar
O'Leary's PCR	O'Leary's plaque control record
$OSCN^-$	Hypothiocyanite
O_2SCN^-	Superoxythiocyanate
O_3SCN^-	Trioxythiocyanate
OTU	Operational taxonomic unit
PCoA	Principal coordinate analysis

PLP	Pyridoxal-5’-phosphate
PPD	Probing pocket depth
VSCs	Volatile sulfur compounds

Chapter 1 General introduction

Among 300-500 bacterial species estimated to be exist in the human oral cavity, there are pathogenic bacteria that can cause various conditions such as dental caries, periodontal disease, and oral malodor (1). An increasing number of studies have indicated an association between periodontal disease and various systemic diseases including diabetes, heart disease, cerebrovascular disease, pneumonia, rheumatoid arthritis, and kidney disease, suggesting that periodontal pathogens may be involved in the mechanisms underlying these conditions (2). As such, emphasis has been placed on prevention of periodontal disease and maintenance of healthy oral function as part of overall health in the second term of National Health Promotion Movement in the 21st century (Health Japan 21, the second term), which was initiated in April 2013. However, a Survey of Dental Diseases (Ministry of Health, Labour and Welfare, 2011) revealed periodontal disease in over 80% of persons aged between 30 and 70 years, suggesting that various issues need to be addressed. Although the number of teeth retained by elderly persons has been increasing, with more than 1 in 3 individuals are still having at least 20 teeth at the age of 80 years, there has also been an increase in the prevalence and severity of periodontal disease. Additional care is required to maintain oral health among the elderly since oral hygiene decline as saliva production decreases with age (3).

Lactoferrin (LF) and lactoperoxidase (LPO) are defensive factors in exocrine secretions, including milk and saliva (4). Table 1-1 summarizes the primary defensive factors and their concentrations in milk and saliva (5–9). LF is an iron-binding glycoprotein with various properties such as antibacterial activity against pathogens (including periodontal bacteria), anti-inflammatory activity, and anti-biofilm activity (10). LPO is a heme-binding glycoprotein that shows a strong antibacterial activity against various pathogens including periodontal bacteria by catalyzing the production of hypothiocyanite ($OSCN^-$) from

thiocyanate (SCN^-) and hydrogen peroxide in saliva (11), an antimicrobial cascade known as the LPO system. It has been reported glucose oxidase (GO) acts a source of hydrogen peroxide (12). Based on these reports, LF and LPO are thought to be important for the maintenance of oral hygiene in the oral cavity (12–14).

$$\text{Glucose} + O_2 \xrightarrow{\text{GO}} H_2O_2 + OSCN^-$$

$$SCN^- + H_2O_2 \xrightarrow{\text{LPO}} OSCN^- + H_2O$$

In this study, novel clinical effects of LF and the LPO system on oral health were explored and the underlying mechanism were investigated. In chapter 2, I report the *in vitro* effects of the LPO system on bacterial lyases of *Fusobacterium nucleatum* and *Porphyromonas gingivalis* involved in production of volatile sulfur compounds, which are major contributors to oral malodor. Chapter 3, I cover the synergistic effects of LF and the LPO system on *Candida albicans*, oral overgrowth of which causes tongue pain, a burning sensation, oral malodor and quality of life impairment. Chapter 4 reports a clinical trial assessing the effects on oral malodor of tablet containing LF and LPO. Finally, chapter 5 discusses I assessed the clinical effects of long-term ingestion of tablets containing LF and LPO on the oral microbiota, based on data obtained by 16S rRNA gene high-throughput sequencing. The results of these investigations demonstrated that LF and LPO were useful for patients with various oral problems.

Table in Chapter 1

Table 1-1. Concentrations of antimicrobial factors in human saliva and bovine milk

	Concentration (μg/ml)	
	Human saliva	Bovine milk
Lactoferrin	200	9-24
Lactoperoxidase	30	2
Immunoglobulin	500-1000	200
Lysozyme	0.4	5-50
β-defensin	0.15	1

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Chapter 2 Lactoperoxidase inactivates lyases related to oral malodor production

2.1 Abstract

The main components of oral malodor have been identified as volatile sulfur compounds (VSCs) including hydrogen sulfide and methyl mercaptan. The lactoperoxidase (LPO) system (consisting of LPO, glucose oxidase, glucose, and thiocyanate) has been reported to exhibit antimicrobial activities against oral bacteria. Although LPO-containing tablets, which utilize thiocyanate present in saliva, were found to have suppressive effects on VSCs in a previous clinical trial, the mechanism of action currently remains unclear. I herein examined the *in vitro* effects of the LPO system on the activities of bacterial lyases related to the production of VSCs by oral anaerobes. The exposure of crude bacterial extracts of *Fusobacterium nucleatum* and *Porphyromonas gingivalis* or purified methionine γ-lyase to the LPO system resulted in the inactivation of their lyase activities through $_{L}$-cysteine and $_{L}$-methionine, which is linked to the production of hydrogen sulfide and methyl mercaptan, respectively. These results indicated that methionine γ-lyase is one of the targets of the LPO system in bacterial lyases. The inactivation of lyase activities was similarly observed when living *F. nucleatum* and *P. gingivalis* cells were exposed to the LPO system. The LPO system did not reduce the number of viable *F. nucleatum* cells at near neutral pH 6.4 and 7.4, but moderately inactivated the lyase activity of the bacterium. The inactivation of the crude bacterial extracts of *F. nucleatum* and purified methionine γ-lyase by the LPO system was partly recovered by the addition of dithiothreitol. Therefore, the LPO system may inactivate bacterial lyases including methionine γ-lyase by reacting with the free cysteine residues of enzymes. These results suggest that the LPO system suppresses the production of VSCs not only through its antimicrobial effects, but also by its inactivating effects on the bacterial lyases of VSC-producing bacteria.

2.2 Introduction

Oral malodor is a common condition and concern amongst a large number of people. Volatile sulfur compounds (VSCs), mainly composed of hydrogen sulfide (H_2S) and methyl mercaptan (CH_3SH), were previously reported to be primarily responsible for oral malodor (1). VSCs have been identified as toxic agents that increase the permeability of the oral mucosa (2), suppress collagen synthesis (3), and inhibit osteoblast proliferation (4). These *in vitro* findings suggest that VSCs accelerate the progression of periodontal disease in addition to being a causative factor of oral malodor.

VSCs are produced from sulfur-containing amino acids by the bacterial lyases of oral anaerobes such as cystathionine γ-lyase (E.C. 4.4.1.1) and methionine γ-lyase (E.C. 4.4.1.11). Cystathionine γ-lyase has been detected in *Fusobacterium nucleatum* (5) while methionine γ-lyase was found in *F. nucleatum* (6) and *Porphyromonas gingivalis* (7). Cystathionine γ-lyase and methionine γ-lyase catalyze the α, β-elimination of $_L$-cysteine to produce H_2S, pyruvate, and ammonia. Methionine γ-lyase also catalyzes the γ-elimination of $_L$-methionine to produce CH_3SH, α-ketobutyrate, and ammonia.

Lactoperoxidase (LPO) is a glycoprotein found in milk, saliva, and other exocrine secretions (8, 9). It catalyzes the hydrogen peroxide-dependent oxidation of thiocyanate (SCN^-) to hypothiocyanite ($OSCN^-$), which is a potent antimicrobial agent against bacteria, fungi, and viruses (8, 9). This antimicrobial system is called the LPO system. $OSCN^-$ reacts with microbial thiol groups (10) and inhibits various functions such as glycolysis (11), the membrane transport of sugars and amino acids (12, 13), respiration (14, 15), and the urease activity of *Helicobacter pylori* (16).

The composition of LPO, glucose oxidase (GO), glucose, and buffer salts was previously shown to exhibit *in vitro* bactericidal activity against *Aggregatibacter actinomycetemcomitans* and *P. gingivalis* through the formation of $OSCN^-$ in the presence of saliva or SCN^- (17). In a recent clinical trial, the ingestion of LPO-containing tablets, which utilize SCN^- present in saliva, led to suppressive effects on the

concentrations of VSCs in mouth air (18). Although the ingestion of the tablet was considered to have suppressed the metabolic activity or cell number of periodontopathic bacteria residing in the oral microflora, the mechanism of action of reducing VSCs has not yet been elucidated in detail. Therefore, the aim of the present study was to examine the *in vitro* effects of the LPO system (consisting of LPO, GO, glucose, and sodium thiocyanate) on the bacterial lyases of *F. nucleatum* and *P. gingivalis* involved in the production of VSCs.

2.3 Material and methods

2.3.1 Bacteria and growth conditions

Fusobacterium nucleatum JCM 6328 was obtained from the Riken BioResource Center. *Porphyromonas gingivalis* ATCC 53978 was obtained from the American Type Culture Collection. These strains were grown and maintained on Trypticase soy agar (TSA; BD) supplemented with 5% (v/v) sheep blood, 10 mg/ml yeast extract, 5 μg/ml hemin, and 0.5 μg/ml menadione in an anaerobic chamber (37°C, 10% CO_2, 5% H_2 and 85% N_2; model 2000, Coy Laboratory Products). Bacteria were incubated at 37°C in Trypticase soy broth (BD) supplemented with 1 mg/ml yeast extract, 5 μg/ml hemin, and 5 μg/ml menadione overnight to prepare the inocula.

2.3.2 Lactoperoxidase system

The reaction mixture of the LPO system consisted of 4.2 units/ml of LPO purified from bovine milk (FrieslandCampina Domo), 0.4 units/ml of GO purified from *Penicillium chrysogenum* (Shin-Nihon Chemical), 1.55 mM glucose, and 0.66 mM sodium thiocyanate. The reaction mixture was prepared in anaerobic environment because the reaction through GO required dissolved oxygen. The reaction mixture in 40 mM phosphate buffer (pH 7.7) was incubated at 37°C for 10 and 30 min without the addition of

bacteria and the concentration of $OSCN^-$ was measured by monitoring the reaction with 5-thio-2-nitrobezonic acid (19).

2.3.3 Preparation of the crude bacterial extract

F. nucleatum or *P. gingivalis* in the stationary phase of growth were harvested by centrifugation at 3,000 × g for 15 min at 4°C, washed, and then suspended in 40 mM phosphate buffer (pH 7.7) containing 50 mM sodium chloride. The bacterial suspensions were mixed in a tube with glass beads (diameter, 0.1 mm; Yasui Kikai) and lysed by shaking with a Multi-Beads Shocker (Yasui Kikai) according to the following program: 3,000 rpm for 30 sec × 3 times with 30-sec intervals at 0°C. The supernatants were obtained as crude bacterial extracts by centrifugation at 16,000 × g for 30 min at 0°C. The protein contents in bacterial cells and crude bacterial extracts were measured by a commercial kit (Pierce BCA Protein Assay Kit; Thermo Scientific) using bovine serum albumin as the standard.

2.3.4. Effects of the LPO system in crude bacterial extracts - Lyase assay

The crude bacterial extract of *F. nucleatum* or *P. gingivalis* at 0.15 mg/ml was incubated with 1 ml of the LPO system in 40 mM phosphate buffer (pH 7.7) at 37°C for 10 and 30 min. The reaction of the LPO system was stopped by the addition of 10 µl of 10 mM sodium azide. The addition of sodium azide at this concentration did not have any inhibitory effects on lyase activities. Lyase activities toward $_L$-cysteine and $_L$-methionine were measured by determining the amounts of pyruvate and α-ketobutyrate using 3-methyl-2-benzothiazolone hydrazine hydrochloride (MBTH), as described previously (7, 20). In brief, 12 µl of the substrate solution of $_L$-cysteine or $_L$-methionine solution was added to 388 µl of the crude bacterial extract solution. The final concentration of the substrates was 10 mM. An incubation was carried out at 37°C for 90 min and the reaction was terminated by mixing with 200 µl of 4.5% (w/v)

trichloroacetic acid. The crude bacterial extract solution was centrifuged and 100 µl of the supernatant was added to 100 µl of 0.05% (w/v) MBTH in 1 M sodium acetate (pH 5.2) and then incubated at 50°C for 30 min. The amount of pyruvate or α-ketobutyrate was determined by spectrophotometry with A_{335}. Controls in which the LPO system was substituted for only SCN^- were simultaneously examined. Purified methionine γ-lyase from *Pseudomonas putida* (recombinant, expressed in *Escherichia coli*; Sigma-Aldrich) at a concentration of 1.0 mg/ml was also tested in the same manner.

2.3.5 Effects of the LPO system on bacterial cells

The LPO system with the same composition was also tested to assess its effects on the viability and lyase activities of bacterial cells. Approximately 10^7 cfu/ml of *F. nucleatum* or *P. gingivalis* was incubated with 30 ml of the LPO system in 40 mM citrate buffer (pH 5.0) at 37°C for 10 and 30 min under anaerobic conditions. Bacterial viability was assayed on TSA plates by spreading aliquots of serial 10-fold dilutions of the suspension. Bacterial colonies were counted after culturing for 6 days. The residual suspension was mixed with 300 µl of sodium azide to terminate the reaction of the LPO system. Bacteria were harvested by centrifugation at 3,000 × g for 15 min at 4°C, washed, and then resuspended in 40 mM phosphate buffer (pH 7.7) with 50 mM sodium chloride to a turbidity of 1.5 at 550 nm. Lyase activities in these bacterial suspensions were measured in the same manner as described above. The influence of pH on the activities of the LPO system was also examined.

2.3.6 Restoration of lyase activities by DTT

The crude bacterial extract of *F. nucleatum* and purified methionine γ-lyase were treated with the LPO system at 37°C for 30 min, and then incubated in the presence of dithiothreitol (DTT; Sigma-Aldrich) at 37°C for 15 min before the lyase assay.

2.3.7 Statistical Analysis

Data are expressed as means ± standard deviations (SD). Statistical analyses were performed between two groups using the two-tailed Student's t-test. *P* values of < 0.05 were considered to indicate a significant difference.

2.4 Results

2.4.1 Effects of the LPO system on lyase activities in crude bacterial extracts

I determined whether the LPO system affected the lyases involved in the production of VSCs in crude bacterial extracts. The LPO system yielded $OSCN^-$ at concentrations of 0.183 ±0.011 (mean ± SD of 4 independent experiments) after a 10-min incubation. The concentration of $OSCN^-$ did not increase significantly after a 30-min incubation (0.202 ± 0.019 mM; P = 0.13 vs. 10 min).

Exposure of the crude bacterial extract of *F. nucleatum* and *P. gingivalis* to the LPO system for 10 min resulted in significantly lower lyase activity toward $_L$-cysteine or $_L$-methionine than that of the control, which was incubated with SCN^- only (Table 2-1, $P < 0.01$). Lyase activity toward $_L$-cysteine or $_L$-methionine has been linked to the production of hydrogen sulfide or methyl mercaptan, respectively. After a 30-min incubation, the lyase activity of *P. gingivalis* toward $_L$-cysteine was significantly lower than that after 10 min ($P < 0.01$). Similar results were obtained for the activity of *F. nucleatum* toward $_L$-methionine ($P < 0.01$).

The lyase activities of purified methionine γ-lyase after a 10-min incubation with the LPO system were significantly lower than those of the control ($P < 0.01$). Lyase activities toward $_L$-cysteine or $_L$-methionine were significantly lower in the LPO system and control at 30 min than at 10 min ($P < 0.05$).

None of the LPO system components (i.e., LPO, GO, or glucose) significantly suppressed the lyase

activities of the crude bacterial extract of *F. nucleatum* to lower than those of the control (Table 2-2).

2.4.2 Effects of the LPO system on bacterial cells

I investigated whether the LPO system exerted bactericidal and lyase-inactivating effects against living bacterial cells. The number of viable bacterial cells of *F. nucleatum* after a 10-min incubation with the LPO system was decreased by less than 1 log unit, and was significantly lower than that of control cells incubated with SCN^- only after 30 min (Figure 2-1a, $P < 0.05$). The LPO system reduced the number of viable cells of *P. gingivalis* by more than 1 log unit after 10 min, and to below the detection limit (< 2.7 $\log_{10}$ cfu/ml) after 30 min (Figure 2-1b). The viability of *P. gingivalis* was more susceptible to the LPO system than that of *F. nucleatum*.

The exposure of bacterial cells to the LPO system also resulted in the inactivation of lyase activities. Lyase activities rapidly decreased during the first 10 min, and then slightly decreased from 10 min to 30 min. The percentages of lyase activities toward $_L$-cysteine and $_L$-methionine at 10 min were 47.2% and 54.7% (*F. nucleatum*, Figure 2-1c and 2-1e) and 29.2% and 28.8% (*P. gingivalis*, Figure 2-1d and 2-1f) of the control respectively. The lyase activities of *P. gingivalis* were more susceptible to the LPO system than those of *F. nucleatum*.

I then examined the influence of pH on the bactericidal and lyase-inactivating effects of the LPO system in *F. nucleatum*. The bactericidal and lyase-inactivating effects of the LPO system both depended on pH (Figure 2-2). The lowest number of viable cells and their lyase activities were observed under weakly acidic conditions (pH 4.2 and 4.6). At near neutral pH 6.4 and 7.4, the number of viable cells was reduced by less than 0.5 log10 cfu/ml of the initial number. The lyase activities for $_L$-cysteine and $_L$-methionine both decreased to approximately 60% of the control incubated at the same pH with only 0.66 mM SCN^-.

2.4.3 Restoration of lyase activities treated with the LPO system by DTT

I investigated whether the inactivation of lyase activities was due to oxidative modifications to the thiol groups of lyases by $OSCN^-$. The inactivation of the lyase activities of the crude bacterial extract from *F. nucleatum* was partly recovered by the addition of DTT in a dose-dependent manner (Figure 2-3a and 2-3c). Although the lyase activities of purified methionine γ-lyase were also recovered by the addition of DTT, the rate of restoration was less than that of the crude bacterial extract (Figure 2-3b and 2-3d).

2.5 Discussion

VSCs are the main causes of oral malodor and result from the degradation of L-cysteine and L-methionine by bacterial lyases (1). A recent clinical trial reported that sucking a LPO-containing tablet for 10 min had short-term continuous suppressive effects on VSCs (18). However, the mechanism of action has not yet been elucidated in detail. In the present study, I demonstrated that the activities of bacterial lyases toward both L-cysteine and L-methionine were significantly lower with the LPO system than with the control after a 10-min incubation. The time required for the *in vitro* inactivation of the lyase agreed with the sucking time of a LPO-containing tablet in the oral cavity in the clinical trial. Therefore, the inactivation effects of the LPO system on lyases may be one of the possible mechanisms of action of reducing VSCs.

The concentration of each component of the LPO system (LPO, GO, and glucose) in the present study was almost the same as that in 1.0 g of the test tablet from the clinical trial dissolved in 100 ml of buffer. This concentration was approximately 10-fold lower than that in saliva during sucking the test tablet. SCN^- was used at a concentration of 0.66 mM, which is within the range reported in human saliva

samples (0.55-1.87 mM for resting whole saliva, 0.36-1.32 mM for stimulated whole saliva; (21)). No significant differences were observed in the concentrations of $OSCN^-$ between the 10-min and 30-min incubations. These results implied that $OSCN^-$ was stable or in equilibrium for 30 min.

None of the components of the LPO system (i.e., LPO, GO, or glucose) inhibited the lyase activities of the crude bacterial extract of *F. nucleatum*. These results suggest that $OSCN^-$, which is a product of the LPO system, had inactivating effects on lyase activities. Hydrogen peroxide is generated at the onset of the reaction through GO. Previous studies reported that hydrogen peroxide was immediately reduced in the presence of peroxidase (17, 22); therefore, the contribution of hydrogen peroxide to the inactivating effects of the LPO system on lyases may have been negligible.

The LPO system also inactivated purified methionine γ-lyase, which degrades $_L$-cysteine and $_L$-methionine. This lyase has been detected in *F. nucleatum* (23) and *P. gingivalis* (7). Therefore, methionine γ-lyase is one of the targets of the LPO system in bacterial lyases. Methionine γ-lyase is considered to be an exploitable target for reducing VSCs (1). The inhibition of this enzyme may not have had a marked effect on human cells because methionine γ-lyase is not found in mammals (1). The inactivation of methionine γ-lyase by the LPO system may represent a safe and effective approach for preventing oral malodor.

The LPO system exhibited bactericidal and lyase-inactivating effects on living *F. nucleatum* and *P. gingivalis* cells. Since the suppressive effects of antimicrobial agents on oral malodor have been reported previously (24), the bactericidal effects of the LPO system appear to be one of the mechanisms of action involved in reducing VSCs.

Lyase activities rapidly decreased during the first 10 min and slightly decreased from 10 min to 30 min, whereas the number of viable cells decreased steadily for 30 min. These results suggested that the inactivation of lyases was not likely to correlate directly with the survivability of bacteria. In a previous

study, the growth rate of a methionine γ-lyase-deficient mutant of *P. gingivalis* was similar to that of the parent strain (7). The inhibition of other bacterial functions such as glycolysis, respiration, and glucose transport may contribute to bactericidal effects.

The viability and lyase activities of *P. gingivalis* were more susceptible to the LPO system than those of *F. nucleatum*. However, a previous study reported that the viabilities of *P. gingivalis* TUPg007 and *F. nucleatum* ATCC 10953 showed similar susceptibilities to the LPO system (25). Therefore, the differences observed in the susceptibilities of *P. gingivalis* and *F. nucleatum* in the present study may not be a general phenomenon.

The bactericidal activity of the LPO system against *F. nucleatum* cells depended on pH, which was consistent with previous findings (17, 26). At low pH, the main product of the LPO system is hypothiocyanous acid (HOSCN), which is in equilibrium with $OSCN^-$ (27). HOSCN is expected to be more reactive than $OSCN^-$ because the uncharged form has been shown to more rapidly penetrate the hydrophobic barrier of biological membranes (27). In the present study, a similar pH dependency was also apparent for the lyase-inactivating effects of the LPO system. Information on the intracellular localization of lyases in oral anaerobes is currently limited. However, these lyases appear to be intracellular enzymes because the crude bacterial extracts obtained by removing cell membranes exhibited lyase activities. Therefore, similar to the dependency of bactericidal effects on pH, the pH dependency of lyase-inactivating effects may be explained by the amount of HOSCN present, which can rapidly penetrate bacterial membranes. Since the LPO system partly inhibited lyase activities at near neutral pH 6.4 and 7.4, the LPO system may affect bacterial lyases in saliva, in which pH is 6 to 7 (28).

Hypothiocyanite is known to react with the thiol groups of bacterial enzymes or proteins (8, 29). In the present study, the inactivation of lyases treated with the LPO system was partly recovered by the addition of DTT. DTT has the ability to reduce the solvent-accessible disulfide bonds of proteins. In a

previous study, the combination of lactoperoxidase-thiocyanate-hydrogen peroxide inhibited sugar transport in *Streptococcus agalactive*, while the addition of DTT restored this ability. In addition, Nakayama *et al*. demonstrated that methionine γ-lyase was inactivated by a chemical modification with the thiol-specific cyanylating reagent, 2-nitro-5-thiocyanobenzonic acid, and this inactivation was reversed by DTT (30). These findings suggest that the inactivation of lyases by the LPO system may, at least partly, be attributed to the oxidative modification of thiol groups in bacterial lyases including methionine γ-lyase.

The cysteine residues in the active sites were found to be conserved in various methionine γ-lyases (Figure 2-4a; (31)). The cysteine residue is located in the catalytic pocket (Figure 2-4b) and is closed to the pyridoxal 5'-phosphate (PLP) co-factor (Figure 2-4c). This free cysteine residue is presumed to play an important role in the γ-elimination reaction of $_{L}$-methionine as well as in substrate recognition (31). Therefore, the LPO system may inactivate methionine γ-lyase by reacting $OSCN^-$ with the cysteine residue in the active site of this lyase.

The cysteine residue in the active site was not generally conserved in cystathionine γ-lyase, which has been detected in *F. nucleatum* (5), but not in *P. gingivalis* (Figure 2-4a). In the present study, I was unable to concretely confirm whether the LPO system inactivated cystathionine γ-lyase. A previous study reported that the LPO system inactivated $_{D}$-lactase dehydrogenase, which is considered to be non-thiol-dependent (14). Although the LPO system may also exert inactivating effects on cystathionine γ-lyase, further studies are needed to clarify the targets of the LPO system.

The results of the present *in vitro* study suggest that the LPO system suppressed the production of VSCs not only through its bactericidal effects on *F. nucleatum* and *P. gingivalis*, but also its inactivating effects on bacterial lyases. Since the elimination reaction of $_{L}$-cysteine and $_{L}$-methionine by lyases is an important process in VSC production, the inactivation of bacterial lyases is assumed to be a more effective

approach than a deodorant or masking for suppressing oral malodor for a long period. I considered the results of the present study to have contributed to a deeper understanding of the mechanism of action of the LPO system in the oral cavity. Tongue coating is the primary source of oral malodor (1, 32). The LPO system exerts its effects under aerobic conditions because the reaction through GO requires dissolved oxygen. Although it is likely that the LPO system affects VSC-producing bacteria in tongue coating utilizing dissolved oxygen in saliva, *in vivo* environments are more complex than the conditions used in the present *in vitro* study. Tongue coating comprises biofilms of various microorganisms and epithelial cells released from the oral mucosa. Therefore, further studies are needed to confirm whether the LPO system affects the bacterial lyases of VSC-producing bacteria on the tongue surface.

2.6 Tables and figures in Chapter 2

Table 2-1. Effects of the LPO system on lyase activities of crude bacterial extracts or a purified enzyme

			Lyase activity (pyruvate or α-ketobutyrate nmol/min/protein mg)		
	Time		Crude bacterial extracts		Methionine γ-lyase
Substrate	(min)	LPO system	*F. nucleatum*	*P. gingivalis*	from *P. putida*
L-Cysteine	10	–	75.0 ± 13.8	2.5 ± 0.6	29.1 ± 0.7
		+	49.5 ± 5.6 *	1.0 ± 0.3 *	1.6 ± 0.1 *
	30	–	68.4 ± 8.3	2.4 ± 0.3	23.0 ± 0.7 #
		+	39.6 ± 8.5 *	0.4 ± 0.2 * #	0.7 ± 0.1 * #
L-Methionine	10	–	41.1 ± 5.3	12.2 ± 0.3	28.0 ± 0.6
		+	2.7 ± 1.1 *	2.1 ± 0.4 *	2.6 ± 0.9 *
	30	–	38.3 ± 6.7	12.2 ± 0.8	24.6 ± 0.7 #
		+	0.3 ± 1.4 * #	2.1 ± 0.5 *	1.7 ± 0.2 * #

The crude bacterial extract of *F. nucleatum* JCM 6328 or *P. gingivalis* ATCC 53978 at 0.15 mg/ml or methionine γ-lyase from *P. putida* (recombinant, expressed in *Escherichia coli*) at 1 mg/ml was treated with the LPO system composed of 4.2 units/ml LPO, 0.4 units/ml GO, 1.55 mM glucose, and 0.66 mM SCN^- in 40 mM phosphate buffer (pH 7.7) at 37˚C for 10 and 30 min. The control (LPO system –) contained 0.66 mM SCN^- in phosphate buffer (pH 7.7). Values represent the mean ± SD of four experiments. *, $P < 0.01$ significantly different from the control. #, $P < 0.05$ significantly different from the result at 10 min.

Table 2-2. Effects of the LPO system or its components on lyase activities of crude bacterial extracts of *F. nucleatum*

	Lyase activity (pyruvate or α-ketobutyrate nmol/min/protein mg)	
	L-Cysteine	L-Methionine
Control	70.2 ± 9.7	40.1 ± 7.8
LPO system	41.4 ± 8.7 *	0.6 ± 0.6 *
LPO	59.5 ± 5.5	37.8 ± 1.6
GO	72.6 ± 10.0	36.2 ± 2.2
Glucose	72.7 ± 9.9	35.6 ± 2.8

The crude bacterial extract of *F. nucleatum* JCM 6328 at 0.15 mg/ml was treated with the LPO system or its component in 40 mM phosphate buffer (pH 7.7) at 37˚C for 30 min. The LPO system composed of 4.2 units/ml LPO, 0.4 units/ml GO, 1.55 mM glucose, and 0.66 mM SCN^-. The control contained 0.66 mM SCN^- in phosphate buffer (pH 7.7). Values represent the mean ± SD of three experiments. *, $P < 0.05$ significantly different from the control.

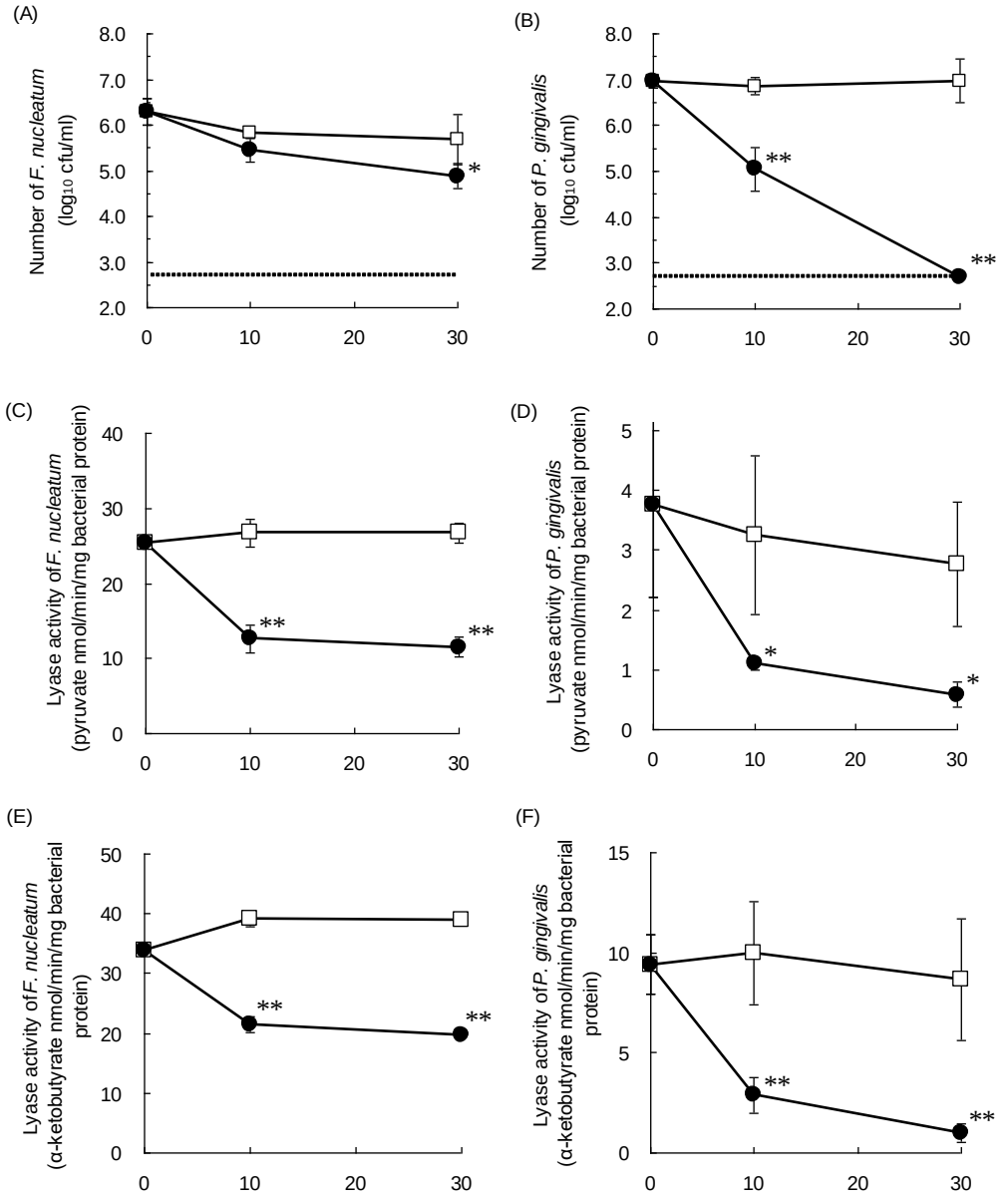


Figure 2-1. Effects of the LPO system on the viability and lyase activities of *F. nucleatum* and *P. gingivalis*

Approximately 7 $\log_{10}$ cfu/ml of *F. nucleatum* JCM 6328 (A, C, E) or *P. gingivalis* ATCC 53978 (B, D, F) was treated with the LPO system (closed circle) or control (open square) in 40 mM citrate buffer (pH 5.0) at 37˚C for 10 or 30 min under anaerobic conditions. Bacterial viability was assayed at the indicated times (A, B). The detection limit was shown as a broken line (2.7 $\log_{10}$ cfu/ml). Lyase activities were performed with L-cysteine (C, D) and L-methionine (E, F) in 40 mM phosphate buffer (pH 7.7) after removal of the LPO system. Data represent the mean ± SD of four experiments. *, $P < 0.05$ significantly different from the control; **, $P < 0.01$ significantly different from the control.

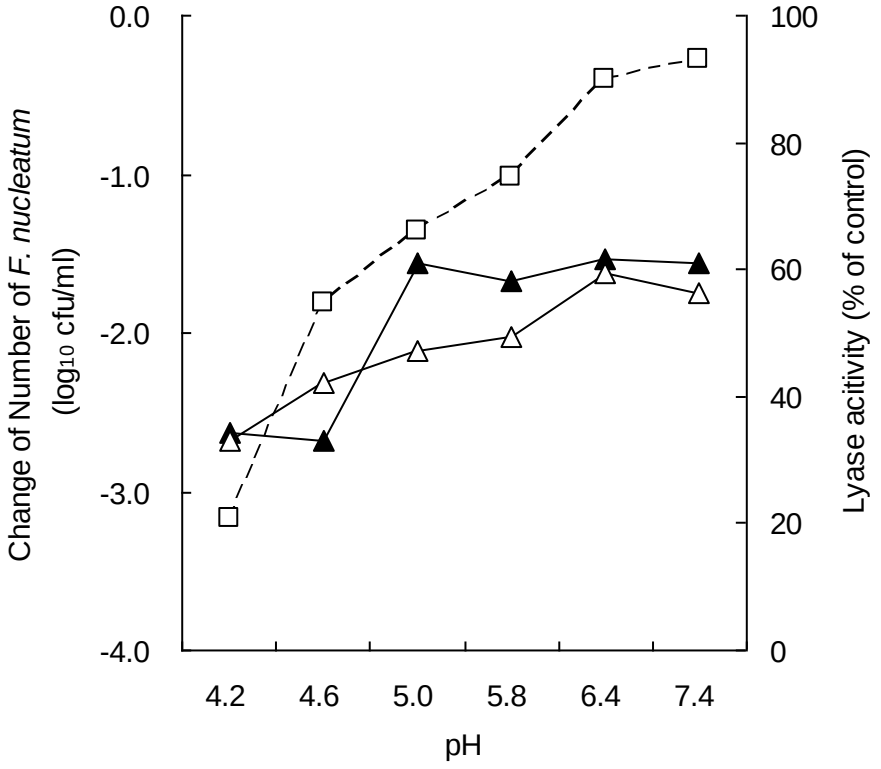


Figure 2-2. Influence of pH on bactericidal and lyase-inactivating effects of the LPO system

Approximately 7 $\log_{10}$ cfu/ml of *F. nucleatum* JCM 6328 was treated with the LPO system in 40 mM citrate buffer or phosphate buffer at 37˚C for 30 min under anaerobic conditions. Changes in the number of viable bacteria are shown (open square, n=4). After removal of the LPO system, lyase activities were performed with $_{L}$-cysteine (closed triangle, n=2) and $_{L}$-methionine (open triangle, n=2) and compared to a control incubated at the same pH with only 0.66 mM SCN^{-}.

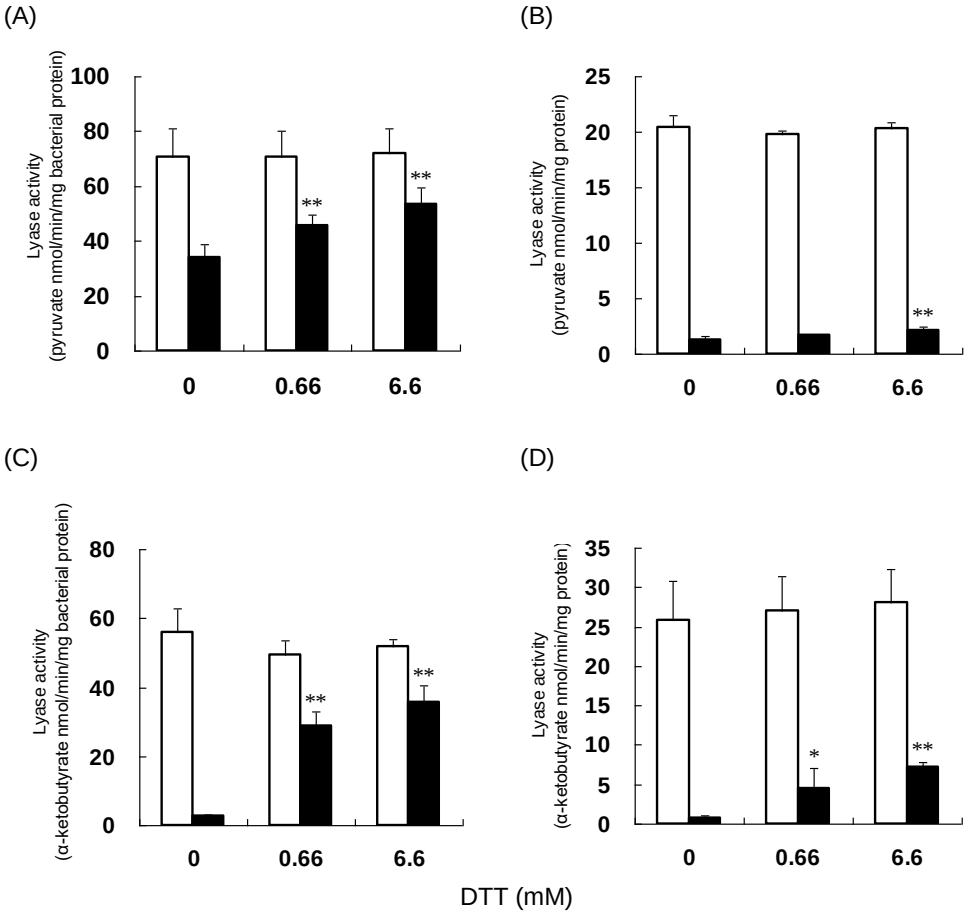


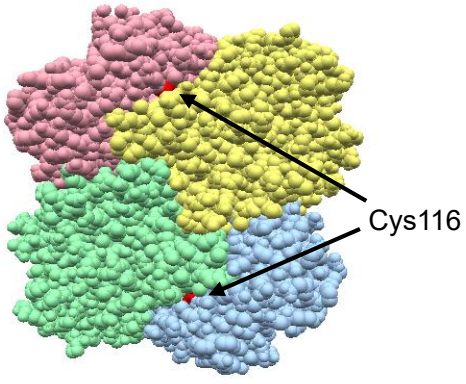
Figure 2-3. Inactivation of lyase activities with the LPO system was partly recovered by DTT

A crude bacterial extract of *F. nucleatum* JCM 6328 at 0.15 mg/ml (A, C) or methionine γ-lyase from *P. putida* (recombinant, expressed in *Escherichia coli*) at 1 mg/ml (B, D) was treated with 0.66 mM SCN^- (white bars) or the LPO system (black bars) in 40 mM phosphate buffer (pH 7.7) at 37˚C for 30 min, followed by a further incubation in the indicated concentrations of DTT for 15 min. Lyase activities were performed with L-cysteine (A, B) and L-methionine (C, D). Data represent the mean ± SD of three experiments. *, $P < 0.05$ significantly different from the results obtained with no DTT; **, $P < 0.01$ significantly different from the results obtained with no DTT.

(A)

	Homologous active site residues														
Methionine γ-lyase															
Pseudomonas putida	59	Y	S	R	114	Y	G	C	240	K	D				
Fusobacterium nucleatum	56	Y	T	R	111	Y	G	C	238	K	D				
Porphyromonas gingivalis	53	Y	T	R	108	Y	G	C	235	K	D				
Fusobacterium periodonticam	56	Y	T	R	111	Y	G	C	238	K	D				
Cystathionine γ-lyase															
Fusobacterium nucleatum	48	Y	S	R	102	Y	G	G	227	T	K				
Fusobacterium periodonticam	48	Y	S	R	102	Y	G	G	227	T	K				

(B)



(C)

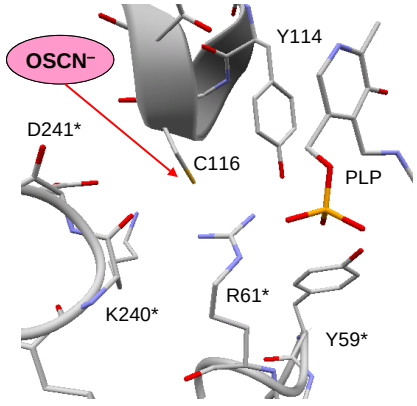


Figure 2-4. A proposed model for the inactivation of methionine γ-lyase by the LPO system

(A) Comparison of amino acid sequences of methionine γ-lyases and cystathionine γ-lyases. Cysteine residues conserved in methionine γ-lyase homologs are boxed. Multiple-sequence alignment was performed with Clustal W, version 2.1. (B) The overall structure of methionine γ-lyase of *P. putida* (PDB code 2O7C). Each subunit with the homotetramer is shown in pink, yellow, light green, and light blue. Cys116 (indicated in red) is located in the catalytic pocket (Kudou et al., 2008). (C) Active site of methionine γ-lyase from *P. putida*. Active site residues and pyridoxal-5'-phosphate (PLP) cofactor are indicated. The residues from the second subunit are marked by an asterisk. Cys116 is presumed to play an important role in the γ-elimination reaction of L-methionine and in substrate recognition (Kudou *et al*., 2008). $OSCN^-$, a reactive intermediate produced by the LPO system, appears to inactivate methionine γ-lyase by reacting with the cysteine residue in the active site. These figures were produced using jV, version 4.4.

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Chapter 3 Synergistic anti-candida activities of lactoferrin and the lactoperoxidase system

3.1 Abstract

Candida albicans is a commensal fungus in human mucosal surfaces, including the oral cavity. Lactoferrin (LF) and the lactoperoxidase (LPO) system, which are host protection components in exocrine secretions, each exhibit weak anti-candida activity. I herein examined the effects of the combination of LF and the LPO system on *C. albicans*. Morphological observations indicated that the combination of LF and the LPO system reduced the mycelial volume of *C. albicans* and changed the size and shape of cells more than each agent alone. The combination of LF and the LPO system also exerted strong inhibitory effects on the cellular metabolic activity and adhesive hyphal form of *C. albicans*. A checkerboard analysis revealed that the anti-candida activity of LF and the LPO system was synergistic. These results suggest that the combination of LF and the LPO system is useful for preventing candidiasis.

3.2 Introduction

Candida albicans is a commensal fungus in human mucosal surfaces, including the oral cavity. Oral candidiasis is an opportunistic infection of the oral cavity caused by the overgrowth of *Candida* spp., which causes tongue pain, a burning feeling, bad breath, and decreased quality of life. Reduced saliva production, poor oral hygiene, the long-term use of antibiotics, smoking, chemotherapy, and aging have been identified as risk factors for oral candidiasis (1). Although antifungal agents are used to treat oral candidiasis, their repeated usage is associated with the creation of resistant microorganisms (2, 3).

Lactoferrin (LF) and lactoperoxidase (LPO) are glycoproteins found in saliva, milk, vaginal secretions, and other exocrine secretions and play a role in host protection (4–6). LF exerts antimicrobial effects against fungi such as *Candida* (7, 8). LPO catalyzes the hydrogen peroxide-dependent oxidation of thiocyanate (SCN^-), a component in saliva, to hypothiocyanite ($OSCN^-$), which is a potent antimicrobial agent against fungi, bacteria, and viruses (5, 6). This antimicrobial system is called the LPO system. The combination of LPO, glucose oxidase (GO), glucose, and buffer salts was previously shown to exhibit *in vitro* antimicrobial activity against periodontal bacteria and *C. albicans* through the formation of $OSCN^-$ in the presence of SCN^- (9–11). The candidacidal activity of LF or the LPO system alone is moderate (8, 10). Although the combined use of LF with antifungal drugs has been reported to enhance anti-candida effects (12), it cannot be taken continuously and safely on a daily basis. In the present study, I assessed the combined effects of LF and the LPO system on the cellular metabolic activity of *C. albicans* using the alamarBlue assay. I also investigated the *in vitro* antifungal effects of the combination of LF and the LPO system against *C. albicans* using a crystal violet staining method to quantify adhesive hyphal cells, which are related to pathogenicity. The aim of the present study was to develop a new method for the prevention of oral candidiasis.

3.3 Materials and Methods

3.3.1 Materials

Bovine LF was produced by Morinaga Milk Industry Co., Ltd. (Tokyo, Japan). Components of the LPO system consisted of 43 mg/g of LPO purified from bovine milk (Tatua, Morrinsville, New Zealand), 430 mg/g of GO purified from *Penicillium chrysogenum* (Shin-Nihon Chemical, Aichi, Japan), 450 mg/g glucose, 24 mg/g citric acid, and 53 mg/g sodium citrate. 66 mM sodium thiocyanate solution was prepared and sterilized with filtration. LF and the LPO system were dissolved in 0.66 mM sodium thiocyanate.

Hypothiocyanite ($OSCN^-$) solution was produced enzymatically as described previously (13). In brief, 0.16 mg/ml LPO solution with 7.5 mM sodium thiocyanate and 3.75 mM H_2O_2 in 10 mM potassium phosphate buffer (pH 6.6) was incubated at room temperature for 15 min. After stopping the reaction by the addition of catalase, LPO and catalase were removed by centrifugation through a 10-kDa molecular mass cut-off filter (Amicon Ultra, Merck, Darmstadt, Germany) at 5000×g for 15 min. The concentration of $OSCN^-$ was measured by monitoring the reaction with 5-thio-2-nitrobezonic acid (14).

3.3.2 Preparation of medium for *C. albicans* hyphal formation

One milliliter of fetal calf serum (FCS, Thermo Fisher Scientific, MA., US) was aseptically added to 39 ml of RPMI 1640 medium (Sigma Chemical Co., Mo., US) with $_L$-glutamine and sodium bicarbonate (Sigma-Aldrich, MO., US), aseptically supplied with 0.03 g of penicillin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 0.03 g of kanamycin (FUJIFILM Wako Pure Chemical Corporation) and 2.39 g of HEPES (4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid, Dojindo, Kumamoto, Japan) in 500 ml of the same medium. This 2.5% FCS containing RPMI 1640 medium was three-fold diluted by adding sterile distilled water. The diluted FCS-RPMI medium was used in this study.

3.3.3 Assay for the inhibition of the cellular metabolic activity of *C. albicans*

C. albicans TIMM1768, a clinically isolated serotype A strain obtained from the Teikyo University Institute of Medical Mycology, was used in experiments. Cells were cultured on Sabouraud dextrose agar, which contained 1% Bactopeptone (Becton Dickinson, MD, US), 2% glucose, and 1.5% agar, and suspended the diluted FCS-RPMI medium. One hundred microliters of the *C. albicans* suspension (5×10^3 cells/ml) in a 96-well microtiter plate was prepared. After the addition of 100-µl serial dilutions of LF and the LPO system, the microtiter plate was incubated at 37°C for 16 h in 5% CO_2 in air. After the incubation, 20 µl of alamarBlue (Thermo Fisher Scientific) was added to each of the wells. Samples were incubated at 37°C for 20 h in 5% CO_2 in air. Absorbance at 570 and 594 nm was measured spectrophotometrically. The percent inhibition of cellular metabolic activity was calculated as follows. 100 − (absorbance (samples)/absorbance (control)) × 100 (%).

3.3.4 Assay for the growth inhibition of *C. albicans* in the hyphal form

The assay for the hyphal growth of *C. albicans* was performed using the method previously reported (15, 16). The assay was performed under the same culture conditions as described above. One hundred microliters of the *C. albicans* suspension (5×10^3 cells/ml) in a 96-well microtiter plate (MS-8096-F, Sumitomo Bakelite, Tokyo, Japan) was prepared. After the addition of 50-µl each of serial dilutions of LF and the LPO system to well of plate, the microtiter plate was incubated at 37°C for 16 h in 5% CO_2 in air. The hyphal growth content of *C. albicans* was assessed by the crystal violet staining assay. The medium in the wells was discarded by inverting the microplate. Adhesive *Candida* mycelia were sterilized by immersion in 70% ethanol. The plate was washed twice in distilled water. Mycelia were stained with 0.01% crystal violet (Merck, Darmstadt, Germany) for 20 min and washed 3 times with water. After drying the microplate, 150 µl of isopropanol containing 0.04 N HCl and 50 µl of 0.25% sodium dodecyl sulfate

were added to the wells. Samples were mixed by a plate mixer for 2 min to extract crystal violet from mycelia. The absorbance at 620 nm of triplicate samples was measured spectrophotometrically. The percent inhibition of *Candida* was calculated as follows: 100 − (absorbance (samples)/absorbance (control)) × 100 (%).

A checkerboard analysis was used to assess anti-candida combinations, and the results obtained were evaluated based on standard criteria (17). Concentrations of the combination of LF and the LPO system showing 50% inhibitory concentration (IC50) against *C. albicans* were assessed by the crystal violet staining method and plotted in closed circles on arithmetic scales. The fractional inhibitory concentration (FIC) index was calculated as follows: (lowest inhibitory concentration of LF in combination/IC50 of LF alone + lowest inhibitory concentration of the LPO system in combination/IC50 of the LPO system alone). FIC index values of ≤0.5, 1.0, and >4.0 represented synergism, additivity, and antagonism, respectively.

3.3.5 Statistical analysis

Statistical analyses were performed between two groups using the two-tailed Student's *t*-test. P values of < 0.05 were considered to indicate a significant difference.

3.4 Results

3.4.1 Effects of LF and the LPO system on the growth morphology of *C. albicans*

The effects of LF and the LPO system on the hyphal form of *C. albicans* were investigated morphologically. Figure 3-1 shows the hyphal growth of *C. albicans* after an incubation at 37°C for 16 h. LF alone at 500 μg/ml did not reduce the mycelial volume of *C. albicans*; however, morphologically, the hyphal length of *C. albicans* under these conditions was shorter than those of the control (Figure 3-1c). The LPO system alone at 500 μg/ml morphologically altered the hyphal shape of *C. albicans* to a slightly more isolated and

smaller colony-like appearance (Figure 3-1g). The combination of 125 μg/ml of LF and 125 μg/ml of the LPO system markedly reduced the mycelial volume of *C. albicans* and altered the size and shape of cells (Figure 3-1e).

3.4.2 Effects of LF and the LPO system on the cellular metabolic activity of *C. albicans*

AlamarBlue is a redox indicator that changes its color and fluoresces in response to metabolic activity (18). This method is commonly used to quantitatively assess the viability and proliferation of microorganisms. Table 3-1 shows the effects of the combination of LF and the LPO system on cellular metabolic activity. No marked inhibition was observed in the presence of 125 μg/ml of LF or the LPO system alone (Table 3-1). LF alone did not exert inhibitory effects, even at 2,000 μg/ml (data not shown). The LPO system alone at 500 μg/ml only exerted weak inhibitory effects (30.7% inhibition, data not shown). In contrast, the combination of more than 7.8 μg/ml of LF and more than 31 μg/ml of the LPO system completely inhibited metabolic activity.

3.4.3 Inhibitory effects of LF and the LPO system on the adhesive hyphal form of *C. albicans*

The *in vitro* antifungal effects of LF and the LPO system on *C. albicans* were also investigated using a crystal violet staining method to quantify adhesive hyphal cells. The IC50 of LF alone and the LPO system alone were 1,000 and 400 μg/ml, respectively (Table 3-2). In contrast, the combination of 7.8 μg/ml of LF and 50 μg/ml of the LPO system exerted inhibitory effects (82.6%). This combined effects of LF and the LPO system were characterized by the checkerboard analysis (Figure 3-2). The points indicate the concentration of each compound achieving more than 50% inhibition against *C. albicans* by the crystal violet staining method. The FIC index of this combination was 0.134, and this value indicated synergy (≤0.5). These results suggest that the combination of LF and the LPO system exerts strong cooperative

effects against the adhesive hyphal form of *C. albicans*.

I also assessed the inhibitory effects of LF combined with $OSCN^-$ solution using the crystal violet staining method. I produced this $OSCN^-$ solution enzymatically, and removed LPO and GO from the solution. When an $OSCN^-$ concentration of 225 μM was used, 500 μg/ml of LF exerted inhibitory effects against the adhesive hyphal form of *C. albicans* (56.7%, data not shown). On the other hand, LF alone at 500 μg/ml did not exert any inhibitory effects against the adhesive hyphal form (0.0%, data not shown). The $OSCN^-$ solution at a concentration of 225 μM exerted weak inhibitory effects (24.0%, data not shown). These results suggest that $OSCN^-$ solution, which is a potent antimicrobial product generated by the LPO system, also functions with LF against *C. albicans.*

3.5 Discussion

LF and LPO are antimicrobial components found in saliva and each exert weak candidacidal effects (8, 10). I herein demonstrated that the combination of LF and the LPO system exerted synergistic inhibitory effects on the cellular metabolic activity and adhesion of the hyphal form of *C. albicans*. The effects on the combination of LF and the LPO system (FIC index = 0.134) was more synergistic than that of the combined use of LF with antifungal drugs (FIC index = 0.187-190) (12). The hyphal form of *C. albicans* invades deeper host tissues and initiates clinical disease (1). Therefore, the inhibition of metabolism and adherence is assumed to be a more effective approach for preventing candidiasis.

LF with $OSCN^-$ solution also exerted inhibitory effects against *C. albicans*. This result suggests that $OSCN^-$, which is a product of the LPO system, is an active component. The activity of the LPO system was more effective than $OSCN^-$ alone, and may be explained by the LPO system also producing short-lived, highly reactive intermediates, such as superoxythiocyanate (O_2SCN^-) or trioxythiocyanate (O_3SCN^-) (19). The reaction through GO, which is a component of the LPO system, generates hydrogen

peroxide, which exhibits antifungal activity (20). Since previous studies reported that hydrogen peroxide was immediately reduced in the presence of peroxidase (21), the contribution of hydrogen peroxide to the effects of the LPO system may have been negligible. Furthermore, hydrogen peroxide exerts toxic effects against mammalian cells (22). The LPO system is safer than hydrogen peroxide alone because lactoperoxidase and thiocyanate protects against the toxic effects of hydrogen peroxide.

Previous studies suggested that the main antifungal mechanism of action of LF was dependent on iron and occurred through the direct interaction of LF with the fungal cell surface, leading to cell membrane damage and leakage (8). On the other hand, $OSCN^-$ reacts with microbial sulfhydryl groups and inhibits various functions, such as the membrane transport of sugars and amino acids, glycolysis, and respiration (19). Alterations in microbial membranes increased this efficacy of the LPO system, possibly by promoting the access of $OSCN^-$ to essential cell compounds (23). Accordingly, I speculate that cell membrane damage caused by LF may increase cell permeability to reactive $OSCN^-$ produced by the LPO system, and increase modifications to essential intracellular components. This mechanism of action may have a role in the synergistic inhibitory effects observed; however, further studies are needed to confirm this.

Reductions in salivary flow rates may increase the risk of opportunistic infections including oral candidiasis (24). The concentrations of LF and LPO in exocrine secretions have been reported to be 5-10 and 2 μg/ml in saliva, respectively (25, 26). These concentrations of LF and LPO in saliva were lower than IC50 in the present study. Hence, LF and the LPO system in saliva do not exert anti-candida effects alone. The synergistic inhibitory effects of LF and the LPO system against saliva may be the reason for the co-localization of LF and LPO in mammalian exocrine secretions.

In conclusion, I herein demonstrated that the combination of LF and the LPO system exerted synergistic inhibitory effects against *C. albicans*. These results suggest that food or a dietary supplement containing LF and the LPO system will be helpful for patients with oral issues caused by the excessive

growth of *C. albicans*. Further clinical trials are needed to assess the preventive effects of this combination of LF and the LPO system on oral candidiasis.

3.6 Tables and figures in Chapter 3

Table 3-1. Effects of the combination of LF and the LPO system on the cellular metabolic activity of *C. albicans* TIMM1768 using the alamarBlue assay.

LPO system (μg/ml)	% inhibition				
	LF (μg/ml)				
	0	2.0	7.8	31	125
125	3.2 ± 3.2	2.2 ± 4.3	101.4 ± 1.1*	101.2 ± 1.1*	102.0 ± 0.3*
31	0.0 ± 3.2	0.0 ± 5.4	98.9 ± 1.1*	97.8 ± 1.1 *	98.9 ± 1.1*
7.8	−3.2 ± 3.2	1.1 ± 12.9	−2.2 ± 5.4	9.7 ± 3.2	78.5 ± 7.5*
2.0	−1.1 ± 5.4	−2.2 ± 5.4	−1.1 ± 8.6	1.1 ± 1.1	1.1 ± 3.2
0	0.0 ± 3.2	−3.2 ± 4.3	−4.3 ± 6.5	4.3 ± 3.2	2.2 ± 3.2

Data represent the mean ± SE of three to six experiments. *, $P < 0.01$ significantly different from the control (no added agents).

Table 3-2. Effects of the combination of LF and the LPO system on the adhesion of the hyphal form of *C. albicans* TIMM1768 using the crystal violet staining assay

LPO system (μg/ml)	% inhibition						
	LF (μg/ml)						
	0	2.0	7.8	31	125	500	1000
600	60.7	68.5	73.4	88.5	89.4	90.6	90.8
400	51.1	64.2	69.1	89.6	89.0	89.8	90.2
300	48.5	45.6	52.3	87.7	88.8	89.2	90.2
200	21.5	38.4	86.5	87.5	88.6	89.6	90.0
100	21.5	11.2	86.9	87.5	88.5	88.5	89.8
50	-19.0	81.6	<u>82.6</u>	85.9	86.5	89.2	89.4
0	0.0	6.8	5.7	-0.8	9.7	44.5	56.3

A checkerboard analysis showed percent inhibition on the adhesion of the hyphal form. The underlined combination was used to calculate the FIC index for the combination of LF and the LPO system.

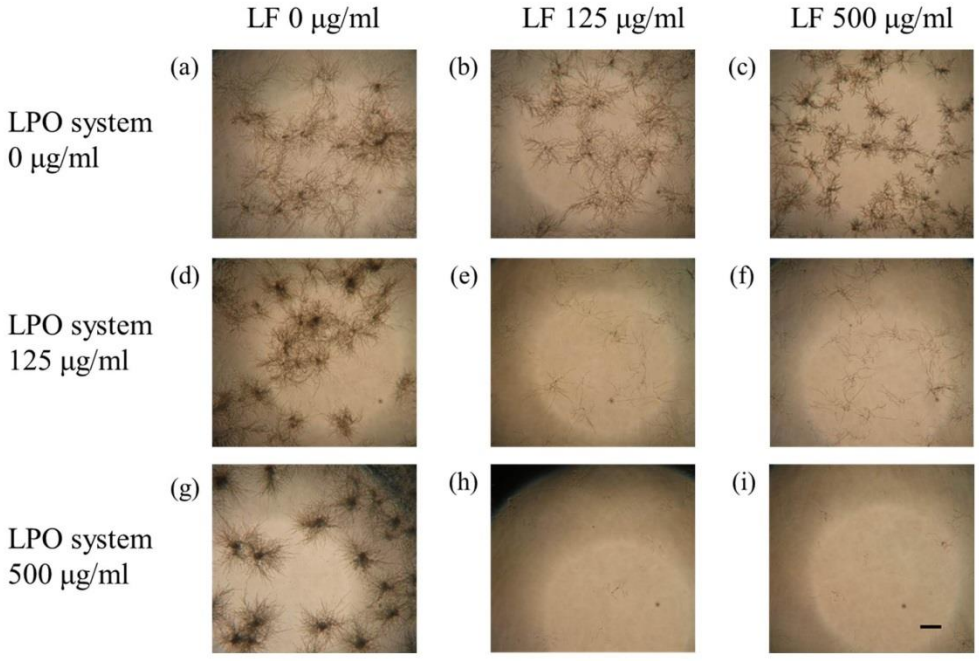

Figure 3-1. Phase-contrast micrographs of the hyphal growth of *C. albicans*

Approximately 5×10^3 cells/ml of *C. albicans* TIMM1768 were incubated with different doses of LF and the LPO system at 37° C for 16 h in 5% CO_2 in air. (a) Control culture showing the prominent development of hyphae; (b) culture with LF (125 μg/ml) alone showing the unchanged development of hyphae; (c) culture with LF (500 μg/ml) alone showing fewer hyphae; (d) culture with the LPO system (125 μg/ml) alone showing a slightly changed hyphal shape; (e, f) culture with LF (125 or 500 μg/ml) and the LPO system (125 μg/ml) showing a marked reduction in the mycelial volume of *C. albicans* and changes in the size and shape of cells; (g) culture with the LPO system (500 μg/ml) alone showing the changed hyphal shape of *C. albicans* to a slightly more isolated and smaller colony-like appearance; (h, i) culture with LF (125 or 500 μg/ml) and the LPO system (500 μg/ml) showing almost no hyphae. Bar, 100 μm.

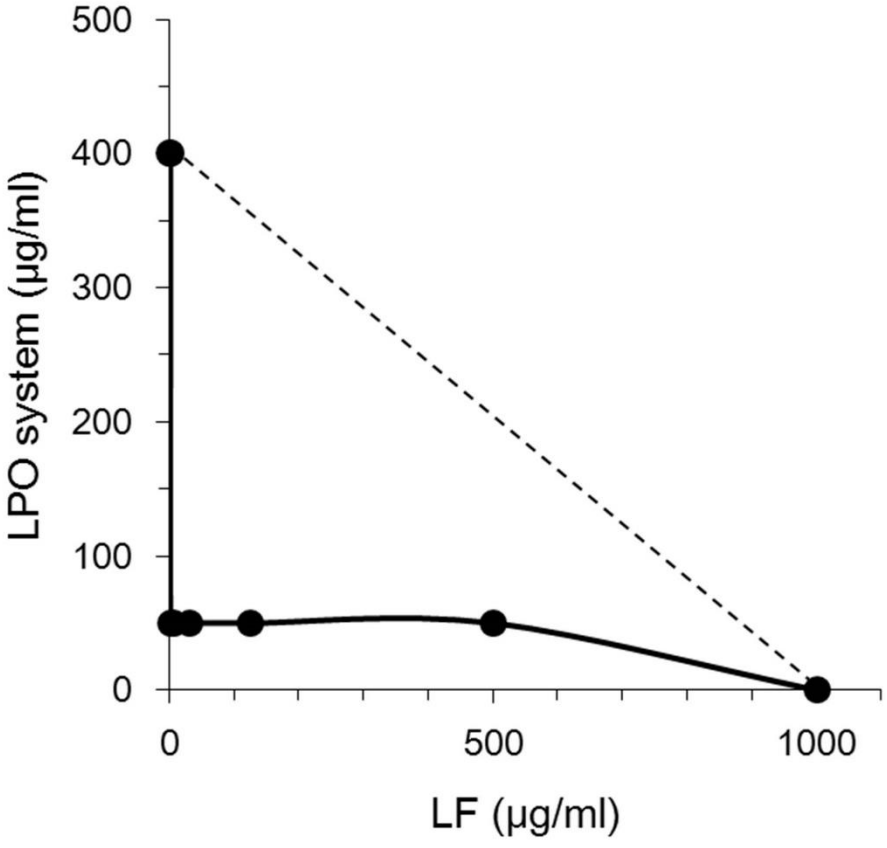

Figure 3-2. The anti-candida activity of the combination of LF and the LPO system was examined using a checkerboard analysis

The IC50 of LF and the LPO system against the adhesive hyphal growth of *C. albicans* TIMM1768 was assessed by the crystal violet staining method and plotted in closed circles on arithmetic scales. A putative additive effect is represented by the dashed line.

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Chapter 4 A randomized, double-blind, crossover, placebo-controlled clinical trial to assess effects of the single ingestion of a tablet containing lactoferrin, lactoperoxidase, and glucose oxidase on oral malodor

4.1 Abstract

Background: The main components of oral malodor have been identified as volatile sulfur compounds (VSCs) including hydrogen sulfide (H_2S) and methyl mercaptan (CH_3SH). VSCs also play an important role in the progression of periodontal disease. The aim of the present study was to assess the effects of the single ingestion of a tablet containing 20 mg of lactoferrin, 2.6 mg of lactoperoxidase, and 2.6 mg of glucose oxidase on VSCs in the mouth.

Method: Subjects with VSCs greater than the olfactory threshold in their mouth air ingested a test or placebo tablet in two crossover phases. The concentrations of VSCs were monitored at baseline and 10 and 30 min after ingestion of the tablets using portable gas chromatography.

Results: Thirty-nine subjects were included in the efficacy analysis based on a full analysis set (FAS). The concentrations of total VSCs and H_2S at 10 min were significantly lower in the test group than in the placebo group (–0.246 log ng/10 ml [95% CI –0.395 to –0.098], $P = 0.002$; –0.349 log ng/10 ml; 95% CI –0.506 to –0.192; $P < 0.001$, respectively). In the subgroup analysis, a significant difference in the concentration of total VSCs between the groups was also observed when subjects were fractionated by sex (male or female) and age (20–55 or 56–65 years). The reducing effect on total VSCs positively correlated with the probing pocket depth ($P = 0.035$).

Conclusions: These results suggest that the ingestion of a tablet containing lactoferrin, lactoperoxidase,

and glucose oxidase has suppressive effects on oral malodor.

Trial registration: This trial was registered with the University Hospital Medical Information Network Clinical Trial Registry (number: UMIN000015140, date of registration: 16/09/2014).

4.2 Introduction

Oral malodor is a common condition and a source of concern among a large number of people. When oral malodor is severe or long-standing, it may have a negative impact on self-confidence and social interactions (1). A previous study indicated that oral malodor is the third most common reason, following dental caries and periodontal disease, to visit the dentist (2). Volatile sulfur compounds (VSCs), mainly composed of hydrogen sulfide (H_2S) and methyl mercaptan (CH_3SH), are the main causes of oral malodor (3). VSCs occur due to the metabolic degradation of sulfur-containing amino acids by the lyases of oral anaerobes (4). A previous study reported a strong correlation between periodontal conditions and the concentrations of VSCs (4). VSCs have also been identified as toxic agents that accelerate the progression of periodontal disease by increasing the permeability of the oral mucosa (5), suppressing the synthesis of collagen (6) and inhibiting the proliferation of osteoblasts (7).

Different treatment strategies including rinsing with antimicrobial agents have been proposed for the management of oral malodor (8, 9). Although an antimicrobial mouth rinse containing chlorhexidine was previously shown to effectively suppress oral malodor (8), the use of chlorhexidine for extended periods of time has been associated with side effects including tooth and tongue staining and a bad taste (8, 10).

Lactoferrin (LF) and lactoperoxidase (LPO) are glycoproteins that are found in milk, saliva, and other exocrine secretions (11-14). LF and LPO have been shown to exert antimicrobial effects against oral pathogens. LF exhibits antibacterial effects against periodontopathic bacteria, including *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* (12, 15, 16), and antibiofilm effects against *P. gingivalis* and *Prevotella intermedia* (17). LPO catalyzes the hydrogen peroxide-dependent oxidation of thiocyanate (SCN^-) to hypothiocyanite ($OSCN^-$), which is a potent antimicrobial agent against bacteria, fungi, and viruses (13, 14). This antimicrobial system is called the LPO system. Glucose oxidase (GO),

which is used in the food industry, has been employed as a source of H_2O_2 (18).

$$\text{Glucose} + O_2 \xrightarrow{\text{GO}} H_2O_2 + OSCN^-$$

$$H_2O_2 + SCN^- \xrightarrow{\text{LPO}} OSCN^- + H_2O$$

A composition containing LPO, GO, glucose, and citrate buffer salts was previously found to exhibit *in vitro* bactericidal activity in the presence of saliva or SCN^- (19). A preliminary *in vivo* study suggested that this composition was effective for reducing oral malodor (19). In a previous clinical trial that enrolled 15 healthy volunteers aged 26–54 years, sucking a trial tablet containing 100 mg of LF, 1.8 mg of LPO, and 24 mg of Sumizyme PGO which was composed of dextrin and GO, had short-term suppressive effects on the concentrations of VSCs in mouth air (20). The underlying mechanisms of action reducing VSCs were suggested to involve the antimicrobial effects of LF and the LPO system (20) in addition to the inactivating effects of the LPO system on bacterial lyases involved in the production of VSCs (21). Based on these findings, a powder composition including 20 mg of LF, 2.6 mg of LPO, and 2.6 mg of GO was developed for functional food products (22). Several commercial food products including this composition were recently launched in Japan.

The aim of the present study was to assess the effects of the single ingestion of a commercial tablet containing 20 mg of LF, 2.6 mg of LPO, and 2.6 mg of GO on oral malodor. I enrolled 40 healthy adults in this trial, which was conducted on a larger scale than a previous related clinical trial (20). I also investigated the relationships between the treatment effects of the tablet and periodontal conditions of the subjects.

4.3 Methods

4.3.1 Study food

A commercial tablet (Morinaga Orabarrier, Morinaga Milk Industry Co., Ltd., Tokyo, Japan) was used as the test tablet. The test tablet contained 20 mg of LF, 2.6 mg (≥ 25 units) of LPO, and 2.6 mg (≥ 25 units) of GO as the active ingredients. The test tablet also contained glucose, citric acid, and sodium citrate, which support the effects of the active ingredients. The other ingredients in the test tablet were reduced palatinose, reduced sugar syrup, sorbitol, cellulose, calcium stearate, silicon dioxide, flavor, and sucralose. A placebo tablet contained cornstarch and coloring materials (gardenia pigment) instead of LF, LPO, GO, glucose, citric acid, and sodium citrate. LF (Morinaga Milk Industry Co., Ltd., Tokyo, Japan) and LPO (Tatua, Morrinsville, New Zealand) were purified from bovine milk. GO (Sumizyme PGO, Shin-Nihon Chemical, Aichi, Japan) was composed of dextrin and GO obtained from *Penicillium chrysogenum*. These test and placebo tablets were circular in shape with a diameter of 15 mm and thickness of 6 mm. The tablets were also identical in weight, texture, and appearance, but had slightly different tastes due to their ingredients.

4.3.2 Study design and subjects

This was a single center, randomized, double-blind, placebo-controlled, crossover study conducted in Japan. The study was conducted in accordance with Helsinki Declaration of 1975 and as revised in 2013. The study protocol was reviewed and approved by the Research Ethics Committee of Kenshokai (no. OBHALI01, on August 8, 2014). The study was performed at Shimizu Dental Clinic in Takasaki between October 2014 and May 2015 by following the CONSORT guidelines for clinical trial. Individuals who had been referred to Shimizu Dental Clinic were recruited, and screened after providing written informed consent. Inclusion criteria were all adults aged 20 to 65 who had VSCs greater than the olfactory threshold

(H_2S >1.5 ng and CH_3SH >0.5 ng/10 ml air) in mouth air, which were measured using portable gas chromatography as described below. Exclusion criteria were: 1) Subjects with severe liver, kidney, heart, lung, gastrointestinal, blood, endocrine, and metabolic diseases. 2) Subjects receiving treatments for dental diseases such as caries and periodontal disease. 3) Subjects treated with antibiotics in the past month. 4) Subjects who participated in other clinical studies in the past month. 5) Subjects with a history of allergies to milk and/or dairy products. 6) Subjects who were pregnant or lactating, or those who were expecting to become pregnant during the study. 7) Subjects judged inappropriate for the study by the investigator. Subjects were randomly assigned to one of two groups, initially at a 1:1 ratio. On the first test day, subjects in group A (n=20) ingested the test tablet, while subjects in group B (n=20) ingested the placebo tablet. After a 1-4-week washout, each subject ingested the alternative tablet to that on the first test day. No significant changes were made to the methods after trial commencement.

4.3.3 Outcome and clinical assessments

Oral malodor measurements were conducted in reference to the protocol described previously (23, 24). On the day of the assessment, each subject was asked to refrain from oral activities including eating, drinking, smoking, and using oral-hygiene practices from the time they woke up until the end of the assessment. Concentrations of VSCs at the baseline were analyzed with a portable gas chromatograph (OralChroma®, FIS Inc., Japan) (24), which was calibrated before starting the study. According to the manufacturer's instructions, subjects were instructed to keep a disposable plastic syringe in their mouth for 30 sec. A sample of their mouth air was taken, and 0.5 ml of sample air was injected into OralChroma. The concentrations of H_2S and CH_3SH were measured in each sample. I confirmed correct peak assignments for VSCs in the chromatograms using relevant software (OralChroma data manager, FIS Inc., Japan) (24). Two OralChroma readings were taken at each time point and the calculated average was

recorded as ng/10 ml. The concentration of total VSCs was obtained as the sum of the H_2S and CH_3SH concentrations. After the VSC analysis at baseline, each subject was instructed to suck the test or placebo tablet without chewing or swallowing it. The sucking time was measured by the examiner. The concentrations of VSCs were measured 10 and 30 min after ingestion of the tablet.

On the first test day, all subjects were also clinically evaluated for the following periodontal measurements after a series of oral malodor assessments; number of teeth, probing pocket depth (PPD), and bleeding on probing (BOP) (25). One well-trained examiner performed these assessments. PPD and BOP were entirely measured in each tooth. PPD measurements were recorded as the deepest pocket depth to the nearest millimeter.

The primary endpoint was the concentration of total VSCs at 10 min. The secondary endpoints were the concentrations of H_2S and CH_3SH.

4.3.4 Randomization and blinding procedure

The independent registration center (Morinaga Milk Industry) registered and randomized the subjects. The registration center generated a random sequence using a computer by the permuted block method (block size of 4). The allocation ratio was 1:1. The allocation sequence was concealed from study subjects, the dentist, co-medicals, data manager, and statistical analyst. According to the allocation sequence, the registration center gave a number to the test foods and chronologically assigned subjects to the test food number. After all data were fixed, the allocation sequence was broken.

4.3.5 Statistical Analysis

The sample size was set to 40, which was the maximum possible entry number during the study period. All analyses were based on the intention-to-treat principle. The concentrations of total VSCs, H_2S,

and CH_3SH did not show a normal distribution and were, thus, transformed logarithmically to approximate a normal distribution and obtain equal variance. I used linear mixed models to analyze data for total VSCs, H_2S, and CH_3SH. The fixed effects were treatment, period, and pretreatment values, while the random effect was subjects. The degree of freedom was adjusted by the Kenward-Roger method. I assessed carry-over effects by adding treatment-by-period interactions to the linear mixed model. In the subgroup analysis, I divided subjects into two groups by the median of continuous background data. I used Pearson's correlation to assess the relationship between the change in total VSCs from the baseline and dental clinical parameters. SAS 9.4 and JMP 9.0 (SAS Institute Inc., North Carolina, US) was used for all analyses. Two-sided $P < 0.05$ was considered significant for all tests.

4.4 Results

The flow of our subjects throughout the study is shown in Figure 4-1. Fifty-eight males and females were enrolled between October 2014 and May 2015. Forty subjects were randomized to group A (n = 20) and group B (n =20). After registration of the 40 eligible subjects, enrollment to the study ended in May 2015, and the study was completed in May 2015. Three of the subjects in group A did not complete the study. Two were unable to visit the clinic because of their circumstances. One did not ingest the tablet on the second test day because the investigator judged the concentration of VSCs at the baseline to be insufficient. Therefore, 39 subjects were included in the efficacy analysis based on a full analysis set (FAS). One subject in group B was excluded from the efficacy analysis because the concentration of VSCs at the baseline on the first test day was below the olfactory threshold.

The demographic and baseline characteristics of the full analysis set are listed in Table 4-1. The mean age was 49.4 years, and 64.1% were female. The mean number of teeth was 26.9, the average PPD was 4.2 mm, and the average BOP was 15.1%. The average sucking times in the test and placebo groups

were 343.7 ± 118.2 sec (179–665 sec) and 341.5 ± 130.3 sec (190–630 sec), respectively. No significant differences were observed in sucking times between the test and placebo tablets.

4.4.1 Efficacy

The effects of the test and placebo tablets on the concentrations of total VSCs, H_2S, and CH_3SH 10 and 30 min after their ingestion are summarized in Table 4-2. The concentration of total VSCs at 10 min was significantly lower in the test group than in the placebo group (adjusted difference –0.246 log ng/10 ml; 95% CI –0.395 to –0.098; $P = 0.002$). No significant differences were observed in the concentrations of total VSCs at 30 min between the test and placebo groups ($P = 0.199$). The concentrations of H_2S at 10 min were significantly lower in the test group than in the placebo group (adjusted difference –0.349 log ng/10 ml; 95% CI –0.506 to –0.192; $P < 0.001$). No significant differences were noted in the concentrations of H_2S at 30 min between the groups ($P = 0.066$). Significant differences were not found in the concentrations of CH_3SH at 10 and 30 min between the two groups. The differences observed in the concentrations of CH_3SH between the groups appeared to be slightly higher at 30 min than at 10 min. The concentrations of total VSCs, H_2S, and CH_3SH in both groups were significantly lower at 10 and 30 min than at the baseline. The average values (not estimated) of total VSCs, H_2S, and CH_3SH are shown in Supplemental Figure 4-S1.

No significant interaction was observed between treatment and period (data not shown). This result suggested that carryover effects were not present. The concentrations of VSCs in groups A and B were inversely related between days 1 and 2, showing almost similar changes (Supplemental Figure 4-S2).

Changes in the concentrations of VSCs in each of the 39 subjects examined are shown in detail in Figure 4-2. The olfactory thresholds of H_2S and CH_3SH were previously reported to be 1.5 and 0.5 ng/10 ml (0.18 and –0.30 log ng/10 ml) mouth air, respectively (26). The number of subjects with concentrations

of H_2S lower than the olfactory threshold was significantly higher in the test group (61.5% or 24/39 at 10 min, 59.0% or 23/39 at 30 min) than in the placebo group (31.4% or 11/35 at 10 min, 28.6% or 10/35 at 30 min), according to the chi-squared test (P = 0.010 at 10 min, P = 0.009 at 30 min). The number of subjects with concentrations of CH_3SH lower than the olfactory threshold was also higher in the test group (51.3% or 20/39 at 10 min, 66.7% or 26/39 at 30 min) than in the placebo group (42.9% or 15/35 at 10 min, 51.4% or 18/35 at 30 min), and no significant differences were observed between the groups at 10 min (P = 0.468) or 30 min (P = 0.183). The percentages of subjects with concentrations of CH_3SH lower than the detection limit (= 0 ng/10 ml) were 17.9% (7/39) at 10 min and 43.6% (17/39) at 30 min in the test group, and 20.0% (7/35) at 10 min and 25.7% (9/35) at 30 min in the placebo group, respectively.

The results of the subgroup analysis for outcomes are shown in Figure 4-3. The difference in the reduction of VSCs from the baseline to 10 min between the test and placebo groups was adopted as the treatment effect. The treatment effect on total VSCs and H_2S did not significantly differ when subjects were fractionated by sex and age.

The results of a correlation analysis among the effects on total VSCs, PPD, BOP, and age of subjects are shown in Table 4-3. The reducing effect on total VSCs positively correlated with PPD (r = 0.358, P = 0.035). A correlation was not detected between the treatment effect on total VSCs and BOP. Age positively correlated with PPD (r = 0.603, $P < 0.001$), whereas no correlation was observed for age and the treatment effect on total VSCs.

I investigated the influence of the sucking time of the test tablet on suppressing the concentration of VSCs by adding a treatment-by-sucking time interaction to the linear mixed model. Regarding the concentration of total VSCs at 10 min, the interaction was not significant (P = 0.196).

4.4.2 Safety

General conditions were obtained by interviewing subjects on each day of the assessment. One subject in the placebo group had cold-like symptoms during one of the washout periods, which did not appear to be related to any treatment administered in the present study. Thus, no adverse events related to the treatment were observed in any of the 40 subjects during the study.

4.5 Discussion

This randomized clinical trial was designed to evaluate the efficacy of the single ingestion of a commercial tablet containing 20 mg of LF, 2.6 mg of LPO, and 2.6 mg of GO on oral malodor. Our results demonstrated that oral malodor in the general population was lower following the single ingestion of a tablet containing LF, LPO, and GO than a placebo tablet. As for the primary outcome, the concentration of total VSCs at 10 min was significantly lower in the test group than in the placebo group. Furthermore, similar results were obtained for the concentrations of H_2S and total VSCs. It was estimated that the test tablet reduced the concentrations of total VSCs and H_2S in the mouth by approximately 57% ($\approx 10^{-0.246}$) and 45% ($\approx 10^{-0.349}$) that of the placebo tablet, respectively. No significant differences were observed in the concentrations of CH_3SH at 10 and 30 min between the groups; however, the difference noted in the concentration of CH_3SH at 30 min appeared to be slightly greater than that at 10 min. The proportion of CH_3SH to total VSCs was smaller than that to H_2S, and may be one of the reasons why a significant difference was not noted in CH_3SH concentrations. The test and placebo tablets both significantly reduced the concentration of VSCs at 10 and 30 min to lower than that at the baseline. The suppressive effects of the placebo tablet may also have been one of the reasons why significant differences were not observed between the two groups at 30 min.

VSCs are produced from sulfur-containing amino acids by oral anaerobes. The effects of many

antimicrobial agents on oral malodor have been reported previously (5). LF and the LPO system, consisting of LPO, GO, glucose, and SCN^-, exert *in vitro* antibacterial effects against oral pathogens (12, 19). I consider these antibacterial effects to be weaker than those of antibiotics which might affects indigenous bacteria and cause superinfections. In a previous clinical trial, terminal restriction fragment length polymorphism findings suggested that a tablet containing LF, LPO, and GO reduced one fragment assigned to bacterial species including VSC-producing bacteria (20). In spite of this moderate antibacterial effect, the suppressive effects of the active ingredients on VSCs were demonstrated in the present and previous studies. A recent *in vitro* study reported that the LPO system exhibited inactivation activity against the bacterial lyases related to the production of VSCs (21). Inactivation activity may be the main contributor to suppressive effects on VSCs. Thus, the test tablet immediately exerted suppressive effects on oral malodor. The reproduction of lyases by viable bacterial cells may gradually weaken suppressive effects.

Previous studies reported that a tablet containing LF, LPO, and GO significantly suppressed the concentration of VSCs 2 h after its ingestion to less than that at the baseline (20, 22). In the present study, the concentration of total VSCs in the test group was constantly low from 10 min to 30 min. The concentrations of H_2S and CH_3SH were lower than the olfactory threshold at 10 min in more than half of the subjects in the test group. The percentage of subjects in the test group with concentrations lower than the olfactory threshold at 30 min was nearly the same as that at 10 min. These results suggest that the suppressive effects of the tablet containing LF, LPO, and GO on oral malodor persisted for some time.

The test tablet contained glucose, citric acid, and sodium citrate, which support the effects of the active ingredients. Glucose, citric acid, and sodium citrate were unlikely to have exerted suppressive effects on VSCs because they are known not to exhibit antibacterial activity against periodontal bacteria (19) or inactivation effects on bacterial lyases involved in the production of VSCs (21). The placebo tablet contained cornstarch and coloring materials (gardenia pigment), which were added as replacements.

Although digested starch may be a source of a cariogenic biofilm, previous *in vitro* oral biofilm and *in vivo* studies reported that starch was markedly less cariogenic than sucrose (27, 28). Furthermore, I considered the influence of cariogenic biofilm formation on VSC production to have been negligible in the short term in the present study because biofilm formation requires a long period of time and most cariogenic species do not play a role in the production of VSCs (2). The placebo tablet exerted some suppressive effects on oral malodor. To the best of our knowledge, the suppressive effects of the ingredients of the placebo tablet including cornstarch and coloring materials on oral malodor have not been reported previously. On the other hand, many studies demonstrated that tongue cleaning reduced the amount of tongue coating and number of oral bacteria, thereby effectively improving oral malodor (2, 5, 29). Another previous clinical trial suggested that a placebo tablet without active ingredients reduced VSCs in the short term through its mechanical cleaning effect (30). Therefore, the suppressive effects of the placebo tablet on oral malodor in the present study may have been due to its mechanical cleaning effects. I assumed that the test tablet suppressed VSCs not only through the antimicrobial activities of LF and the LPO system against VSC-producing bacteria, but also by mechanical cleaning during sucking.

The concentration of total VSCs was significantly lower 10 min after ingestion of the previous trial tablet, which contained 100 mg of LF, 1.8 mg of LPO, and 24 mg of Sumizyme PGO, than the placebo tablet (20). In the present study, the concentration of VSCs was significantly lower 10 min after ingesting the commercial tablet containing 20 mg of LF, 2.6 mg of LPO, and 2.6 mg of GO than the placebo tablet. These results suggested that the efficacy of this commercial tablet, which had less LF, in suppressing oral malodor was similar to that of the previous trial tablet in the short term.

A limitation of our study is the slight difference in taste between the tablets, which was due to their ingredients. Although our subjects may have been able to distinguish between these tablets, they were unable to control the concentrations of VSCs measured by OralChroma. Therefore, I considered the

robustness against a measurement bias to have been sustained.

The treatment effects of the test tablet on total VSCs and H_2S did not differ when subjects were fractionated by sex and age. Furthermore, a correlation was not observed between age and the treatment effect on VSCs. A previous survey reported no significant differences in VSCs between males and females or among ages (31). It appears reasonable to extrapolate the results obtained in the present study to general populations.

The reducing effect on total VSCs positively correlated with PPD. These results suggested that treatment effects were greater in subjects with deep periodontal pockets. Tanaka *et al*. found positive coefficients between the percentages of probing pocket depth ≥ 4 mm and the population of periodontal pathogens including *P. gingivalis* on the tongue dorsum, and the population of these pathogens positively correlated with the concentration of total VSCs (32). In the present study, subjects with deep pocket depths may have had a larger population of VSC-producing bacteria, which are considered the target of LF and the LPO system.

In order to allow active ingredients to function effectively in the mouth, subjects were asked to suck the tablets without biting or swallowing them. The time for tablets to dissolve completely was approximately 3 to 11 min. I investigated the influence of the sucking time of the test tablet on suppressing the concentration of VSCs. The sucking time did not interact with the treatment effects. These results suggested that the suppressing effects on oral malodor occurred when the subject sucked the test tablet for at least 3 min. In a previous *in vitro* study, a composition containing LPO, GO, glucose, and SCN^- reduced the number of *A. actinomycetemcomitans* by more than 1 log unit after 3.75 min (19), and was found to inactivate the bacterial lyase related to VSC production after 10 min (21). These *in vitro* bactericidal and inactivating effects in a short time period may contribute to the immediate effects of the test tablet on oral malodor.

No adverse events related to the treatment were observed in any of the 40 subjects during the study. All ingredients in the current test tablet including LF, LPO, and GO have been permitted as a food or food additive in Japan. Furthermore, the long-term ingestion of previous trial tablets containing LF (300 mg/day), LPO (5.4 mg/day) and Sumizyme PGO (72 mg/day) for 12 weeks had no adverse events in general or on the oral condition (33). Therefore, the test tablet in the present study may be continuously and safely taken on a daily basis.

Severe or long-standing oral malodor has a negative impact on self-confidence and social interactions (1). Therefore, a daily treatment for oral malodor is considered important. A previous study demonstrated that VSC concentrations decreased after a meal and then gradually increased between meals (23). Since the test tablet has excellent portability and immediately suppresses oral malodor, it has potential as a daily treatment, particularly for suppressing VSCs between meals. Furthermore, VSCs have been shown to affect the progression of periodontal disease (5-7). The long-term administration of this tablet may potentially contribute to maintaining the oral hygiene status.

The results of the present clinical trial suggested that the single ingestion of a tablet containing 20 mg of LF, 2.6 mg of LPO, and 2.6 mg of GO exhibited suppressive effects on VSCs in mouth air in the general population.

4.6 Tables and figures in Chapter 4

Table 4-1. Demographic and baseline characteristics of the full analysis set (FAS)

Parameters	Values
Age (mean ± SD)	49.4 ± 15.3
Sex (%)	
Female	64.1 (25/39)
Male	35.9 (14/39)
Clinical parameters (mean ± SD)	
Number of teeth	26.9 ± 2.8
PPD[1]	4.2 ± 0.5
BOP[2]	15.1 ± 20.0

[1] Probing pocket depth (mm).

[2] Bleeding on probing (% of teeth).

[3] Obtained from the *t*-test.

Table 4-2. Effects of test and placebo tablets on total VSCs, H_2S, and CH_3SH 10 and 30 min after their ingestion

Outcomes	Group	LS mean ± SE[1]	Difference[2] (95% CI)	*P*-value
Primary outcome				
Total VSCs				
10 min	Test	0.115 ± 0.078	-0.246	0.002
	Placebo	0.362 ± 0.081	(-0.395 to 0.098)	
30 min	Test	0.173 ± 0.086	-0.136	0.199
	Placebo	0.309 ± 0.090	(-0.348 to 0.075)	
Secondary outcomes				
H_2S				
10 min	Test	-0.085 ± 0.083	-0.349	< 0.001
	Placebo	0.263 ± 0.086	(-0.506 to 0.192)	
30 min	Test	0.056 ± 0.080	-0.171	0.066
	Placebo	0.226 ± 0.084	(-0.353 to 0.012)	
CH_3SH				
10 min	Test	-0.635 ± 0.129	-0.044	0.747
	Placebo	-0.591 ± 0.134	(-0.321 to 0.233)	
30 min	Test	-1.070 ± 0.141	-0.265	0.163
	Placebo	-0.806 ± 0.149	(-0.641 to 0.112)	

[1] The concentration (log ng/10 ml) of VSCs estimated using a linear mixed model.

[2] Difference (log ng/10 ml) in the LS mean.

LS: least square

Table 4-3. Pearson correlations between effects on total VSCs and parameters for the clinical assessment

Parameter	vs. Parameter	r	*P*-value
PPD[1]	ΔΔTotal VSCs[2]	0.358	0.035
BOP[3]	ΔΔTotal VSCs	0.273	0.113
Age	ΔΔTotal VSCs	0.200	0.249
Age	PPD	0.603	< 0.001

[1] The mean value of the probing pocket depth (mm) of each tooth.

[2] Reducing effect on total VSCs; Differences between the test and placebo groups in the extent of reductions in the concentration of total VSCs from the baseline to 10 min after ingestion of the tablets.

[3] Bleeding on probing (% of teeth).

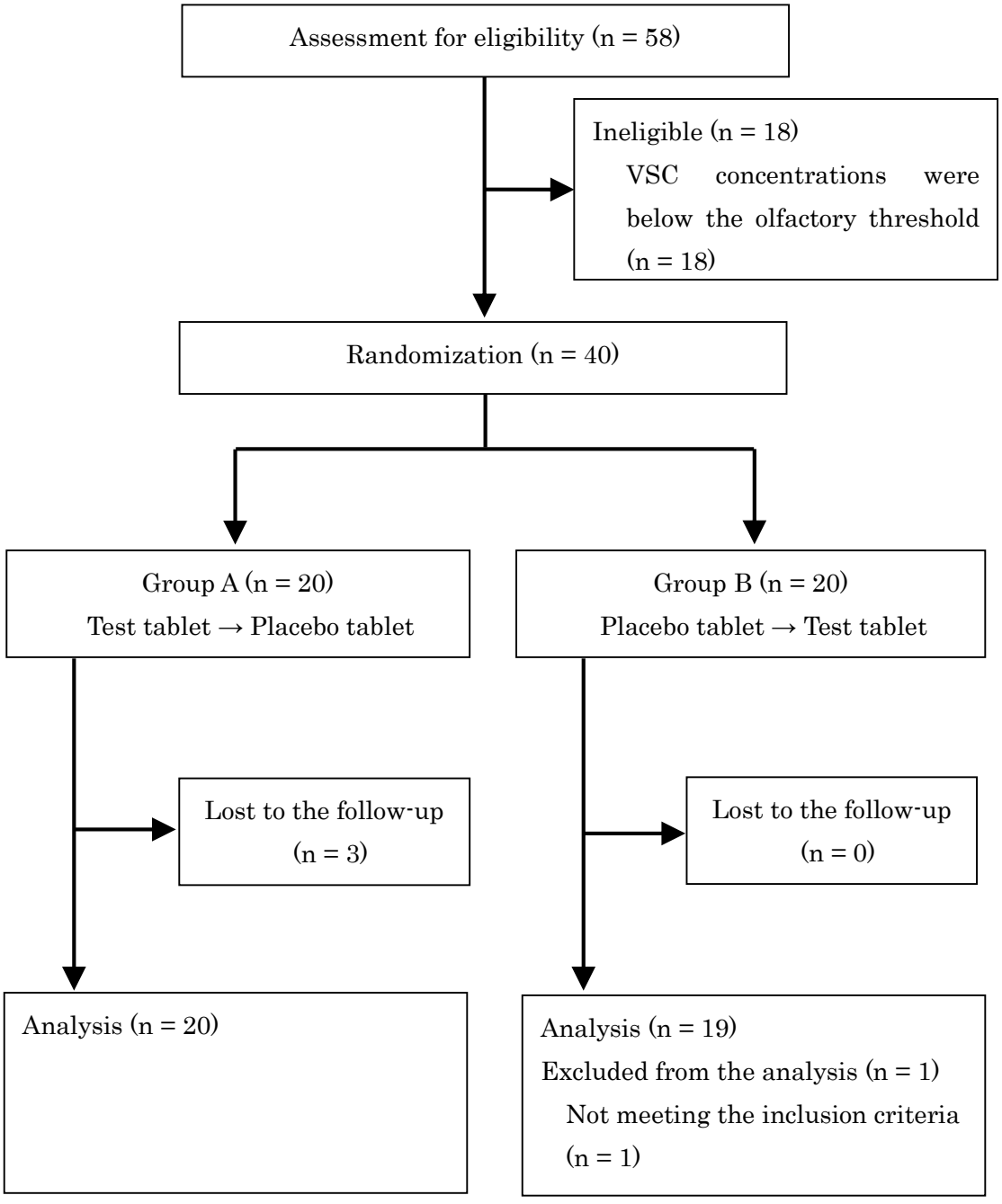


Figure 4-1. Flow diagram of subjects throughout the study

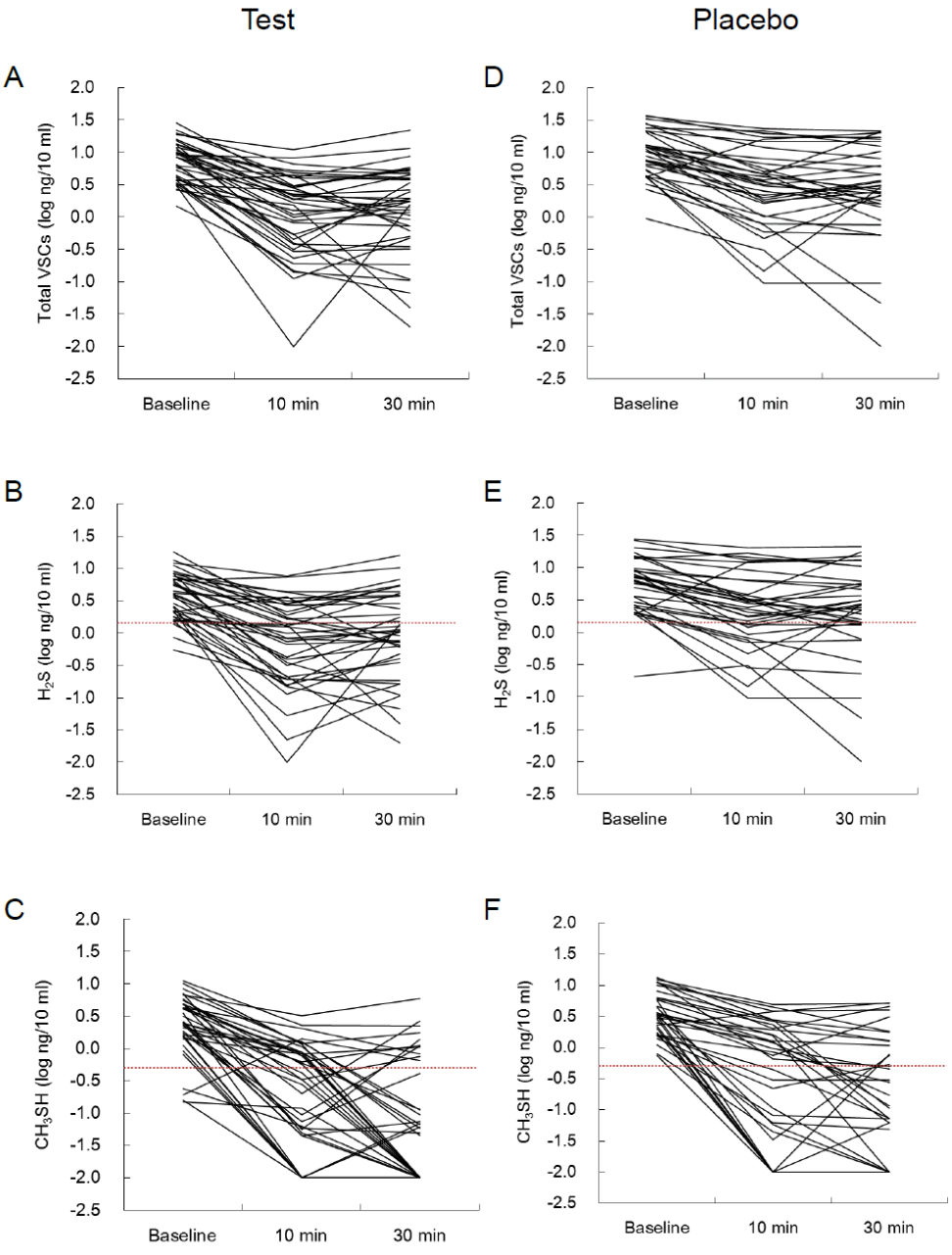


Figure 4-2. Changes in VSC concentrations in each of the 39 subjects

(A, B) Total VSCs, (C, D) H_2S, and (E, F) CH_3SH were measured at the baseline and 10 and 30 min after the ingestion of the test tablet (A, C, E) and placebo tablet (B, D, F). The olfactory thresholds of H_2S (1.5 ng/10 ml ≈ 0.18 log ng/10 ml) and CH_3SH (0.5 ng/10 ml ≈ –0.30 log ng/10 ml) are shown in broken lines.

Subgroup	Number of data	Difference[1] (log ng/10 ml, 95% CI)
Total VSCs		
Overall	74	−0.246 (−0.395 to −0.098)
Sex		
Women	48	−0.194 (−0.378 to −0.009)
Men	26	−0.283 (−0.561 to −0.004)
Age		
56-65	39	−0.280 (−0.546 to −0.013)
20-55	35	−0.195 (−0.370 to −0.020)
H_2S		
Overall	74	−0.349 (−0.506 to −0.192)
Sex		
Women	48	−0.346 (−0.551 to −0.141)
Men	26	−0.213 (−0.516 to 0.090)
Age		
56-65	39	−0.453 (−0.706 to −0.201)
20-55	35	−0.234 (−0.436 to −0.031)

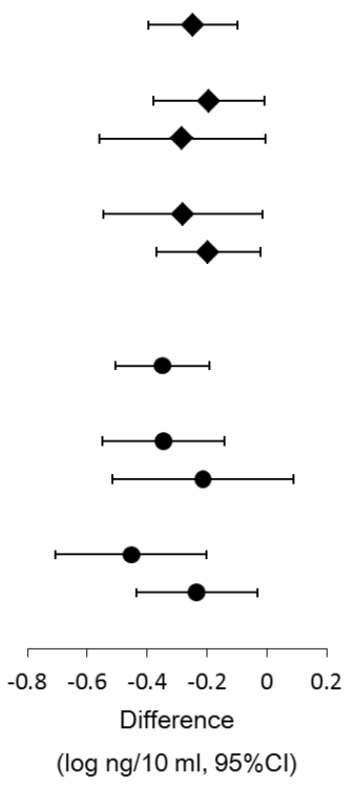
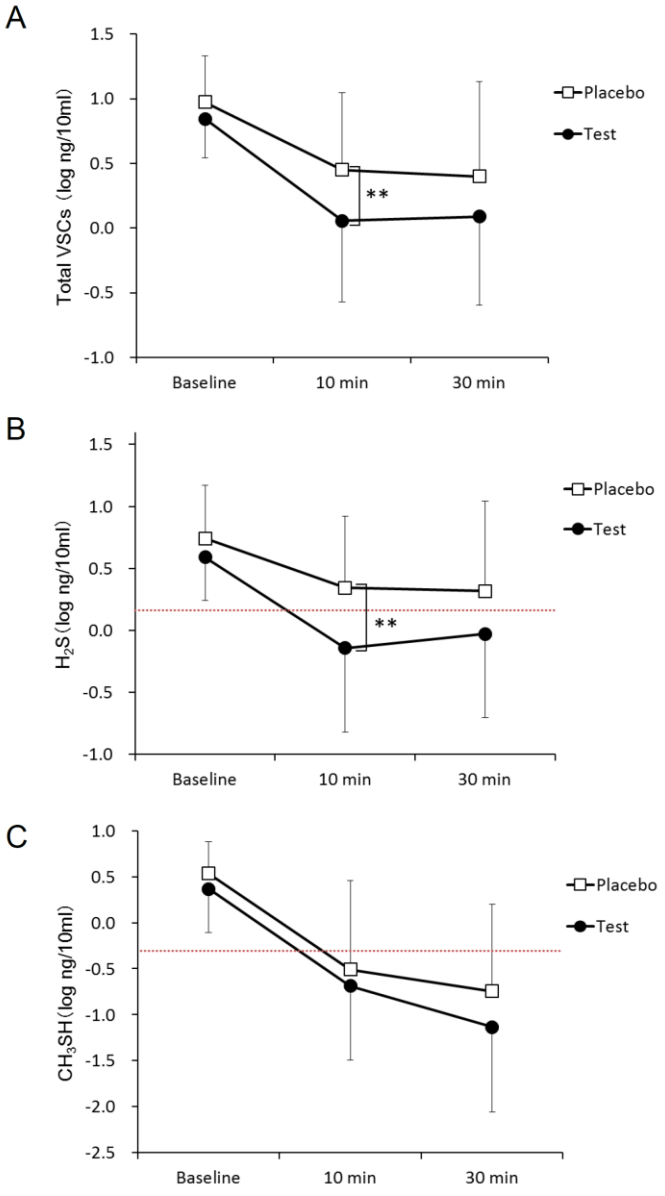
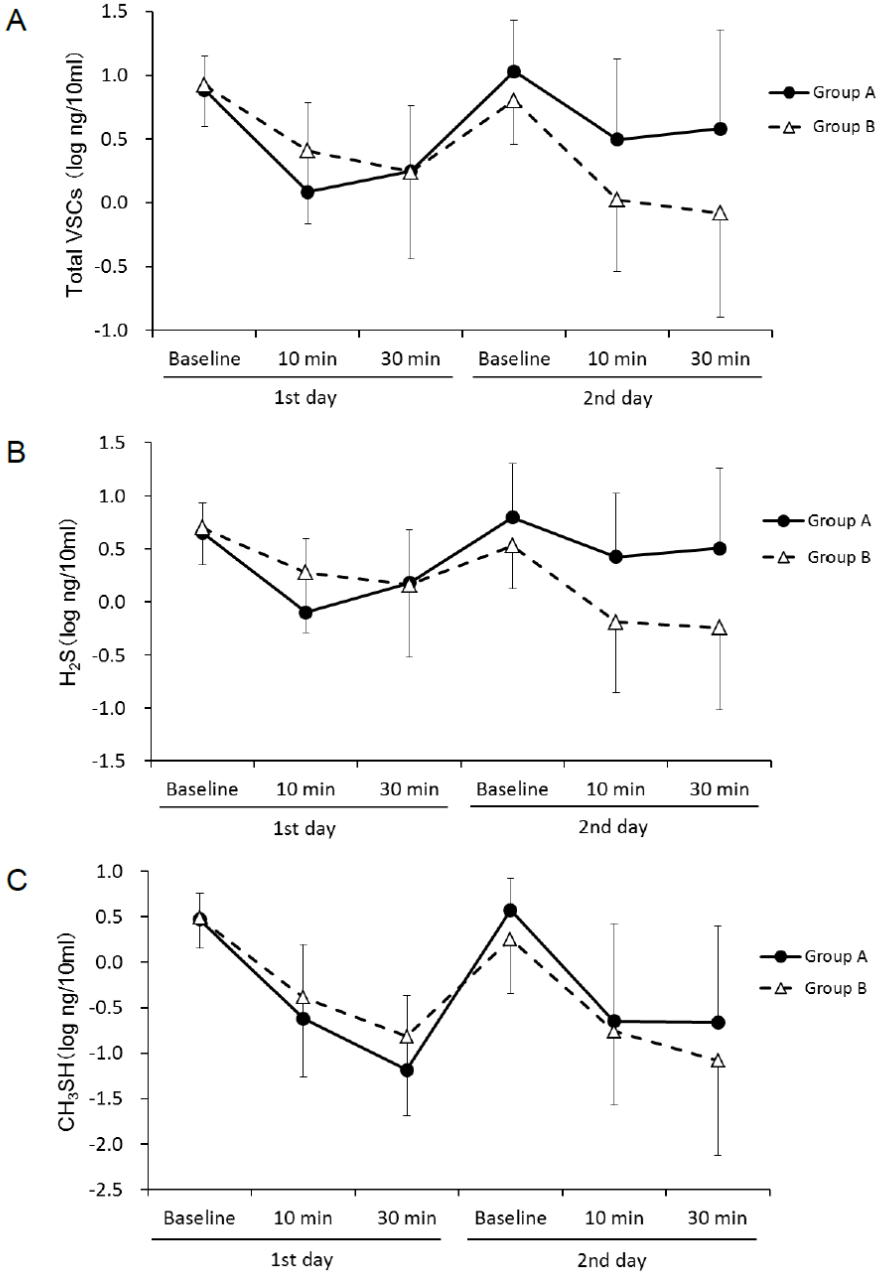


Figure 4-3. Subgroup analyses for primary and secondary outcomes

[1] Difference between the test and placebo groups in the extent of reductions in the concentration of total VSCs from the baseline to 10 min after the ingestion of tablets.



Supplemental figure 4-S1. Effects of test tablets on the concentration of VSCs in mouth air. (A) Total VSCs, (B) H_2S, and (C) CH_3SH were measured at the baseline and 10 and 30 min after the ingestion of tablets. Data represent the average (not estimated) ± SD. The olfactory thresholds of H_2S (1.5 ng/10 ml ≈ 0.18 log ng/10 ml) and CH_3SH (0.5 ng/10 ml ≈ –0.30 log ng/10 ml) are shown in broken lines. **: Significant differences between the groups ($P < 0.01$).



Supplemental figure 4-S2. Effects of test tablets on the concentration of VSCs categorized by the allocation group.

Subjects in group A (closed circle) ingested the test tablet on the first test day and the placebo tablet on the second test day, while subjects in group B (open triangle) ingested the placebo tablet on the first test day and the test tablet on the second test day.

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Chapter 5 Effects of lactoferrin and lactoperoxidase-containing food on the oral microbiota in the elderly

5.1 Abstract

The oral microbiota influences health and disease states. Some Gram-negative anaerobic bacteria play an important role in tissue destruction associated with periodontal disease. Lactoferrin (LF) and lactoperoxidase (LPO) are antimicrobial proteins found in saliva; however, their influence on the whole oral microbiota currently remains unknown. I herein assessed the effects of the long-term ingestion of LF- and LPO-containing tablets on the microbiota of supragingival plaque and tongue coating in a randomized, double-blinded, placebo-controlled study. Forty-six elderly subjects ingested placebo or test tablets after every meal for 8 weeks. The relative abundance of bacterial species was assessed by 16S rRNA gene high-throughput sequencing. Most of the bacterial species in supragingival plaque and tongue coating that exhibited significant decreases in the test group were Gram-negative bacteria including periodontal pathogens. The decrease in the total relative abundance of Gram-negative group in supragingival plaque and tongue coating correlated with improvements in the parameters of the oral health assessment such as oral malodor and plaque accumulation. Furthermore, the microbiota diversity of supragingival plaque at 8 weeks was significantly lower in the test group than in the placebo group, and low microbiota diversity correlated with improvements in the parameters of the oral health assessment. These results suggest that LF- and LPO-containing tablets promote a shift from a highly diverse and Gram-negative-dominated to a Gram-positive-dominated community in the microbiota of supragingival plaque and tongue coating. This microbial shift may contribute to improvements in oral health including oral malodor and gingival conditions.

5.2 Introduction

The human oral cavity is colonized by approximately 500 species of bacteria (1, 2). Indigenous microorganisms are necessary for the maintenance of oral health. However, periodontal pathogens, mainly Gram-negative bacteria, play an important role in tissue destruction and the production of oral malodor (3, 4). A decrease in the amount of saliva and immune responses in the elderly leads to colonization by anaerobic Gram-negative pathogens (3, 5). Oral health care management by medical professionals has been shown to effectively reduce the risk of diseases (3). Although the use of antimicrobial agents is also regarded as an effective strategy for the prevention of aspiration pneumonia (3), the repeated usage of antibiotics is not recommended because of potential issues associated with the creation of resistant microorganisms and microbial substitution, as described in the position paper by the Research, Science and Therapy Committee of the American Academy of Periodontology (6). Therefore, the development of safe and daily methods for oral care is needed in order to maintain health conditions and microbial homeostasis, particularly in the elderly.

Lactoferrin (LF) and lactoperoxidase (LPO) are glycoproteins found in saliva, milk, and other exocrine secretions (7–9). LF has been shown to exert *in vitro* antimicrobial effects against oral pathogens such as *Porphyromonas gingivalis*, and antibiofilm effects against *P. gingivalis* and *Prevotella intermedia* (7). LPO catalyzes the hydrogen peroxide-dependent oxidation of thiocyanate (SCN^-) to hypothiocyanite ($OSCN^-$), which exerts bactericidal effects on periodontal pathogenic bacteria (10) and inhibitory effects on bacterial lyases related to the production of oral malodor (11). Based on these properties, LF and LPO have been used in oral health care products and foods (12, 13). Morita *et al*. showed that the long-term ingestion of LF- and LPO-containing tablets significantly reduced the number of *P. gingivalis* and *Fusobacterium nucleatum* in supragingival plaque and tongue coating in the elderly (14). These findings suggest that LF and LPO have the potential to suppress several oral pathogens. The targeted DNA

sequencing of bacterial 16S rRNA gene regions recently revealed relationships between oral health-related conditions and the oral microbiota (15, 16). However, the effects of LF and LPO on the whole oral microbiota have not yet been examined in detail. In the present study, I assessed the effects of LF and LPO on the microbiota of supragingival plaque and tongue coating collected in a previous clinical trial conducted by Morita *et al.* (14) using 16S rRNA gene high-throughput sequencing.

5.3 Material and methods

5.3.1 Study design

This was a single-center, randomized, double-blinded, placebo-controlled, parallel group comparative study performed in Japan. This study was conducted in accordance with the Helsinki Declaration of 1975 and as revised in 2013. The study design was reviewed and approved by the research Ethics Committee of the School of Dentistry, Showa University (no. 2014-016). This study was enrolled in the UMIN clinical trial registration system (ID: UMIN000015706).

Forty-seven subjects were recruited and screened after providing informed consent. Inclusion criteria were adults aged 65 and older with tongue coating. Exclusion criteria were as follows: 1) eating pureed and finely-chopped meals; 2) receiving parenteral nutrition; 3) receiving treatment for dental disease (except adjustment of denture, oral hygiene instructions); 4) history of allergy to milk; 5) received antibiotic treatment in the past 1 month, or expected to receive it in the near future; 6) use of oral care products for prevention of oral malodor or improvement of oral hygiene; 7) regular consumption of LF or LPO-containing food or oral care products; and 8) presence of exacerbated diseases of the liver, kidney, heart, lung, gastro-intestine, blood, endocrine system, and metabolic system.

The test tablet contained 80 mg of LF+LPO powder (Orabarrier®, Morinaga Milk Industry Co., Ltd., Tokyo, Japan) including 20 mg of LF, 2.6 mg of LPO, and 2.6 mg of glucose oxidase as active ingredients.

LF and LPO were purified from bovine milk. Glucose oxidase was obtained from *Penicillium chrysogenum*. The LF+LPO powder also contained glucose and pH-adjusting agents that support the effects of the active ingredients. The placebo tablet contained dextrin and coloring materials instead of LF+LPO powder. These placebo and test tablets were identical in weight, texture, and appearance. The tablets were the readily soluble perforated type in order to prevent asphyxia and aspiration. Subjects were randomly assigned to receive either placebo or test tablets, and were asked to suck on the tablet after every meal for 8 weeks. The subjects were instructed not to change their oral hygiene regimens throughout the study period.

5.3.2 Oral assessments

Oral assessments were conducted 2 hours after lunch at baseline, 4, and 8 weeks. Three trained and calibrated dentists performed the assessment including O'Leary's plaque control record (O'Leary's PCR) (17), probing pocket depth (PPD), bleeding on probing (BOP) (18), and total volatile sulfur compounds (VSCs) in oral air (19). O'Leary's PCR, PPD, and BOP were measured entirely in each tooth. PPD measurements were recorded as the deepest pocket depth to the nearest millimeter. The concentrations of VSCs in oral air were analyzed with a portable gas chromatograph (OralChroma®, FIS Inc., Japan) according to the manufacturer's instructions (19). The concentration of total VSCs was obtained as the sum of hydrogen sulfide, methylmercaptan, and dimethyl sulfide concentrations.

5.3.3 Oral sample collection and DNA extraction

Tongue coating and supragingival plaque were collected using sterile swabs (Puritan Medical Products Company LLC, US) with constant pressure. Tongue coating was collected from a 2-cm^2 area in mid-dorsum of tongue, and supragingival plaque was taken from the cervical region on the buccal surface

of maxillary molars. The swab was suspended in 1 ml of sterile saline stored in vials (20). The same sampling site in each subject was used at all sampling period. Bacterial DNA was extracted from the samples using a commercial kit (QIAamp, Qiagen, Hilden, Germany) according to the manufacturer's protocol. The remaining sample was inoculated into Sabouraud agar (BD, US) for the detection of *Candida* sp. Following incubation for 48 h in a 5% CO_2 atmosphere, *Candida* sp. was identified using a commercial kit (Candida check, LSI Medience Corporation, Japan).

5.3.4 PCR amplification and DNA sequencing of bacterial 16S rRNA

A 16S rRNA gene sequencing analysis was performed as described previously with minor modifications (21). The V1-V2 region of bacterial 16S rRNA genes was amplified using PCR with the TaKaRa Ex Taq HS kit (TaKaRa Bio, Shiga, Japan) and the primer set of Tru27F (5′-CGCTCTTCCGATCTCTGAGRGTTTGATYMTGGCTCAG-3′) and Tru354R (5′-CGCTCTTCCGATCTGACCTGCCTCCCGTAGGAGT-3′). DNA was amplified according to the following program: 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s; 72 °C for 5 min. After verifying amplified DNA using the QIAxcel system (Qiagen, Valencia, CA, USA), triplicate samples were combined. A 1-μl sample of combined PCR products was amplified using the following barcoded primers adapted for the Illumina MiSeq: Fwd 5′-AATGATACGGCGACCACCGAGATCTACAC XXXXXXXX ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG-3′, Rev 5′-CAAGCAGAAGACGGCATACGAGAT XXXXXXXX GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAC-3′, where X represents a barcode base. DNA was amplified according to the program described above, except that only 8 cycles were conducted. After validating the second amplified DNA product with the QIAxcel system, PCR products were purified

using the QIAquick 96 PCR Purification Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Purified products were quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, Carlsbad, CA, USA). Equal amounts of amplicons from multiple samples were pooled and primer dimers were removed via the GeneRead Size Selection Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Pooled libraries were sequenced using an Illumina MiSeq instrument and the MiSeq v3 Reagent Kit (Illumina Inc, San Diego, CA, USA).

After the acquisition of Illumina paired-end reads, the bowtie-2 program (ver. 2-2.2.4) (22) was used to remove the reads mapped to PhiX 174 sequence and Genome Reference Consortium human build 37 (GRCh37). Thereafter, the 3′ region of each read with a PHRED quality score of less than 17 was trimmed. Trimmed reads less than 150 bp in length with an average quality score of less than 25 or those lacking paired reads were also removed. Trimmed paired-end reads were combined using the fastq-join script in EA-Utils (ver. 1.1.2–537) (23). Potential chimeric sequences were removed by reference-based chimera checking in USEARCH (ver. 5.2.32) (24) and the gold database (http://drive5.com/otupipe/gold.tz).

Non-chimeric sequences were analyzed in the QIIME software package version 1.8.0 (25, 26). Sequences were assigned to operational taxonomic units (OTUs) using USEARCH with a parameter (Minimum cluster size: 2). A representative sequence was selected from each OTU, and the representative sequence set was then classified taxonomically using the BLAST program (ver. 2.2.22) with a 98.6% pairwise identity threshold and DDBJ 16S ribosomal RNA sequence data of prokaryotes (published October 2015).

5.3.5 Diversity analysis

α-Diversity indices (Shannon index, Chao1, number of observed species (the number of OTUs) and phylogenetic distance (PD) whole tree) and distances between samples (UniFrac distance) were estimated

using QIIME version 1.8.0 software.

5.3.6 Statistical analysis

All statistical analyses were conducted using SPSS version 23.0 statistical software (IBM, Armonk, NY, USA). Inter-group differences in relative abundance were analyzed using the Wilcoxon rank-sum test. Differences in the relative abundance of each bacterial taxon between the baseline and each time point in each group were analyzed using the Wilcoxon signed-rank test. Differences between the clusters in PCoA were analyzed using chi-squared test. Intra- and inter-group differences in α-diversity were analyzed by the Student's *t*-test. The relationship between bacterial abundance and oral assessment parameters was analyzed using Spearman's correlation. Two-sided $P < 0.05$ was considered significant for all tests.

5.4 Results

5.4.1 Subjects

The flow of subjects throughout the study is shown in Figure 5-1. Forty-seven subjects were assessed for eligibility to participate in the study, and one subject was considered ineligible according to the exclusion criteria. Forty-six subjects were randomized to the placebo group (n = 22) and test group (n = 24). One of the subjects in the test group did not complete the study. Five subjects in the placebo group and three subjects in the test group failed to comply with the suggested intake rate (less than 75%). Therefore, 37 subjects were included in the efficacy analysis based on a full analysis set. The demographic and baseline characteristics of the full analysis set are listed in Table 5-1. Since 3 subjects in the placebo group and 4 subjects in the test group used antibiotics during the study period, their data after taking antibiotics were regarded as missing values. Supragingival plaque was collected from dentate subjects only. Four subjects in the placebo group and three subjects in the test group were edentulous. The number

of sample of supragingival plaque and tongue coating are summarized in Supplemental table 5-S1.

5.4.2 Microbiota composition at the phylum level

The 16S rRNA gene amplicon analysis identified a total of 1,012,766 and 1,302,024 high-quality paired sequences obtained from 84 samples of supragingival plaque and 103 samples of tongue coating, with 12,056.7 ± 3,400.0 and 12,641.0 ± 3,322.6 (average ± standard deviation) reads per sample, which were assigned 985 and 1,034 OTUs, respectively.

The phylum compositions of the microbiota in supragingival plaque and tongue coating are shown in Figure 5-2. The compositions of the microbiota in supragingival plaque and tongue coating included five predominant phyla: *Firmicutes*, *Bacteroidetes*, *Fusobacteria*, *Proteobacteria*, and *Actinobacteria*. Although supragingival plaque and tongue coating had similar compositions at the phylum level, significant differences were observed between supragingival plaque and tongue coating in the UniFrac principal coordinate analysis (PCoA, Supplemental figure 5-S2). These results suggested that the compositions of the microbiota differed between supragingival plaque and tongue coating. The composition of the microbiota at the phylum level did not markedly change after 4 and 8 weeks of ingesting the placebo and test tablets.

5.4.3 Changes in the relative abundance of bacterial species

Although there was no significant change at the phylum level, the relative abundance of several bacterial species significantly changed between the baseline and 8 weeks. Bacterial species with significant intra-group changes are summarized in Tables 5-2 (supragingival plaque) and 5-3 (tongue coating). In supragingival plaque, the test group showed 10 and 11 bacterial species with significant decreases and increases in relative abundance, respectively. In contrast, the placebo group showed only 1 and 2 bacterial

species with significant decreases and increases in the relative abundance. In tongue coating, the test group showed 14 and 15 bacterial species with significant decreases and increases, respectively. The placebo group showed 4 and 13 bacterial species with significant increases and decreases, respectively. In supragingival plaque and tongue coating, most of the bacterial species with significant decreases in the test group were Gram-negative bacteria. On the other hand, most of the bacterial species with significant increases in the test group were Gram-positive bacteria. Each relative abundance is shown in Supplemental table 5-S3. Bacterial species with significant inter-group differences are shown in Supplemental table 5-S4.

I divided bacterial taxa into Gram-positive group including *Firmicutes* (excluding the family *Veillonellaceae*) and *Actinobacteria* and Gram-negative group including *Firmicutes* (including the family *Veillonellaceae*), *Bacteroidetes*, *Fusobacteria*, *Proteobacteria* and *Spirochaetes*. The total relative abundance of Gram-positive group in tongue coating was significantly greater at 4 and 8 weeks than at baseline in the test group (Figure 5-3c). In contrast, the total relative abundance of Gram-negative group in the test group showed significant decreases at 4 and 8 weeks (Figure 5-3d). Significant changes were not observed in supragingival plaque during the test period, with the median of the total relative abundance of Gram-positive and -negative group in the test group being higher and lower at 8 weeks that at baseline, respectively (Figure 5-3a, 5-3b).

The relative abundance of caries-associated bacteria (*Streptococcus mutans* and *S. sobrinus*) did not significantly increase in supragingival plaque and tongue coating after the ingestion of each tablet (Supplemental table 5-S5). Most opportunistic bacteria (*Haemophilus parainfluenzae*, *Moraxella catarrhalis*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Staphylococcus aureus*, and *S. pneumoniae*) did not significantly increase (Supplemental table 5-S5). The relative abundance of *Klebsiella pneumonia*, which is an opportunistic pathogen, in tongue coating was significantly lower at 8 weeks that at baseline

in the test group. The detection rate of *Candida* sp. determined by culturing was low at the baseline in the both group. The rate did not change and remained low level during the test period.

5.4.4 Diversity of the microbiota

Changes in one of the α-diversity indices, the Shannon index, in the microbiota of supragingival plaque and tongue coating are shown in Figure 5-4. The Shannon index is an α-diversity measure accounting for both the abundance and evenness of the species present. In the microbiota of supragingival plaque, the Shannon index was significantly lower in the test group than in the placebo group at 4 and 8 weeks (Figure 5-4A). In the microbiota of tongue coating, no significant differences were observed between the groups (Figure 5-4B). There was no significant intra-group difference between the groups at either site. The other α-diversity indices including chao1, the number of observed species, and PD whole tree showed similar results to those obtained for the Shannon index (Supplemental figure 5-S6).

5.4.5 Oral microbiota and health conditions

I conducted a correlation analysis between bacterial abundance and oral assessment parameters in the full analysis set (Table 5-4). In supragingival plaque, the total relative abundance of Gram-positive group negatively correlated with the parameters of the oral assessment (O'Leary's PCR, BOP, and total VSCs). In contrast, the total relative abundance of Gram-negative group positively correlated with O'Leary's PCR, BOP and total VSCs. The Shannon index positively correlated with O'Leary's PCR, and PPD. The results were consistent when I used other α-diversity indices (Supplemental table 5-S7). In tongue coating, the total relative abundance of Gram-positive group correlated with PPD and total VSCs. Furthermore, the relative abundance of several bacterial species with significant changes in supragingival plaque and tongue coating also correlated with the parameters of the oral assessment (Supplemental table

5-S8). For example, the relative abundances of 4 periodontal species (*Prevotella oris*, *Capnocytophaga gingivalis*, *Treponema sp. oral taxon 231*, *Prevotella buccae*) with significant decreases in supragingival plaque positively correlated with some parameters of the oral assessment including O'Leary's PCR, PPD, and BOP. These bacteria were identified as periodontal pathogens. On the other hand, the relative abundances of 8 species (*human intestinal firmicute CS13*, *Bifidobacterium pseudocatenulatum*, *[Ruminococcus] gnavus*, *bacterium IARFR575*, *[Clostridium] leptum*, *Roseburia inulinivorans*, *bacterium ic1379*) with significant increases in supragingival plaque negatively correlated with some parameters of the oral assessment. The some α-diversity score negatively correlated with the total relative abundance of Gram-positive group and positively correlated with that of Gram-negative group in supragingival plaque and tongue coating (Supplemental table 5-S9).

5.5 Discussion

LF and LPO are antimicrobial proteins in saliva. Our previous clinical trial showed that the long-term ingestion of LF- and LPO-containing tablets significantly reduced the total number of bacteria as well as *P. gingivalis* in supragingival plaque and tongue coating (14). However, limited information is available on the effects on other oral bacteria including pathogens, indigenous bacteria, and opportunistic bacteria. Therefore, I conducted a comprehensive analysis on the oral microbiota of samples taken from this clinical trial (14) using a 16S rRNA gene sequencing approach.

I compared the microbial compositions of supragingival plaque and tongue coating. UniFrac PCoA showed that supragingival plaque and tongue coating possessed different microbial communities. This result is consistent with previous findings demonstrating that supragingival plaque communities differed more from other oral sites including tongue coating than microbial communities among seven oral sites (27). Bacteria in supragingival plaque have been implicated in periodontal disease such as gingival

inflammation. Bacteria in tongue coating have been identified as a major source of oral malodor (28). Therefore, the compositions of the microbiota of supragingival plaque and tongue coating need to be examined in more detail in order to evaluate its effects on oral health.

In supragingival plaque and tongue coating, most bacterial species with significant decreases and increases in the test group were Gram-negative and -positive bacteria, respectively. Similarly, the total relative abundance of Gram-negative and -positive group in tongue coating significantly decreased and increased in the test group, respectively. These results suggest that the test tablet promotes a microbial shift in oral microbiota of supragingival plaque and tongue coating from a Gram-negative-dominated community to a Gram-positive-dominated community. Previous *in vitro* studies reported that the LPO system exerts bactericidal effects against Gram-negative bacteria and bacteriostatic effects against Gram-positive bacteria (29). The authors suggested that the cell wall of Gram-positive organisms is a more effective barrier against hypothiocyanite produced by the LPO system than that of Gram-negative organisms. Moreover, Streptococci, which are Gram-positive bacteria that predominate in the oral microbiota, are capable of neutralizing hypothiocyanite. On the other hand, LF exerts antimicrobial effects against a wide range of pathogens (9); however, the selectivity of LF against Gram-positive and -negative bacteria has not yet been investigated. Therefore, I consider the microbial shift induced by the test tablet to have occurred due to the selectivity of the LPO system rather than that of LF.

There is insufficient agreement on the criteria for a healthy oral microbiota; however, recent studies revealed a relationship between the oral microbiota and health conditions (1, 2, 4, 15, 16). For example, Takeshita *et al*. performed a large-scale population-based study on the salivary microbiome (16). An important finding in that study was that high bacterial richness in a salivary microbiome correlated with poor oral health such as periodontitis, poor oral hygiene, and the presence of decayed teeth. Liu *et al*. reported that the oral microbiota in healthy samples was dominated by Gram-positive bacteria, whereas

that in periodontal disease was dominated by Gram-negative bacteria, including many pathogenic species (15).

In the present study, the decrease observed in the total relative abundance of the Gram-negative group in supragingival plaque correlated with reductions in the parameters of the oral assessment including O'Leary's PCR, BOP, and total VSCs. In addition, BOP in the test group at 8 weeks had significantly reduced from baseline (14). These results suggest that the microbial shift induced by the test tablet from a Gram-negative-dominant to a Gram-positive-dominant community contributes to improvements in oral health conditions.

Microbiota diversity indices of supragingival plaque positively correlated with the parameters of the oral assessment such as O'Leary's PCR and PPD. These results are consistent with the previous findings of a large-scale clinical study in which the lower α-diversity of the salivary microbiota was associated with better conditions for oral health such as a lower plaque index, less gingival bleeding, and shallower periodontal pockets (16). In the present study, the microbiota diversity indices of supragingival plaque were significantly lower in the test group than in the placebo group at 8 weeks. These results suggest that the long-term ingestion of the test tablet suppressed increases in the microbial diversity of supragingival plaque related to the deterioration of oral conditions.

The repeated usage of antibiotics is not recommended because of potential issues associated with microbial substitution (6). I confirmed that the test tablet did not induce increases in the relative abundance of opportunistic bacteria and the detection rate of *Candida* sp. The results obtained from comparisons of the phylum composition suggest that the overall structure of the microbiota in supragingival plaque and tongue coating was not markedly affected by the ingestion of the test tablet. No adverse events related to the tablets were observed in any of the 46 subjects examined during this study (14). Therefore, the long-term use of the test tablet does not appear to result in microbial substitution. I consider it possible to take

the test tablet continuously and safely on a daily basis because LF and LPO are antimicrobial components present in saliva.

Although previous studies evaluated the oral microbiota after the use of food or probiotics as described below, none demonstrated beneficial effects on the whole oral microbiota. The use of xylitol gum decreased the abundance of S. *mutans*, but did not affect the salivary microbial composition (30). The short-term consumption of lozenges containing *Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* subsp. *Lactis* BB-12 decreased the amount of plaque without affecting the microbiota (31). A 12-week supplementation with *Lactobacillus reuteri* DSM 17938 and PTA 5239 induced a shift in the microbiota composition of tooth biofilm; however, the biological relevance of this microbial shift has not yet been elucidated (32). To the best of our knowledge, the present study is the first to demonstrate improvements in the microbiota of supragingival plaque and tongue coating through the long-term ingestion of food using 16S rRNA gene high-throughput sequencing.

In the present clinical study, I collected tongue coating and supragingival plaque, not subgingival plaque and saliva. This was a limitation of our study. A previous study suggested that the long-term ingestion of test tablets (LF 300 mg/day, LPO 5.4 mg/day) reduced the number of *Fusobacterium* in subgingival plaque (33). Therefore, I consider LF and the LPO-containing tablets to exert some effects on the microbiota of subgingival plaque. Further clinical studies are needed in order to demonstrate these effects.

In conclusion, the results of the present clinical study suggest that the long-term ingestion of LF- and LPO-containing tablets promotes a shift from a highly diverse and Gram-negative-dominated to a Gram-positive-dominated community in the microbiota of supragingival plaque and tongue coating. This microbial shift may contribute to improvements in oral health including oral malodor and gingival conditions.

5.6 Tables and figures in Chapter 5

Table 5-1. Demographic and baseline characteristics of the full analysis set

Parameters	Placebo group (n = 17)	Test group (n = 20)
Age (mean ± SD)	85.9 ± 6.7	80.4 ± 6.4
Male/Female	4/13	8/12
Edentulous/Dentate	4/13	3/17
Remaining teeth (mean ± SD)	10.8 ± 8.8	13.5 ± 10.5
Parameters of the oral assessment (mean ± SD)		
O'Leary's PCR[a] (%)	72.9 ± 27.7	60.0 ± 31.4
PPD[b] (mm)	3.3 ± 0.8	3.5 ± 0.7
BOP[c] (% of teeth)	52.1 ± 35.0	34.5 ± 32.0
Total VSCs[d] in oral air (ng/10 ml)	1.6 ± 2.7	2.5 ± 2.9

[a] O'Leary's Plaque Control Record (indicator of plaque accumulation)

[b] Probing pocket depth (indicator of the severity of periodontal disease)

[c] Bleeding on probing (indicator of gingival inflammation)

[d] Volatile sulfur compounds (main components of oral malodor)

Table 5-2. Bacterial species with significant changes in relative abundance between baseline and 8 weeks in supragingival plaque

Group		Bacterial species	
		Significant decrease	Significant increase
Placebo	Gram-positive	*Streptococcus salivarius*	
	Gram-negative		*Terrahaemophilus sp. oral taxon G25* *Haemophilus sp. T13*
	Unknown		
Test	Gram-positive	*[Clostridium] aminophilum*	*human intestinal firmicute CS13* *Streptococcus sp. oral taxon 071* *Bifidobacterium pseudocatenulatum* *Bifidobacterium longum* *[Ruminococcus] gnavus* *Roseburia inulinivorans* *[Clostridium] leptum* *Streptococcus sp. oral taxon 057* *Candidatus Dorea massiliensis AP6*
	Gram-negative	*Campylobacter gracilis* *Prevotella oris* *Capnocytophaga gingivalis* *Treponema sp. oral taxon 231* *Leptotrichia sp. oral taxon 212* *Enterobacter sp. AR451* *Prevotella buccae* *Neisseria cinerea* *Prevotellaceae bacterium DJF_LS10*	
	Unknown		*bacterium IARFR575* *bacterium ic1379*

Each relative abundance is shown in Supplemental table 5-S3.

Bacterial species with significant inter-group differences are shown in Supplemental table 5-S4.

Table 5-3. Bacterial species with significant intra-group differences between baseline and 8 weeks in tongue coating

Group		Bacterial species	
		Significant decrease	Significant increase
Placebo	Gram-positive	*Lachnoanaerobaculum orale*	*Gemella sanguinis* *Actinomyces sp. 'ARUP UnID 85'* *Actinomycetaceae bacterium 'ARUP UnID 87'* *Clostridiales bacterium oral taxon 085* *Streptococcaceae bacterium 'ARUP UnID 627'* *Streptococcus sp. oral taxon C14* *Actinomyces sp. oral taxon 172*
	Gram-negative	*Leptotrichia wadei* *Leptotrichia sp. oral taxon 223* *Stenotrophomonas maltophilia*	*Veillonella parvula* *Leptotrichia sp. oral taxon 213* *Neisseria meningitides* *Bacteroides plebeius*
	Unknown		*No blast hit* *Bacterium endosymbiont of Otiorhynchus armadillo*
Test	Gram-positive	*Lachnospiraceae bacterium oral taxon 096* *Lachnoanaerobaculum sp. S6-P3*	*Streptococcus mitis* *Gemella haemolysans* *Megasphaera micronuciformis* *Actinomycetaceae bacterium 'ARUP UnID 87'* *Streptococcus tigurinus* *Bifidobacterium longum* *Faecalibacterium prausnitzii* *Ruminococcus sp. CB3* *Lactobacillus gasseri* *Lactobacillus salivarius* *Gemella sp. oral taxon G07* *Eubacterium rectale*

Gram-negative	*Neisseria flavescens*	*Veillonella parvula*
	Leptotrichia sp. oral taxon 221	*Neisseria sp. ChDC B321*
	Klebsiella pneumoniae	*Bacteroides sp. S327*
	Neisseria perflava	
	Enterobacter sp. AR451	
	Neisseria sp. 260	
	Campylobacter showae	
	Stenotrophomonas maltophilia	
	Haemophilus sp. T13	
	Treponema maltophilum	
	Halospirulina sp. EA11(2012)	
Unknown	*bacterium MS4*	

Each relative abundance is shown in Supplemental table 5-S3.

Bacterial species with significant inter-group differences are shown in Supplemental table 5-S4.

Table 5-4. Relationships between bacterial abundance and oral assessment parameters in full analysis set

Bacterial abundance		Oral assessment			
		O'Leary's PCR (%)	PPD (mm)	BOP (%)	Total VSCs (ng/10ml)
Supragingival plaque					
Relative abundance					
Gram-positive group	r	**−0.474**	−0.189	**−0.388**	**−0.293**
	P	< 0.001	0.084	< 0.001	0.007
Gram-negative group	r	**0.513**	0.185	**0.375**	**0.320**
	P	< 0.001	0.092	<0.001	0.003
α-diversity score					
Shannon index	r	**0.373**	**0.298**	0.109	0.156
	P	< 0.001	0.006	0.325	0.114
Tongue coating					
Relative abundance					
Gram-positive group	r	−0.184	**−0.268**	−0.076	**−0.344**
	P	0.097	0.014	0.497	<0.001
Gram-negative group	r	0.107	0.133	0.022	**0.275**
	P	0.337	0.230	0.844	0.007
α-diversity score					
Shannon index	r	0.139	0.162	−0.051	**0.205**
	P	0.209	0.144	0.645	0.038

Bold numbers indicate significant coefficient values.

The relationships between other α-diversity scores and the parameters of the oral assessment are shown in Supplemental table 5-S7.

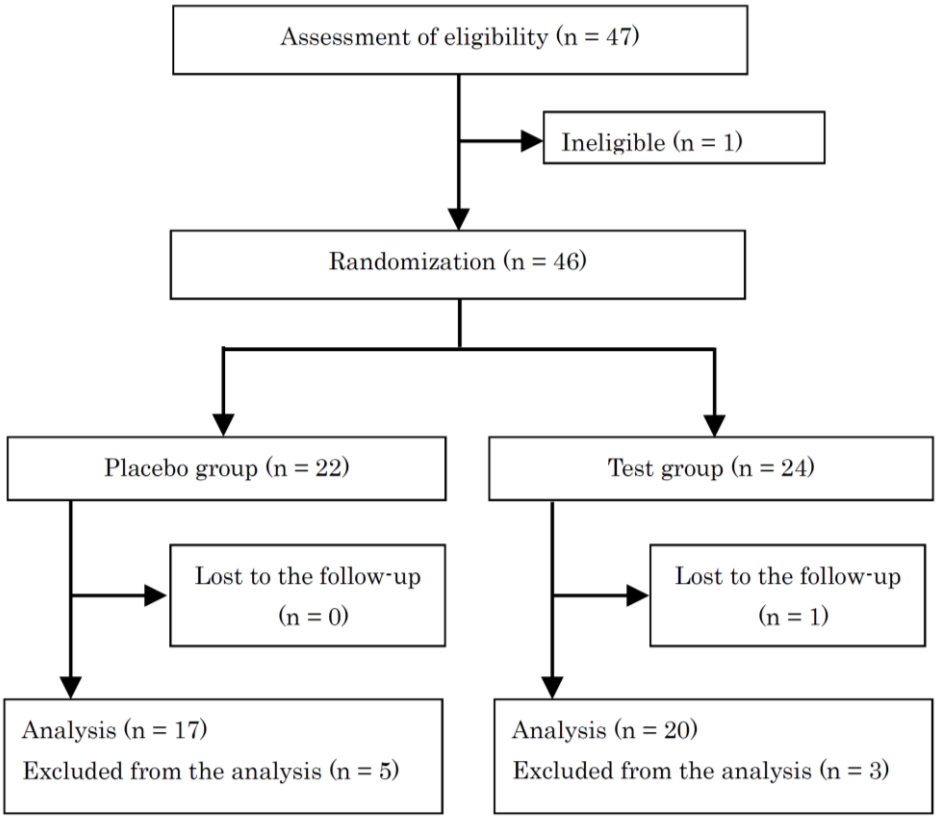


Figure 5-1. Flow diagram of subjects throughout the study

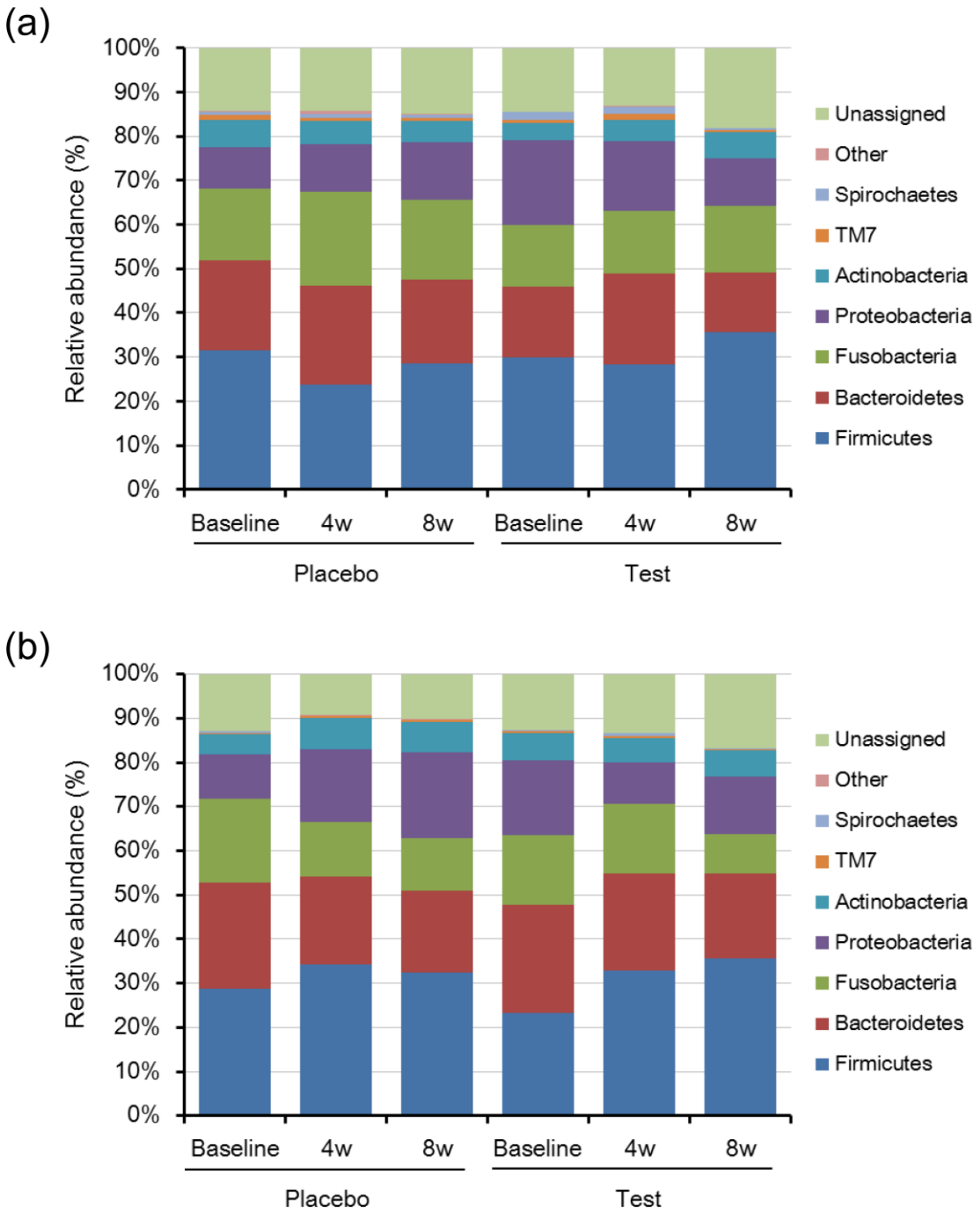


Figure 5-2. Microbial compositions of supragingival plaque (a) and tongue coating (b) at the phylum level

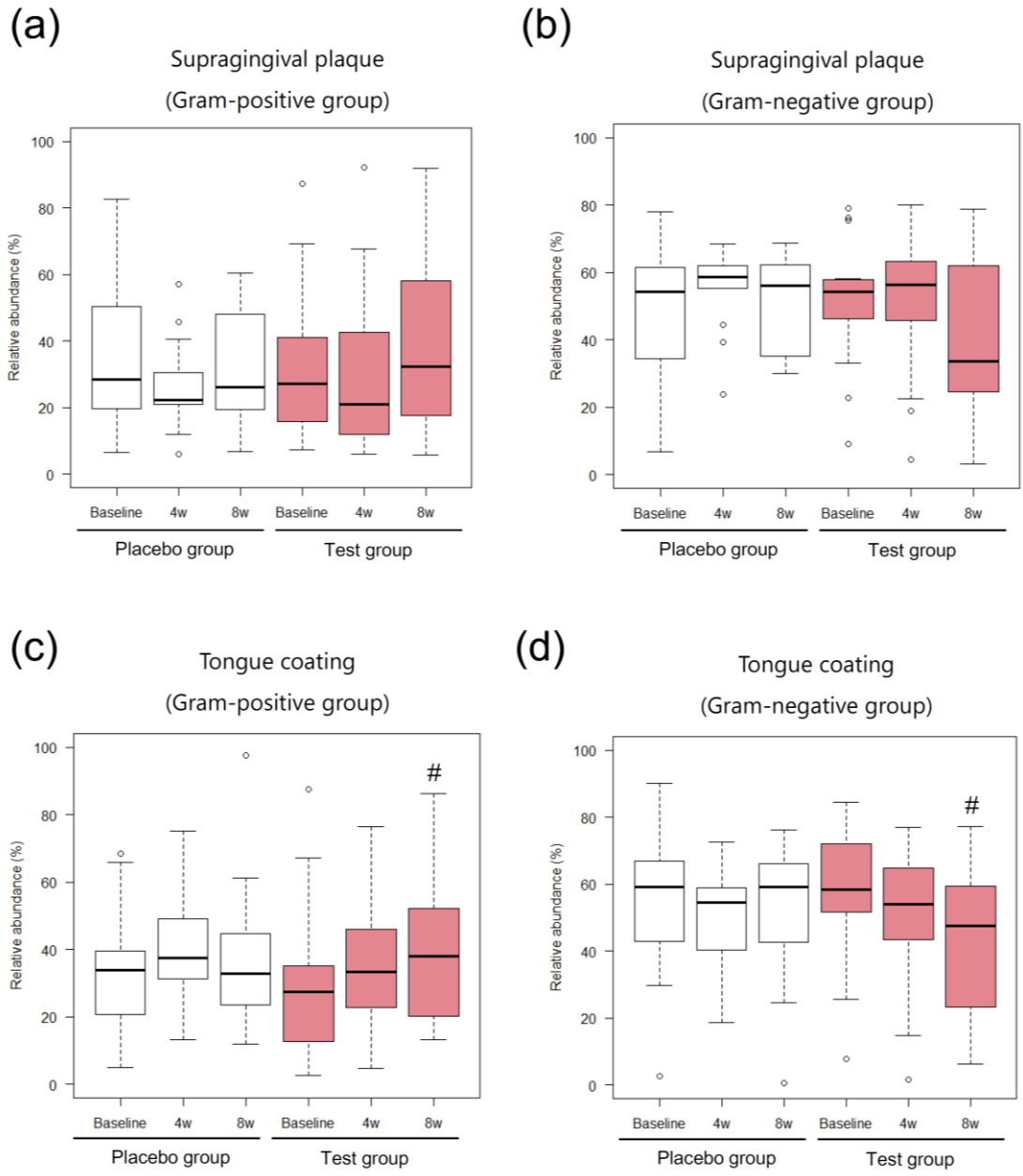
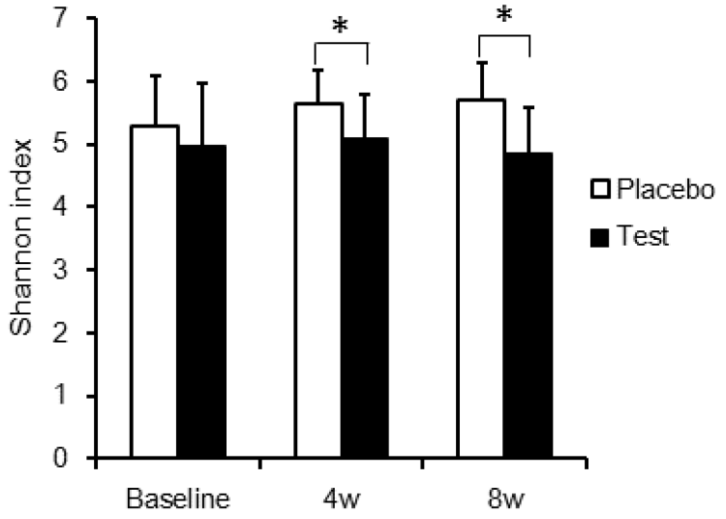


Figure 5-3. Changes in the total relative abundance of Gram-positive and -negative group in supragingival plaque and tongue coating

Gram-positive group contains Firmicutes and Actinobacteria. Gram-negative group contains Bacteroidetes, Fusobacteria, and Proteobacteria. Data are expressed as the sample minimum (lower edge of the whisker), lower quartile (lower edge of the box), median (traverse line in the box), upper quartile (upper edge of the box), sample maximum (lower edge of the whisker), and outlier (circles) in box-and-whisker plots. #: $P < 0.05$ significantly different from the baseline.

(a)



(b)

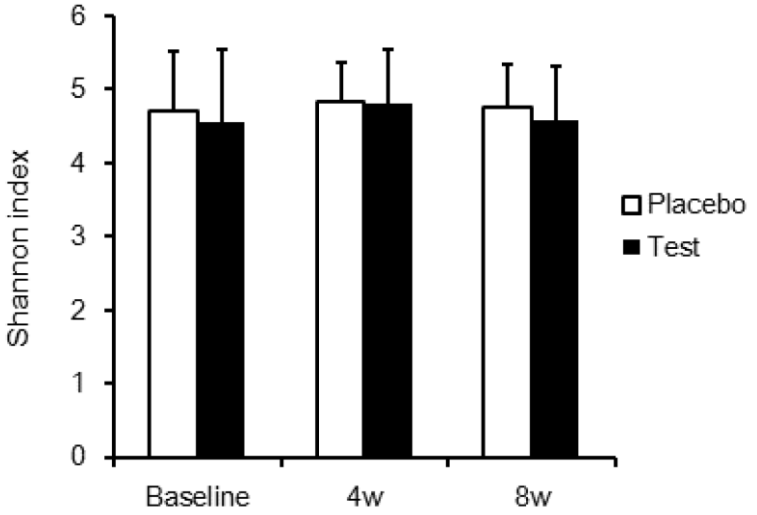


Figure 5-4. Changes in the alpha diversity index (Shannon index) of supragingival plaque (a) and tongue coating (b)

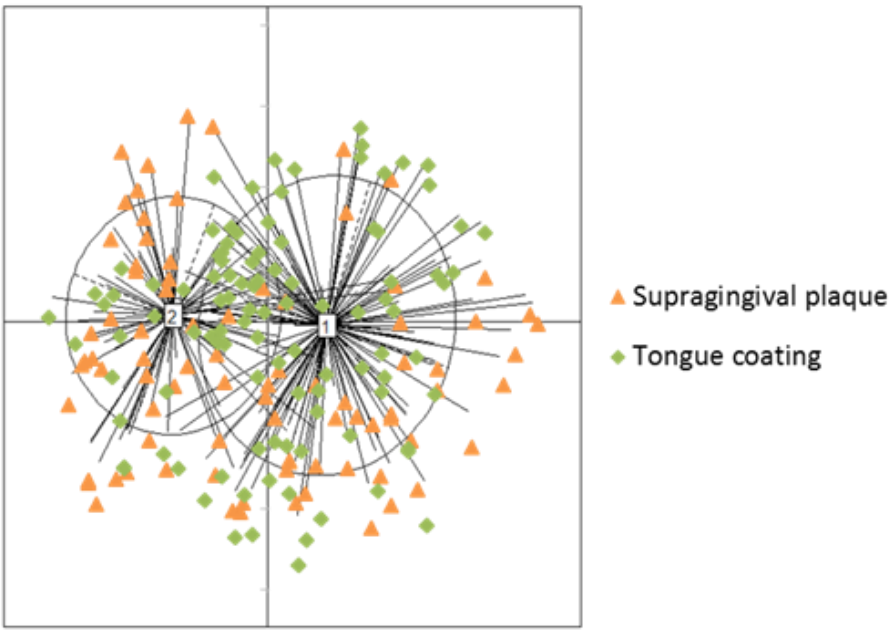
Data are expressed as the mean ± SD. *: $P < 0.05$ significant difference between the placebo and test groups.

5.7 Supplemental table and figure in chapter 5

Supplemental table 5-S1. Number of sample of supragingival plaque and tongue coating

Bacterial abundance	Number of sample			
	Baseline	4w	8w	Total
Supragingival plaque				
Placebo group	13	13	10*	**36**
Test group	17	17	14*	**48**
Tongue coating				
Placebo group	17	16*	14*	**47**
Test group	20	20	16	**56**

*Three subjects in the placebo group and 4 subjects in the test group used antibiotics during the study period. Their data after taking antibiotics were regarded as missing values.



Supplemental figure 5-S2. Difference in the microbiota between supragingival plaque and tongue coating using a UniFrac principal coordinate analysis (PCoA)

Clusters 1 and 2 included 37 and 47 bacterial species from supragingival plaque and 78 and 25 from tongue coating, respectively ($P < 0.01$, chi-squared test).

Supplemental table 5-S3. Changes in the relative abundance of bacterial species with significant intra-group differences

Species	Relative abundance (%, median)				Gram stain
	Placebo group		Test group		
	Baseline	8w	Baseline	8w	
Supragingival plaque					
Streptococcus salivarius	0.141	**0.082**#	0.264	0.992	P
Terrahaemophilus sp. oral taxon G25	0.016	**0.328**#	0.038	0.348	N
Haemophilus sp. T13	0.000	**0.022**#	0.000	0.000	N
[Clostridium] aminophilum	0.000	0.000	0.000	**0.000**#	P
Campylobacter gracilis	1.507	1.135	1.382	**0.609**#	N
Prevotella oris	0.730	0.553	0.884	**0.135**#	N
Capnocytophaga gingivalis	0.124	0.137	0.151	**0.047**#	N
Treponema sp. oral taxon 231	0.000	0.000	0.079	**0.004**#	N
Leptotrichia sp. oral taxon 212	0.000	0.296	0.011	**0.000**#	N
Enterobacter sp. AR451	0.010	0.000	0.015	**0.000**#	N
Prevotella buccae	0.000	0.003	0.011	**0.000**#	N
Neisseria cinerea	0.000	0.000	0.000	**0.000**#	N
Prevotellaceae bacterium DJF_LS10	0.000	0.000	0.000	**0.000**#	N
human intestinal firmicute CS13	0.024	0.046	0.038	**0.124**#	P
Streptococcus sp. oral taxon 071	0.016	0.023	0.014	**0.143**#	P
Bifidobacterium pseudocatenulatum	0.012	0.027	0.014	**0.059**#	P
Bifidobacterium longum	0.012	0.015	0.010	**0.082**#	P
[Ruminococcus] gnavus	0.012	0.005	0.000	**0.020**#	P
Roseburia inulinivorans	0.000	0.000	0.000	**0.008**#	P
[Clostridium] leptum	0.000	0.000	0.000	**0.011**#	P
Streptococcus sp. oral taxon 057	0.000	0.000	0.000	**0.004**#	P
Candidatus Dorea massiliensis AP6	0.000	0.000	0.000	**0.003**#	P
bacterium IARFR575	0.000	0.000	0.000	**0.020**#	–
bacterium ic1379	0.000	0.000	0.000	**0.007**#	–
Tongue coating					
Lachnoanaerobaculum orale	0.155	**0.044**#	0.095	0.090	P
Leptotrichia wadei	0.120	**0.033**#	0.051	0.035	N
Leptotrichia sp. oral taxon 223	0.009	**0.000**#	0.018	0.000	N
Stenotrophomonas maltophilia	0.000	**0.000**#	0.000	**0.000**#	N
Gemella sanguinis	0.177	**0.968**#	0.794	0.745	P
Actinomyces sp. 'ARUP UnID 85'	0.234	**0.483**#	0.109	0.261	P

Actinomycetaceae bacterium 'ARUP UnID 87'	0.049	**0.098**#	0.027	**0.095**#	P
Clostridiales bacterium oral taxon 085	0.018	**0.052**#	0.084	0.072	P
Streptococcaceae bacterium 'ARUP UnID 627'	0.000	**0.010**#	0.011	0.021	P
Streptococcus sp. oral taxon C14	0.000	**0.019**#	0.000	0.000	P
Actinomyces sp. oral taxon 172	0.000	**0.005**#	0.000	0.000	P
Veillonella parvula	0.052	**0.267**#	0.071	**0.092**#	N
Leptotrichia sp. oral taxon 213	0.021	**0.023**#	0.027	0.024	N
Neisseria meningitidis	0.009	**0.062**#	0.015	0.011	N
Bacteroides plebeius	0.000	**0.007**#	0.000	0.000	N
No blast hit	0.730	**1.145**#	1.141	0.827	
Bacterium endosymbiont of Otiorhynchus armadillo	0.000	**0.011**#	0.000	0.000	–
Lachnospiraceae bacterium oral taxon 096	0.000	0.000	0.005	**0.000**#	P
Lachnoanaerobaculum sp. S6-P3	0.000	0.000	0.000	**0.000**#	P
Neisseria flavescens	0.560	0.984	0.723	**0.082**#	N
Leptotrichia sp. oral taxon 221	0.035	0.174	0.094	**0.035**#	N
Klebsiella pneumoniae	0.030	0.031	0.033	**0.020**#	N
Neisseria perflava	0.188	0.294	0.092	**0.024**#	N
Enterobacter sp. AR451	0.010	0.000	0.027	**0.007**#	N
Neisseria sp. 260	0.021	0.085	0.029	**0.000**#	N
Campylobacter showae	0.007	0.047	0.006	**0.000**#	N
Haemophilus sp. T13	0.000	0.000	0.000	**0.000**#	N
Treponema maltophilum	0.000	0.000	0.000	**0.000**#	N
Halospirulina sp. EA11(2012)	0.000	0.000	0.000	**0.000**#	N
bacterium MS4	0.000	0.000	0.000	**0.000**#	–
Streptococcus mitis	0.594	0.649	0.265	**0.928**#	P
Gemella haemolysans	0.138	0.270	0.167	**0.612**#	P
Megasphaera micronuciformis	0.024	0.041	0.080	**0.137**#	P
Streptococcus tigurinus	0.009	0.004	0.013	**0.015**#	P
Bifidobacterium longum	0.000	0.008	0.000	**0.015**#	P
Faecalibacterium prausnitzii	0.000	0.014	0.000	**0.010**#	P
Ruminococcus sp. CB3	0.009	0.008	0.000	**0.008**#	P
Lactobacillus gasseri	0.000	0.004	0.000	**0.009**#	P
Lactobacillus salivarius	0.000	0.000	0.000	**0.003**#	P
Gemella sp. oral taxon G07	0.000	0.000	0.000	**0.007**#	P
Eubacterium rectale	0.000	0.000	0.000	**0.007**#	P
Neisseria sp. ChDC B321	0.000	0.004	0.000	**0.005**#	N

Bacteroides sp. S327	0.000	0.000	0.000	**0.008**[#]	N

#: $P < 0.05$ (vs. baseline).

Gram stain, P: Gram-positive, N: Gram-negative, −: unknown.

Supplemental table 5-S4. Changes in the relative abundance of bacterial species with significant inter-group differences at 8 weeks

Species	Relative abundance (%, median)				Gram stain
	Placebo group		Test group		
	Baseline	8w	Baseline	8w	
Supragingival plaque					
No blast hit	1.396	1.584	0.476	**0.354***	
Treponema socranskii	0.047	0.249	0.096	**0.012***	N
Streptococcus sp. oral taxon 057	0.000	0.000	0.000	**0.004***	P
Leptotrichia sp. oral taxon 212	0.000	0.296	0.011	**0.000***	N
Porphyromonas catoniae	0.036	0.177	0.000	**0.000***	N
TM7 phylum sp. oral taxon 349	0.036	0.077	0.000	**0.000***	P
Fusobacterium canifelinum	0.016	0.062	0.000	**0.000***	N
Selenomonas noxia	0.000	0.013	0.000	**0.000***	N
TM7 phylum sp. oral taxon 352	0.000	0.010	0.000	**0.000***	P
Actinomyces johnsonii	0.000	0.009	0.000	**0.000***	P
Neisseria mucosa	0.000	0.009	0.000	**0.000***	N
Tongue coating					
Prevotella sp. CD3_34	1.455	1.760	0.266	**0.265***	N
Neisseria flavescens	0.560	0.984	0.723	**0.082***	N
Neisseria meningitidis	0.009	0.062	0.015	**0.011***	N
Porphyromonas sp. KUFDS01	0.000	0.115	0.014	**0.005***	N
Neisseria sp. 260	0.021	0.085	0.029	**0.000***	N
Porphyromonas sp. oral taxon 279	0.014	0.079	0.010	**0.007***	N
Veillonella sp. BS32b	0.034	0.019	0.045	**0.092***	N
Solobacterium sp. oral taxon A05	0.045	0.096	0.000	**0.004***	P
Fusobacterium hwasookii ChDC F300	0.000	0.005	0.000	**0.000***	N

*: $P < 0.05$ (vs. placebo).

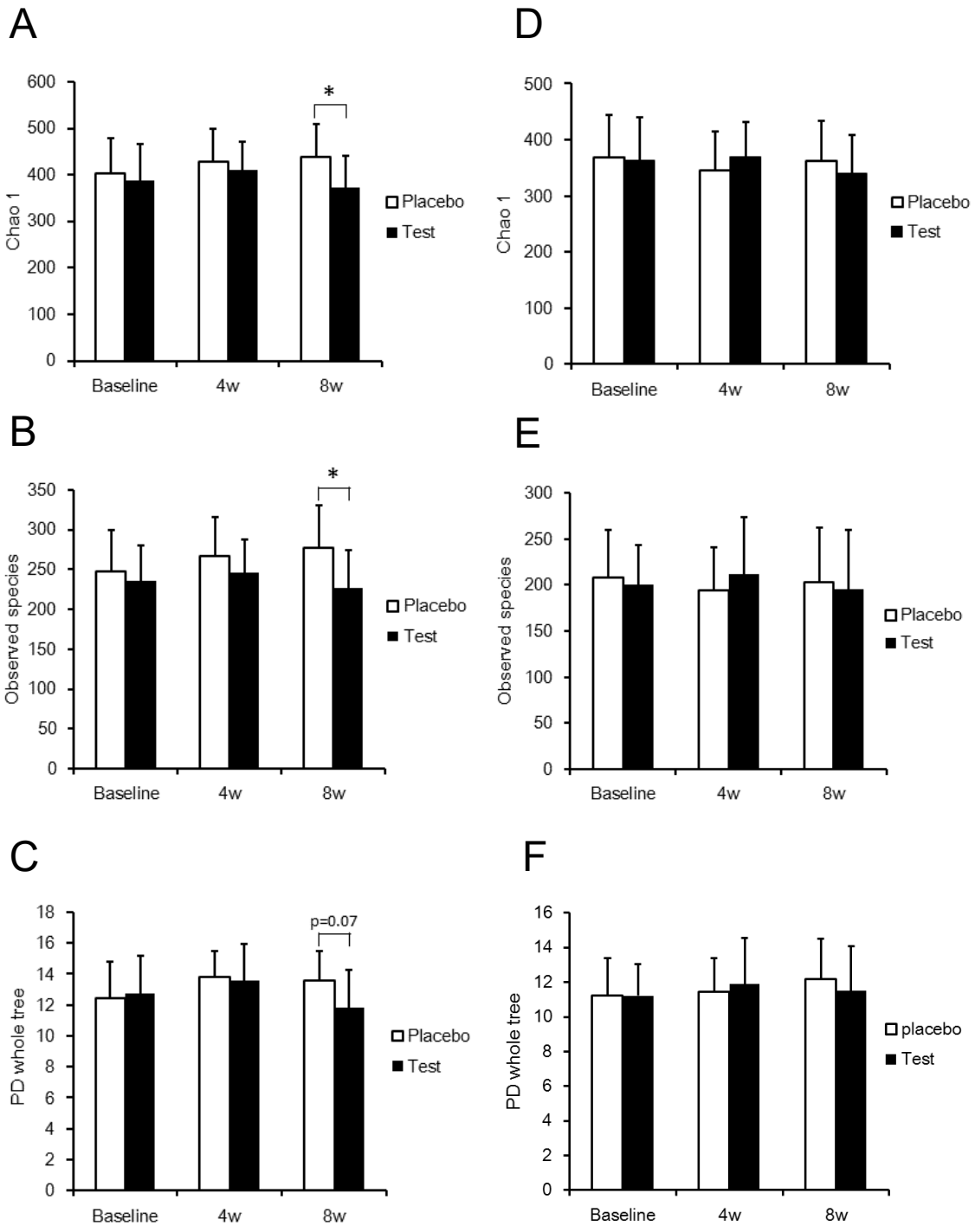
Gram stain, P: Gram-positive, N: Gram-negative, −: unknown.

Supplemental table 5-S5. Relative abundance of caries-associated bacteria and opportunistic pathogens

Species	Relative abundance (%, median)			
	Placebo group		Test group	
	Baseline	8w	Baseline	8w
Supragingival plaque				
Streptococcus mutans	0.009	0.000	0.013	0.003
Streptococcus sobrinus	0.000	0.000	0.000	0.000
Haemophilus parainfluenzae	0.027	0.080	0.091	0.077
Klebsiella pneumoniae	0.016	0.037	0.032	0.016
Moraxella catarrhalis	N.D.	N.D.	N.D.	N.D.
Pseudomonas aeruginosa	0.000	0.000	0.000	0.000
Serratia marcescens	N.D.	N.D.	N.D.	N.D.
Staphylococcus aureus	N.D.	N.D.	N.D.	N.D.
Streptococcus pneumoniae	0.000	0.004	0.000	0.000
Tongue coating				
Streptococcus mutans	0.000	0.000	0.000	0.000
Streptococcus sobrinus	0.000	0.000	0.000	0.000
Haemophilus parainfluenzae	0.000	N.D.	N.D.	N.D.
Klebsiella pneumoniae	0.030	0.031	0.033	0.020#
Moraxella catarrhalis	N.D.	N.D.	N.D.	N.D.
Pseudomonas aeruginosa	0.000	0.000	0.000	0.000
Serratia marcescens	0.000	N.D.	0.000	N.D.
Staphylococcus aureus	N.D.	N.D.	N.D.	N.D.
Streptococcus pneumoniae	N.D.	N.D.	N.D.	N.D.

N.D.: not detected.

#: $P < 0.05$ (vs. baseline).



Supplemental figure 5-S6. Changes in the α-diversity index (chao1, observed species, and PD whole tree) of supragingival plaque (A–C) and tongue coating (D–F).

Data are expressed as the mean ± SD. *: $P < 0.05$ significant difference between the placebo and test groups.

Supplemental table 5-S7. Relationships between α–diversity scores (chao1, observed species, PD whole tree) and parameters of the oral assessment

α-diversity score		Oral assessment			
		O'Leary's PCR (%)	PPD (mm)	BOP (%)	Total VSCs (ng/10 ml)
Supragingival plaque					
Chao1	r	**0.227**	**0.249**	−0.037	**0.224**
	P	0.038	0.022	0.740	0.040
Observed species	r	**0.285**	**0.267**	−0.028	**0.225**
	P	0.009	0.014	0.803	0.040
PD whole tree	r	**0.416**	**0.427**	0.117	0.142
	P	< 0.001	< 0.001	0.289	0.149
Tongue coating					
Chao1	r	**0.280**	**0.347**	0.107	**0.316**
	P	0.010	0.001	0.334	0.001
Observed species	r	**0.289**	**0.315**	0.044	**0.323**
	P	0.008	0.004	0.696	0.001
PD whole tree	r	**0.310**	**0.319**	0.042	**0.304**
	P	0.004	0.003	0.707	0.002

Bold numbers indicate significant coefficient values.

Supplemental table 5-S8. Relationships between parameters of the oral assessment and the relative abundance of bacterial species with significant changes

Bacterial species		Oral assessment			
		O'Leary's PCR (%)	PPD (mm)	BOP (%)	Total VSCs (ng/10 ml)
Supragingival plaque					
Significant decrease					
Prevotella oris	r	**0.265**	0.177	**0.316**	-0.058
	P	0.015	0.108	0.003	0.600
Capnocytophaga gingivalis	r	0.005	0.021	**0.255**	0.031
	P	0.964	0.847	0.019	0.782
Treponema sp. oral taxon 231	r	0.194	**0.234**	0.144	0.120
	P	0.077	0.032	0.193	0.278
Prevotella buccae	r	**0.262**	0.152	0.087	0.092
	P	0.016	0.169	0.430	0.405
Significant increase					
human intestinal firmicute CS13	r	**-0.269**	-0.194	-0.123	-0.171
	P	0.013	0.077	0.265	0.119
Bifidobacterium pseudocatenulatum	r	**-0.287**	**-0.222**	-0.149	-0.199
	P	0.008	0.043	0.177	0.069
[Ruminococcus] gnavus	r	**-0.272**	-0.201	-0.128	-0.190
	P	0.012	0.067	0.246	0.084
bacterium IARFR575	r	**-0.243**	**-0.217**	-0.114	-0.160
	P	0.026	0.048	0.300	0.145
[Clostridium] leptum	r	**-0.243**	-0.198	-0.140	-0.185
	P	0.026	0.071	0.203	0.092
Roseburia inulinivorans	r	**-0.282**	-0.219	-0.154	-0.181
	P	0.009	0.045	0.162	0.099
bacterium ic1379	r	**-0.243**	-0.208	-0.117	-0.165
	P	0.026	0.058	0.290	0.134
Tongue coating					
Significant decrease					
Candidatus Dorea massiliensis AP6	r	**-0.258**	-0.186	-0.129	-0.144
	P	0.018	0.090	0.242	0.192
Neisseria flavescens	r	-0.139	**-0.220**	-0.200	-0.133
	P	0.210	0.046	0.070	0.180
Campylobacter showae	r	0.213	**0.257**	**0.230**	**0.352**

	P	0.053	0.019	0.036	0.000
Porphyromonas sp. KUFDS01	r	0.002	-0.154	**-0.252**	0.102
	P	0.983	0.166	0.021	0.307
Treponema maltophilum	r	**0.226**	**0.322**	0.188	**0.341**
	P	0.040	0.003	0.089	0.000
bacterium MS4	r	**-0.273**	**-0.217**	-0.054	-0.162
	P	0.013	0.048	0.630	0.102
Lachnoanaerobaculum sp. S6-P3	r	**-0.258**	0.034	0.071	-0.084
	P	0.019	0.763	0.523	0.396
Significantly increase					
Megasphaera micronuciformis	r	0.059	0.162	**0.272**	-0.101
	P	0.597	0.143	0.013	0.310
Veillonella parvula	r	-0.186	**-0.400**	**-0.326**	-0.041
	P	0.092	0.000	0.003	0.682
Gemella sp. oral taxon G07	r	**0.237**	0.000	-0.142	0.015
	P	0.031	0.998	0.199	0.884
Eubacterium rectale	r	0.069	0.031	**-0.218**	-0.029
	P	0.535	0.778	0.047	0.769
Neisseria sp. ChDC B321	r	0.127	-0.058	0.092	**0.194**
	P	0.251	0.601	0.406	0.050

Bold numbers indicate significant coefficient values.

Supplemental table 5-S9. Relationships between the α-diversity score and the relative abundance of Gram-positive and -negative group in full analysis set

Bacterial abundance		α-diversity score			
		Shannon index	Chao1	Observed species	PD whole tree
Supragingival plaque					
Gram-positive group	r	**−0.217**	−0.075	−0.120	**−0.319**
	P	0.047	0.498	0.278	0.003
Gram-negative group	r	0.209	0.033	0.072	**0.258**
	P	0.057	0.767	0.513	0.018
Tongue coating					
Gram-positive group	r	−0.096	**−0.222**	**−0.251**	−0.167
	P	0.334	0.024	0.011	0.091
Gram-negative group	r	0.077	0.186	**0.198**	0.097
	P	0.440	0.060	0.045	0.330

Bold numbers indicate significant coefficient values.

5.8 References

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Concluding remarks

LF and LPO are host defense factors in exocrine secretions, such as milk and saliva. This study identified the following novel clinical effects of LF and LPO on oral health, as well as the mechanisms of action.

1. The main contributors to oral malodor have been identified as volatile sulfur compounds (VSCs), including hydrogen sulfide and methyl mercaptan. The LPO system suppressed production of VSCs through a bactericidal effect on periodontal pathogens (*F. nucleatum* and *P. gingivalis*), and also by rapid inactivation of bacterial lyases.
2. The combination of LF and the LPO system exerted a strong synergistic inhibitory effect on cellular metabolic activity and the adhesive hyphal form of *C. albicans*. These results suggested that the combination of LF and the LPO system could be useful for preventing candidiasis.
3. A single dose of a tablet containing LF and LPO reduced VSCs in oral air in the general population.
4. Long-term ingestion of the tablets containing LF and the LPO system promoted a shift from a highly diverse and Gram-negative-dominated to a Gram-positive-dominated community in the microbiota of supragingival plaque and tongue coating. This microbial shift may contribute to improvement of oral health including oral malodor and gingival conditions.

Good oral health care has been shown to effectively reduce the risk of various diseases. Use of antimicrobial agents is also regarded as an effective strategy, but the repeated administration of antibiotics is not recommended because of potential issues associated with the development of resistant microorganisms and microbial substitution. LF and LPO exhibit the above mentioned oral health benefits with fewer side effects. It is hoped that the findings of this study will contribute to improvement of oral diseases.

List of papers related to this study

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