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Effects of French Pine Bark Extract Chewing Gum on Oral Malodor and Salivary Bacteria

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Summary Frequent or persistent malodor (halitosis) represents a considerable embarrassment to those affected. French pine bark extract, Pycnogenol® (PYC), has displayed antibacterial activity against a broad range of bacterial species. In the present study, anticipated benefits of PYC on diminishing halitosis were investigated. Ten healthy males and 11 females, aged 40.1±12.3 y, were recruited based on threshold breath sulfur compounds presence, diagnosed by portable gas chromatography. Subjects were randomly assigned to either sugar-free gums, or gums bearing an additional 2.5 mg PYC per piece. The subjects were required to consume two pieces of PYC or placebo gum six times daily for 15 min. The levels of volatile sulfur compounds (VSCs), measured by OralChroma™, and tongue-coating score were recorded at baseline, 2, and 4 wk. Hydrogen sulfide-producing bacteria in saliva were cultured on Brucella blood agar plates containing 0.05% cysteine, 0.12% glutathione, and 0.02% lead acetate. The group consuming PYC chewing gum reduced exhaled hydrogen sulfide, methyl mercaptan and dimethyl sulfide significantly ($p<0.01$) after 2 wk versus baseline. Continuation of daily PYC-gum consumption for 4 wk remarkably lowered the tongue-coating score and exhaled hydrogen sulfide was significantly decreased compared to the placebo group. PYC chewing gum significantly reduced hydrogen sulfide-producing bacteria in saliva after 4 wk ($p<0.01$), with no effects observed in the placebo control. The results suggest that PYC chewing gum is effective in reducing oral malodor by decreasing the accumulation of tongue coating and the number of hydrogen sulfide-producing bacteria in saliva.

Key Words Pycnogenol®, chewing gum, oral malodor, salivary bacteria, volatile sulfur compounds

Oral malodor represents a major hygiene concern for the majority of people. Volatile sulfur compounds (VSCs), predominantly hydrogen sulfide, methyl mercaptan and dimethyl sulfide, are the principal volatile elements constituting malodor. The etiology of malodor comprises insufficient saliva flow, periodontal diseases, excessive microbial colonization of the tongue and poor oral hygiene (*1–3*). Malodorous volatile sulfur compounds originate from putrefactive activity of microorganisms in oral cavities, saliva, and periodontal pockets and on the tongue surface (*1*, *4*, *5*). Among the large number of bacterial species populating the oral cavity, Gram-negative anaerobic bacteria, including *Porphyromonas* spp., *Veillonella* spp., *Prevotella* spp., *Fusobacterium* spp., *Tannerella forsythia*, and *Treponema denticola* represent the microorganisms predominantly involved in VSC generation (*6–8*).

Reducing the number of bacteria where they accumulate in the oral cavity is effective for the treatment of oral malodor. A number of studies have reported effects of antimicrobial additives to dentifrices (*9*, *10*) and mouth rinses (*11–13*) for improving malodorous breath. Most of these studies examined agents such as chlorhexidine and triclosan to reduce malodor. However, controversies persist on the use of antimicrobial agents, as related to unpleasant taste, staining effects, and especially harm to normal oral microflora.

Chewing gum after meals contributes to oral hygiene owing to the mechanical dislodging of food particles (*14*). Xylitol-bearing chewing gums lower salivary counts of *Streptococcus mutans* versus a placebo (*15*). Other groups have focused on possibilities for using natural substances for controlling malodor (*16*, *17*). Tanaka et al. (*18*) showed long-term effects of eucalyptus-extract chewing gums, decreasing tongue coating accumulation; however, effects on microbial flora in the oral cavity remained unclear.

Pycnogenol® (PYC) is the standardized bark extract of the French maritime pine (*Pinus pinaster* Aiton), which grows in the coastal region of southwest France. PYC is standardized to contain 70±5% procyanidins, comprised of condensed catechin and epicatechin units of varying chain length, and the presence of defined phenolic acids, in accordance with USP requirements (*19*).

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The extract is monographed in the United States Pharmacopeia (USP 39) as a dietary supplement (*20*). The extract was shown to have potent antioxidant activity and anti-inflammatory activity (*21*, *22*). Torras et al. (*23*) found that PYC possesses marked bacteriostatic activity against a broad range of microorganisms, including cariogenic and periodontopathic bacteria. Kimbrough et al. (*24*) reported that PYC-containing chewing gum minimized gingival bleeding and plaque formation. Recently, Sugimoto et al. (*25*) demonstrated that PYC inhibited alveolar bone resorption caused by *P. gingivalis*, which is considered to be one of the major periodontopathic bacteria, resulting from PYC antibacterial potency inflicted on this microorganism. Since PYC exhibits antibacterial activity against cariogenic and periodontopathic bacteria, this extract may be effective to improve periodontal health as a natural bacteriostatic agent. However, to date the anticipated effects of PYC chewing gum on oral malodor and bacterial flora, responsible for VSC generation in halitosis, have not been elucidated. The purpose of the present study was to evaluate effects of long-term regular Pycnogenol® chewing gum consumption on oral malodor and the presence of hydrogen sulfide-generating microorganisms in the saliva of healthy adult volunteers.

MATERIALS AND METHODS

Subjects. A total of 44 healthy volunteers (18 males and 26 females) aged 18 to 59 y, were screened for suitability as subject volunteers. Cigarette smokers and subjects who were suffering from systemic diseases or undergoing periodontal treatment were excluded from the study. Individuals were examined the periodontal probing depth (PD) before the trial and no subject had more than one site with PD (>5 mm). Subjects were required not to have received antibiotic therapy within the previous 30 d. Volunteers were examined for oral malodor, measuring breath volatile sulfur compounds (VSCs), using the portable gas chromatograph OralChroma™ (Abimedical Co., Osaka, Japan). Halitosis was ascertained, meeting recruitment criterion, when all relevant breath sulfur compounds reached the following threshold values, which are organoleptically objectionable (*26*):

Hydrogen sulfide (H_2S)	≥112 ppb
Methyl mercaptane (CH_3SH)	≥26 ppb
Dimethyl sulfide [$(CH_3)_2S$]	≥8 ppb

Twenty-one subjects (10 males and 11 females, mean age 40.1±12.3 y) met the recruitment criteria and were randomly assigned to one of two groups, receiving either Pycnogenol® (PYC) gums or placebo gums. The subjects were divided into the groups with a stratified randomization method based on age and gender. All subjects provided verbal and written informed consent for participation in the study.

The study was conducted in accordance with the Declaration of Helsinki. Approval for this study was obtained from the Ethical Committee for Clinical Research of Kanagawa Dental University (No. 131) and the research has been conducted at Kanagawa Dental University from December 2011 to August 2012. The study is registered in UMIN Clinical Trials Registry (UMIN-CTR), ID UMIN000018305.

Table 1. Proportion of compounds in gum.

Ingredients	PYC gum	Placebo gum
Pycnogenol (%)	0.42	0.00
(mg/cpr[1])	(2.52)	(0.00)
Gum base (%)	38.22	38.22
Sweeteners (%)		
Isomaltose	37.50	37.50
Mannitol	10.23	10.85
Sorbitol	2.63	2.63
Aspartame	0.30	0.30
Acesufame K	0.15	0.15
Flavors (%)	6.16	6.16
Anticaking agents (%)		
Talc	2.90	2.90
Silicon dioxide	1.00	1.00
E473[2] (%)	0.50	0.30
Total (%)	100	100

[1] cpr (comprimido)=tablet.
[2] Sucrose ester of fatty acids.
Chewing gum used in this study was supplied by Gum Base Co. S.p.A., Milano, Italy.

Chewing gum. Pycnogenol® (PYC) powder was provided by Horphag Research Ltd. (Geneva, Switzerland) and chewing gum, with or without PYC, was supplied by Gum Base Co. S.p.A. (Milano, Italy). The weight of each tablet was 600 mg and PYC gum contained 0.42% PYC (2.52 mg) per gum piece. The PYC gum and placebo gum were indistinguishable from each other by visible appearance, weight, taste and smell. Table 1 presents the proportion of compounds in the PYC and placebo gum.

Study design. The subjects were randomly assigned to one of the following groups: an experimental group with eleven participants consuming twelve pieces of PYC-bearing gum per day, corresponding to total daily intake of 30.24 mg PYC and a placebo group (n=10). The daily dose of PYC intake as a supplement is around 1 mg per 1 kg of body weight (*19*). During the intake period (4 wk), subjects chewed two pieces of gum for 15 min, six times daily. Subjects were instructed to chew gum after their three main meals and between the meals, and to record the time of meals and chewing gum consumption in a diary. Participants were advised to refrain from changing their oral hygiene habits and regimens and not to consume other chewing gum throughout the experimental period. Measurements of oral malodor, tongue coating, and examination of salivary bacteria, were carried out at baseline and again after week 2 and week 4.

Oral malodor assessment. Oral malodor was assessed using the OralChroma™ portable gas chromatograph. Briefly, subjects closed their lips tightly and waited for 1 min, followed by gas sampling applying an aspirating syringe. A volume of 0.5 mL mouth air was subse-

quently injected into the inlet of the chromatograph. The levels of VSCs were analyzed and displayed as concentration in part per billion (ppb). Measurements were carried out at approximately the same time for each subject, mainly from 4:30 pm to 5:30 pm. Before each oral odor assessment, subjects were requested to refrain from eating, drinking, and chewing gums for 2 h.

Tongue-coating score. Tongue coating was assessed applying semi-quantitative conventional scores: 0, no tongue coating; 1, thin tongue coating covering less than one-third of the tongue dorsum; 2, thick tongue coating covering approximately one-third of the tongue dorsum or thin tongue coating covering one-third to two-thirds of the tongue dorsum; and 3, tongue coating covering greater than two-thirds of the tongue dorsum (5). Examination of tongue-coating score was performed by one trained examiner, who was masked to the group assignments.

Microbiological study. Stimulated whole saliva was collected from each subject after the measurement of VSCs by chewing standardized paraffin wax (chewing pellet; Willdent Co., Ltd., Osaka, Japan) for 5 min, and diluted 10-fold serially with phosphate buffered saline (PBS: pH 7.4). Aliquots (100 μL) of the diluted samples were plated onto brain heart infusion agar (BHI agar; Becton Dickinson Co., Sparks, MD) supplemented with 5 mg/mL yeast extract, 5 μg/mL hemin, 1 μg/mL vitamin K_1, and 5% sheep blood and cultivated for 7 d to determine total number of colony forming units (CFUs) of salivary bacteria. For cultivation of hydrogen sulfide-producing organisms (OHOs), saliva samples were cultured on Brucella agar (Becton Dickinson) with 5% sheep blood containing 0.05% cysteine, 0.12% glutathione, and 0.02% lead acetate, according to the method of Paryavi-Gholami et al. (*8*) with minor modifications. For visualization of H_2S-producing bacteria, lead acetate was added to the medium, staining colonies on agar gray. To determine the bacterial genus responsible for oral malodor, salivary samples were cultured on KVLB (Kanamycin-Vancomycin Laked Blood) agar: Brucella agar with 75 μg/mL kanamycin, 7.5 μg/mL vancomycin, 1 μg/mL vitamin K_1, and 5% laked sheep blood, modified FM agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), and Veillonella agar (Becton Dickinson) to examine the number of black pigmented anaerobic rods (BPARs), *Fusobacterium* spp. and *Veillonella* spp., respectively.

Antibacterial activity of Pycnogenol® against oral bacteria. The bacterial strains used in the current study were *Porphyromonas gingivalis* ATCC 33277, *Prevotella nigrescens* ATCC 25261, *Fusobacterium nucleatum* ATCC 25586, *Streptococcus mutans* Ingbritt and *Actinomyces viscosus* ATCC 15987. Bacterial strains were grown in BHI broth supplemented with 5 mg/mL yeast extract, 5 μg/mL hemin, and 1 μg/mL vitamin K_1 at 37˚C for 18 h under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2). Exponentially growing bacterial cells were washed, suspended, and adjusted to approximately 1×10^9 CFU/mL in PBS. Bacterial suspension of 10 μL was exposed to 1 mL of 10 μg/mL, 100 μg/mL, or 1,000 μg/mL PYC for 10 min, or the same volume of PBS as a control. After the PYC treatment, the bacterial cell suspension was serially diluted and 100 μL of each dilution was spread onto a BHI sheep blood agar plate. CFUs were determined after 7 d of incubation in an anaerobic

Table 2. Baseline demographics.

Demographics	PYC group (n=11)	Placebo group (n=10)	p-value[1]
Males/females (n)	5/6	5/5	1.000
Age (y)	40.0±12.5	40.3±12.7	0.972
(ranges)	(22–59)	(18–58)	
H_2S (ppb)	226.1±132.9	263.0±166.5	0.725
CH_3SH (ppb)	81.1±49.5	71.1±72.1	0.573
$(CH_3)_2S$ (ppb)	30.6±29.2	15.5±11.6	0.105
Tongue-coating score	1.09±0.54	1.30±0.68	0.398

Data are shown as mean±SD.
[1] Comparison between the experimental group and placebo group. Chi-squared exact test with Yates correction was used for proportion and the Mann-Whitney U test was used for differences.

Table 3. Mean values of volatile sulfur compounds (ppb).

Variable	Group	Baseline[1]	2 wk	4 wk	
H_2S	PYC	226.1±132.9	83.3±79.4**	32.2±33.7**	†
	Placebo	263.0±166.5	141.0±88.7	147.1±144.4	
CH_3SH	PYC	81.1±49.5	28.5±21.9**	10.1±14.4**	
	Placebo	71.1±72.1	33.6±32.2	18.5±22.9	
$(CH_3)_2S$	PYC	30.6±29.2	14.7±17.2**	11.5±22.5**	
	Placebo	15.5±11.6	16.5±11.3	16.2±28.9	

Data are shown as mean±SD (ppb).
[1] No statistical difference was observed between the groups at baseline.
** $p<0.01$ significantly different from baseline.
† $p<0.05$ significantly different between groups.
Comparison with the PYC gum group and placebo gum group was analyzed by the Mann-Whitney U test. The Wilcoxon matched pairs signed rank test was used to analyze the difference between baseline and the observed point.

atmosphere. Each experiment was carried out three times, and the results are shown with the mean value of the experiments.

Statistical analyses. Statistical evaluation of the data was performed using software program STATVIEW version 5.0 (Abacus Concept, Inc., Berkeley, CA). Differences in variables between groups at baseline were compared with Chi-squared exact test with Yates correction for proportion and the Mann-Whitney *U* test for differences. Variations between the experimental group and placebo group were analyzed by the Mann-Whitney *U* test and significance of change from baseline at designated time points was compared with the Wilcoxon matched pairs signed rank test within the group. A value of $p<0.05$ was considered statistically significant.

The sample size was estimated using an expected mean value change of total volatile sulfur compounds of 200 ppb in experimental group, a significance level of 5%, and a power of 80%.

RESULTS

Demographic characteristics at baseline

The two groups at baseline were comparable in number, distribution of gender, age range, and presentation of breath sulfur compounds (Table 2). Threshold levels of hydrogen sulfide, methyl mercaptan and dimethyl sulfide are described as 112 ppb for H_2S, 26 ppb for CH_3SH and 8 ppb for $(CH_3)_2S$, respectively. At baseline, the mean values for each VSC exceeded the threshold levels in both groups, presenting manifest malodor. The VSC levels and tongue-coating score at baseline were not significantly different between groups.

Effects of Pycnogenol® gum on oral malodor

The mean oral volatile sulfur compounds measured in subjects are presented in Table 3. At baseline, breath sulfur compounds exceeded threshold values. PYC gum consumption for 2 wk significantly reduced all VSC levels compared to baseline ($p<0.01$) and further decreased H_2S, CH_3SH and $(CH_3)_2S$ levels after 4 wk of PYC gum consumption. The mean values of VSCs in the PYC gum group reached concentrations significantly below halitosis diagnostic criteria, which are presenting normal mouth odors. In particular, the H_2S concentration in the PYC gum group was significantly different from that in the placebo gum group at 4 wk. On the other hand, placebo chewing gum did not decrease H_2S and CH_3SH values to the same extent and exhaled $(CH_3)_2S$ values remained unchanged.

Tongue-coating score

At baseline, there was no statistically significant difference in the mean value of the tongue-coating score between the PYC gum group and the placebo gum group (Table 2). However, remarkable reduction in the tongue coating was observed at 4 wk in the PYC gum group compared to baseline. In contrast, the mean score in the placebo gum group did not undergo any recognizable changes throughout the experimental period (Fig. 1).

Effects of Pycnogenol® gum on salivary bacteria responsible for oral malodor

Salivary bacteria, which produce VSCs, were examined at baseline, 2 wk and 4 wk (Table 4). The total number of salivary bacteria at baseline in the experi-

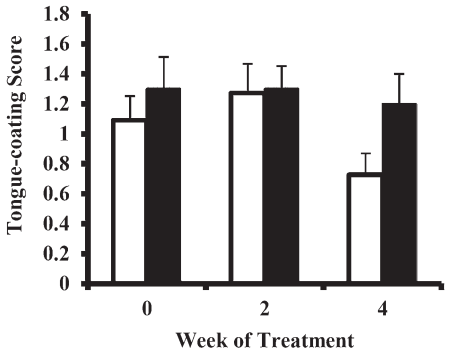

Fig. 1. Effect of Pycnogenol® (PYC) gum on tongue-coating score. Data are shown as mean±SE. □: PYC gum group, ■: placebo gum group.

Table 4. Number of hydrogen sulfide-producing organisms in saliva.

		Baseline[1]	2 wk	4 wk
Total bacterial counts	PYC	28.16±26.66	24.66±19.24	16.99±8.45
	Placebo	23.45±34.70	17.60±12.85	20.02±18.87
Oral hydrogen sulfide-producing organisms	PYC	15.06±12.84	12.55±10.06	7.75±3.97**
	Placebo	9.80±13.15	10.38±10.22	10.56±14.57
Black pigmented anaerobic rods	PYC	0.29±0.27	0.26±0.26	0.14±0.17*
	Placebo	0.26±0.27	0.13±0.11	0.24±0.24
Genus *Fusobacterium*	PYC	0.90±0.88	0.65±1.09	0.23±0.26**
	Placebo	0.37±0.40	0.23±0.20	0.34±0.39
Genus *Veillonella*	PYC	5.28±5.43	5.56±5.84	2.81±2.57
	Placebo	3.17±2.92	3.25±2.79	3.31±2.98

Data are shown as mean±SD of 1×10^7 CFU/mL.

[1] No statistical difference was observed between the groups at baseline.

* $p<0.05$; ** $p<0.01$ comparison with the baseline value. The Wilcoxon matched pairs signed rank test was used for statistical analysis.

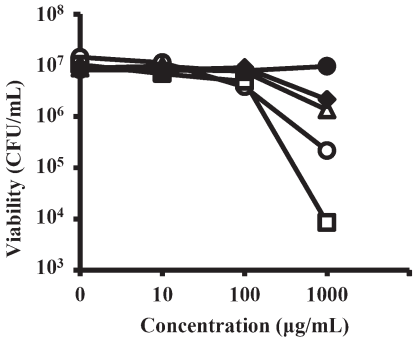

Fig. 2. Antibacterial activity of Pycnogenol® (PYC) against oral bacteria. *Porphyromonas gingivalis* (○), *Prevotella nigrescens* (□), *Fusobacterium nucleatum* (△), *Streptococcus mutans* (◆) and *Actinomyces viscosus* (●) were treated with 0 μg/mL, 10 μg/mL, 100 μg/mL, or 1,000 μg/mL of PYC for 10 min and bacterial suspension was serially diluted and spread onto a BHI sheep blood agar plate. The number of CFUs was determined after 7 d of incubation in an anaerobic atmosphere.

mental and placebo group was 28.16±26.66 (10^7 CFU/mL) and 23.45±34.70 (10^7 CFU/mL), respectively. There was no significant difference between the groups at baseline and no significant change was observed with total number of bacteria during the experimental period in either groups. Approximately half of salivary bacteria produced hydrogen sulfide in the current study. PYC gum chewing reduced the number of oral hydrogen sulfide-producing organisms (OHOs) in a time-dependent manner. After 4-wk intake of PYC gum, OHOs in saliva decreased significantly compared to baseline ($p<0.01$), whereas no effect was observed in the placebo gum group. Black pigmented anaerobic rods (BPARs), which produce hydrogen sulfide and methyl mercaptan, in the PYC gum group decreased at 4 wk compared to the baseline ($p<0.05$). *Fusobacterium* and *Veillonella* species were detected from all subjects during the experimental period. The number of *Fusobacterium* spp. decreased at 4 wk significantly versus baseline in the experimental group ($p<0.01$). Neither PYC gum or placebo gum chewing significantly affected the number of *Veillonella* species; however, there appeared a tendency toward decrease in the number of *Veillonella* species in the PYC gum group.

Antibacterial activity of Pycnogenol® against oral bacteria

Five oral species including cariogenic and periodontopathic bacteria were treated with 10–1,000 μg/mL PYC solution for 10 min. The treatments of *P. gingivalis*, *P. nigrescens*, *F. nucleatum* and *S. mutans* with PYC reduced the number of viable cells in a dose-dependent manner (Fig. 2). After 1,000 μg/mL PYC treatment for 10 min, the number of CFUs of *P. gingivalis*, *P. nigrescens*, *F. nucleatum* and *S. mutans* remarkably decreased by 1.49%, 0.08%, 15.1%, and 23.9%, respectively. However, no antibacterial activity of PYC against *A. viscosus* was observed during the 10-min treatment.

DISCUSSION

Volatile sulfur compounds are primarily produced through the putrefactive activity of bacteria present in saliva, in periodontal pockets, on the tongue surface, and in other areas (*1*, *3*–*5*). In particular, the tongue dorsum has been implicated as a major cause for oral malodor (*5*). Therefore, a reduction of tongue coating score may contribute to the reduction of VSCs in mouth air of the subjects who have been chewing PYC gum.

Chewing gum is an effective way to increase salivary flow, which is elicited by a combination of gustatory and mechanical stimulations (*27*, *28*). Generally, saliva plays an important role in eliminating oral malodor. Mastication increases saliva flow, with concomitant cleansing of the oral cavity and reduction in malodor (*1*, *28*, *29*). These recent studies demonstrated that chewing gums with flavors stimulated salivary flow and prolonged chewing was effective to maintain the flow rate. Moreover, regular use of chewing gum with normal oral hygiene procedures revealed significant reduction in plaque scores (*14*, *30*). In the present study, both PYC gum and placebo gum included the same flavor and the subjects chewed the gum for 15 min every time. Therefore, stimulation of salivary flow and mechanical cleaning of the oral cavity by gum chewing might have evoked the reduction of VSCs in both groups to the same extent.

The primary causative microorganisms of oral malodor are Gram-negative, anaerobic bacteria that are similar to the bacteria causing periodontitis (*31*, *32*). Awano et al. (*31*) indicated a relationship between the presence of periodontopathic bacteria, such as *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia* and *Toreponema denticola*, in saliva and oral malodor. In addition to periodontopathic bacteria, many oral bacteria can produce H_2S and CH_3SH in vitro (*33*). In the current study, PYC gum chewing for 4 wk resulted in a significant decrease for oral hydrogen sulfide-producing organisms (OHOs) in saliva compared with the baseline (Table 4), whereas no effect was observed in the placebo gum group. The total numbers of salivary bacteria did not show significant differences during the experimental period. The results suggest that the decrease of OHOs in saliva may cause the reduction of H_2S in exhaled air in the PYC gum group. Among H_2S-producing bacteria, black pigmented anaerobic rods (BPARs), including *Porphyromonas* spp. and *Prevotella* spp., were reduced by PYC gum chewing for 4 wk. These microorganisms are responsible for producing both H_2S and CH_3SH.

PYC possessed marked bacteriostatic activity against a broad range of microorganisms including cariogenic and periodontopathic bacteria (*23*). Kimbrough et al. (*24*) reported that PYC-containing chewing gum minimized gingival bleeding and plaque formation, when the subjects had worn an acrylic stent while they brushed their teeth over a period of 14 d. Their results show antibacterial activity of PYC to inhibit plaque formation. Sugimoto et al. (*25*) reported the bactericidal activity against *P. gingivalis* with 1,000 μg/mL PYC treatment

for 60 min. To confirm the effects of PYC on various oral bacteria, we further examined the antibacterial activity of PYC against five microorganisms including cariogenic- and periodontitis-related bacteria. Each bacterium was treated with 10 to 1,000 μg/mL PYC. In particular, remarkable antibacterial activity against Gram-negative bacteria was observed. These microorganisms belong to the bacterial genera which were reduced in saliva by PYC-gum chewing. The subjects in the experimental group chewed PYC gum containing 5.04 mg PYC for 15 min each time. Therefore, the PYC gum contained an effective concentration to reduce the number of microorganisms that are responsible for malodor in saliva.

In the present study, we evaluated the reduction of oral malodor together with the examination of salivary microbial flora by chewing PYC gum. The results demonstrated the effectiveness of PYC-gum chewing in reducing oral malodor and decreasing odorigenic bacteria in saliva. In conclusion, the use of a chewing gum containing PYC is effective to reduce oral malodor by decreasing the number of bacteria producing volatile sulfur compounds in saliva as well as the accumulation of tongue-coating bacteria.

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