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# Proteases, actinidin, papain and trypsin reduce oral biofilm on the tongue in elderly subjects and in vitro

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ABSTRACT

*Objective:* Dental plaque is a causative factor for oral disease and a potential reservoir for respiratory infection in the elderly. Therefore, there is a critical need for the development of effective methods to remove oral biofilm. The objective of this study was to investigate the effect of proteases on oral biofilm formation andremoval.
*Design:* The in vivo effect of actinidin, a cysteine protease, on the removal of tongue coating was assessed after orally taking a protease tablet. Effects of the proteases trypsin, papain and actinidin on Actinomyces monospecies biofilm and multispecies biofilm that was reconstructed using a plaque sample from the tongue coating were investigated using the microtiter plate method. Antimicrobial tests and limited proteolysis of fimbrial shaft proteins were also performed to clarify underlying mechanisms of oral biofilm removal.
*Results:* Tablets containing actinidin removed tongue coating in elderly subjects. Oral Actinomyces biofilm was significantly reduced by the proteases papain, actinidin and trypsin. Papain and trypsin effectively digested the major fimbrial proteins, FimP and FimA, from Actinomyces. Actinidin, papain and trypsin reduced multispecies biofilm that was reconstructed in vitro. Papain and trypsin inhibited formation of multispecies biofilm in vitro.
*Conclusions:* This study shows that proteases reduced oral biofilm in vivo in elderly subjects and in vitro, and suggests that protease digests fimbriae and inhibits biofilm formation.

## 1. Introduction

The accumulation of oral plaque biofilms is thought to induce various localized and systemic infectious diseases such as periodontal disease, dental caries and infectious endocarditis (Kumar, 2013). Many types of natural or synthetic agents have been tested to prevent formation of oral biofilm (Rahmani-Badi, Sepehr, & Babaie-Naiej, 2015; Tada et al., 2016; Nakano, Shimizu, Wakabayashi, Yamauchi, & Abe, 2016; Howlin et al., 2015). The tongue dorsum especially retains a large amount of biofilm in the oral cavity as a tongue coating, which is thought to be associated with oral halitosis (Tonzetich & Ng, 1976; Tonzetich, 1977; Miyazaki, Sakao, & Katoh, 1995; Bollen & Beikler, 2012), periodontal disease (De Geest, Laleman, Teughels, Dekeyser, & Quirynen, 2000; Yaegaki & Sanada, 1992; Van Tornout, Dadamio, Coucke, & Quirynen, 2013), opportunistic infection (Pieralisi, de Souza Bonfim-Mendonca, Negri, Jarros, & Svidzinski, 2016), and aspiration pneumonia (Takeshita et al., 2010). Especially in edentate elderly, tongue coating has been identified as a risk indicator for aspiration pneumonia. The relative risk of developing pneumonia in the good tongue hygiene group compared with the poor tongue hygiene group was 0.12 (Abe, Ishihara, Adachi, & Okuda, 2008). Although formation of the tongue coating is a normal phenomenon that is observed even in healthy individuals, accumulation of a tongue coating is affected by various factors such as oral hygiene habitats, smoking, presence of a denture, periodontal status, and dietary habits (Van Tornout et al., 2013). It has also been reported that an increase in salivary viscosity and a decrease in salivary flow accelerates formation of the tongue coating, suggesting that the saliva also plays an important role in tongue coating accumulation (Ueno, Takeuchi, Takehara, & Kawaguchi, 2014; Suzuki et al., 2016). Although mechanical scraping is the main treatment for a tongue coating (Blom, Slot, Quirynen, & Van der Weijden, 2012; Slot, De Geest, van der Weijden, & Quirynen, 2015; Erovic Ademovski et al., 2012), scraping sometimes wounds the taste buds and induces the gag reflex (Rowley, Schuchman, Tishk, & Carlson, 1987; Christensen, 1998). Therefore, chemical and biochemical removal methods have been studied.

Oral biofilm formation starts with an initial colonizer that adheres to oral tissue and dental surfaces. This process involves multiple factors such as 36 fimbriae and fimbriae receptors on host cells, and extracellular polymeric substances (EPSs). The initial colonizer, Actinomyces

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species (Li et al., 2004; Nyvad & Fejerskov, 1987; Kilian, Larsen, Fejerskov, & Thylstrup, 1979), has two types of adhesive fimbriae that are essential for adherence to solid surfaces (Neeser, Chambaz, Del Vedovo, Prigent, & Guggenheim, 1988). Attached bacteria then develop into higher order structures with cell-to-cell communication, and they produce EPSs to accelerate the physical strength of the biofilm (Flemming & Wingender, 2010). EPSs are mostly composed of polysaccharides, DNA, lipid and proteins (Flemming & Wingender, 2010).

Agents that degrade and inhibit oral biofilm have been developed, and several types of antibiofilm agents and enzymes targeting the biofilm extracellular matrix have been reported. Many of them target polysaccharides (Singh, Parsek, Greenberg, & Welsh, 2002; Di Bonaventura et al., 2012), but recently DNA-targeting agents have been studied (Cavaliere, Ball, Turnbull, & Whitchurch, 2014; Rice et al., 2007; Fredheim et al., 2009). Additionally, the effect of proteases on removing biofilm has been studied (Gilan & Sivan, 2013; Meyle et al., 2010; Niazi et al., 2014, 2015). Trypsin, a serine protease, has been intensively studied because of its detachment effect (Niazi et al., 2014, 2015; Chaignon et al., 2007; Harris, Nigam, Sawyer, Mack, & Pritchard, 2013). Nohno, Yamaga, Kaneko, and Miyazaki, 2012 showed that tablets containing actinidin, a cysteine protease from kiwi fruit, reduces oral malodor. However, the underlying mechanisms of the proteases' oral biofilm removal effects are unclear. In this study, we assessed the reduction and antibiofilm activity of several proteases *in vivo* and *in vitro*.

## 2. Materials and methods

### 2.1. Subjects and study design

There were 20 healthy young adults (9 males, 11 females; mean age, 30.8 ± 1.69 years; age range, 18–41 years) and 20 elderly adults who were receiving daily 58 nursing care (8 males and 12 females; mean age, 71.3 ± 3.40 years; age range 63–88 years) who were enrolled into this study. Informed consent was obtained from each participant before enrolment. The study protocol was approved by the Ethics Committees of Osaka Dental University (#110821). This study was a double-blind, placebo-controlled cross-over trial with a 1-week washout period between the crossover phases. The subject flow from the initial screening to final analysis is shown in Fig. S1. All subjects were requested not to eat, drink, smoke, rinse or perform oral hygiene for at least 2 h before their appointment. In this experiment, we used two types of tablets: one with actinidin (a cysteine protease from kiwi fruits; test), and the other with no actinidin (placebo; Table S1). They were identical in size and shape, and were prepared by Ezaki Glico (Osaka, Japan). The actinidin tablets were made from freeze-dried extract of kiwi fruit was added, and they were molded without boiling during the process. After assessment of the tongue coating by taking photos of the tongue dorsum (see below) using a digital camera (EXILIM HS EX-ZR20, Casio Computer, Tokyo, Japan) at the baseline of first test phase, the subjects were randomly divided into two groups. In each crossover phase, each group was instructed to take two tablets of either test or placebo. Patients placed one of the tablets on their tongue dorsum, and approximately 5 min later, they placed the second tablet there. The total time was approximately 10 min, and both of the tablets were of the same type. We then reassessed the amount of tongue coating. One week later, the subjects were allocated to the alternate group and the protocol was repeated. All subjects were studied twice with a 1-week interval between test and control treatment, and the subjects did not take any tablets during the interval. Experimental details are shown in the Supplemental file (Fig. S1).

### 2.2. Tongue coating assessment

The tongue coating was assessed and compared using the Tongue Coating Index (TCI), according to previous studies (Shimizu, Ueda, & Sakurai, 2007). Briefly, each tongue dorsum surface was divided into nine equal areas from the circumvallate papillae to the tip, and the tongue coating was assessed in each of these areas as follows: 0, no coating; 1, light coating; and 2, heavy coating (Fig. S2). There was no significant difference in the TCI value among nine sections of the tongue (data not shown). The TCI was calculated based on all nine areas, and a higher TCI score indicated a greater amount of tongue coating.

### 2.3. Reagents

Because purified actinidin was unavailable, we used a freeze-dried extract from kiwi fruit, which was supplied as a tablet formulation and provided by Ezaki Glico. The kiwi fruit extract is thought to contain over 50% actinidin out of the total soluble protein component. The pH of the extracted supernatant (A1), which represented the equivalent activity of approximately 1.8 mg/mL papain (Fig. S4), was adjusted to 6.5 by adding a small amount of 1 M NaOH. Purified papain from carica papaya and trypsin from porcine pancreas were purchased from Wako Pure Chemical Industries (Osaka, Japan).

L-Pyroglutamyl-L-phenylalanyl-L-leucine *p*-nitroanilide (PFLNA) was purchased from Peptide Institute (Osaka, Japan). E-64 (a cysteine protease inhibitor) was from Merck Millipore Corporation (Darmstadt, Deutschland). All other chemicals were obtained from commercial sources and were of the highest available purity.

### 2.4. Bacterial strains and growth conditions

Bacteria and plasmids used in this study are listed in Table 2. *Actinomyces oris* and *Escherichia coli* were cultured at 37 °C in heart infusion broth (HIB) and Luria-Bertani (LB) broth, respectively. Unless otherwise specified, ampicillin was used at a concentration of 100 μg/mL.

### 2.5. Effect of protease on monospecies and multispecies biofilm

Monospecies biofilm was constructed using the *A. oris* strain MG-1. Strain MG-1 was grown overnight in HIB with shaking, and then diluted to a final optical density of 1.0 at 600 nm (OD600) using fresh HIB with 1% sucrose. Diluted culture (1 mL) was added to each well of a 24-well polystyrene microtitre plate (Nunc). Plates were incubated at 37 °C without shaking for 18 h. The plates were washed once with 200 μL of dH2O to remove planktonic cells. Two hundred microliters of ten-fold protease solution serial dilutions (10, 1, 10 − 1, 10 − 2, 10 − 3 and 10 − 4 113 mg/mL papain and trypsin) or A1 with or without 10 μg/mL E-64 in 0.1 M phosphate buffer (pH 6.5) with 0.5 M sucrose was added. After 10 or 30 min incubation at 37 °C without shaking, the plates were carefully washed once with 200 μL of dH2O to remove peeled biofilm and the attached protease-treated biofilms were not removed. Retained biofilm was stabilized with 200 μL of 2.5% glutaraldehyde for 5 min, stained with 250 μL of 0.5% crystal violet for 5 min, and then washed three times with 10% ethanol to remove unbound excess dye. The crystal violet was solubilized by adding 200 μL 95% ethanol and mixing using a shaker. After 5 min, 50 μL aliquot was transferred to each well of a 96-well polystyrene microtitre plate (Nunc) and the amount of residual biofilm was determined by measuring the absorbance at 590 nm.

Multispecies biofilm was constructed using samples collected from the tongue dorsum. All individuals gave written informed consent and the study protocol was approved by the Osaka Dental University Ethics Committee (approval no. 110864). The tongue coating was incubated overnight in HIB without shaking and was resuspended at a final OD600 of 0.1 in fresh HIB with 1% sucrose. A 1-mL aliquot of the sample was added to each well of a 24-well polystyrene microtiter plate and incubated at 37 °C without shaking for 2 weeks, with 1 mL fresh media exchanged every other day. After incubation, the plates were

washed once with 200 μL of dH2O to remove planktonic cells. The bacterial flora of multispecies biofilm was evaluated using 16S rRNA gene sequencing on the Illumina MiSeq platform, and six genera (*Streptococcus*, *Veillonella*, *Lactobacillus*, *Prevotella*, *Peptostreptococcus* and *Haemophilus*) were detected more than 2% each and *Actinomyces* was detected at 0.4% (data not shown). Protease solutions (200 μL) at various concentration in 0.1 M phosphate buffer (pH 6.5) and 0.5 M sucrose were added and incubated at 37 °C without shaking. After 30 min of incubation, the plates were washed twice with 200 μL of dH2O to remove peeled biofilm. Bacteria retained in the residual biofilm were measured using the same method as above.

### 2.6. Antibacterial assay

Strain MG-1 was grown overnight in HIB with shaking, and then pelleted by centrifugation. The cell pellet was suspended using HIB containing 1% sucrose to a final OD600 of 0.5. A 0.4-mL aliquot of the diluted culture was mixed with 50 mg/mL papain or trypsin in 0.1 M phosphate buffer (pH 6.5) containing 0.5 M sucrose, and incubated at 37 °C with shaking for 60 min. A 10 μL aliquot of the incubating solutions was temporally fractionated, serially diluted and spotted on Brucella agar plates. The number of colony forming units (CFU/mL) was determined and compared.

### 2.7. Overexpression and purification of FimP and FimA

A total DNA sample was extracted from an overnight culture of strain MG-1 using the NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's protocol. The T5 expression vector pQE80L (Qiagen, Hilden Germany) was used for overproduction of the FimP and FimA proteins that were fused to the His6 tag at their N terminus. The *fimP* and *fimA* genes were PCR-amplified using the primers PFP1 plus PFP2 and PFA1 plus PFA2, respectively (Table 2). The linearized vectors of pQE80L were obtained by PCR using primers PFP3 plus PFP4 and PFA3 plus PFA4 (Table 2). The amplified fimP and fimA fragments were fused to the linearized vectors using In-Fusion HD cloning kit (TaKaRa, Tokyo, Japan) to obtain pNMP1 and pNMA1, respectively (Table 3).

*E. coli* DH5a cells harboring one of the His-tagged fusion plasmids were grown at 37 °C in LB medium containing 100 μg/mL ampicillin (Table 3). When the cell growth reached the mid-exponential phase, isopropyl-b-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM and incubation was continued for a further 5 h. The cells were harvested by centrifugation, and his-tagged proteins were purified using Capturem™ His-Tagged Purification Miniprep Kit (Takara, Tokyo, Japan), according to the manufacturer's instructions. Protein concentrations were measured using a protein assay kit (Takara BSA Protein Assay Kit, Takara, Tokyo, Japan). Purified protein was stored at −30 °C until further use.

### 2.8. Limited proteolysis of purified FimP and FimA

Limited proteolysis of purified FimP and FimA by papain and trypsin were performed in 500 mM phosphate buffer (pH 6.5) at 37 °C. FimP (70 μg/mL) and FimA (60 μg/mL) were incubated with papain (17.5 μg/mL) and trypsin (17.5 μg/mL). After starting the 168 incubation, samples were collected at 5, 15, 30, 60, 120 and 180 min and SDS loading buffer was added. Boiled papain and trypsin were added to samples as the zero time point. After SDS-PAGE, the gels were stained with Coomassie brilliant blue.

### 2.9. Prevention of biofilm formation by protease

The tongue coating, which was collected using swabs from the tongue dorsum, was incubated overnight in HIB without shaking. The culture was diluted to a final OD600 of 0.1 using fresh HIB plus 1% sucrose. Papain and trypsin were added to final concentrations of 10, 1, 10–1, 10–2 and 10–3 mg/mL, and actinidin was also added to final concentration of 1–10-4177 -fold serial dilutions of A1. A 1-mL aliquot of the diluted culture was added to each well of a 24-well polystyrene microtiter plate (Nunc). Plates were incubated at 37 °C without shaking for 18 h. After incubation, the plates were washed once with 200 μL of dH2O to remove planktonic cells. Bacteria retained in the residual biofilm were stabilized with 200 μL of 2.5% glutaraldehyde for 5 min, stained with 250 μL of 0.5% crystal violet for 5 min, and then washed three additional times with 10% ethanol to remove unbound excess dye. The crystal violet was solubilized by additional of 200 μL of 95% ethanol using a shaker. After 5 min, a 50-μL aliquot was transferred to each well of 96-well polystyrene microtiter plate and the amount of residual biofilm was determined by measuring the absorbance at 590 nm.

### 2.10. Statistical analysis

All experiments were performed in duplicate or triplicate for each condition and was repeated at least three times. All the statistical analyses were performed using the IBM SPSS statistical software package (version 21.0). The pre-specified research objective was to determine whether taking test tablets removed the tongue coating in healthy young adults and in elderly adults who required daily nursing care. The pre-specified outcome for the study was change in tongue coating after taking the test tablet compared to the change after placebo. The mean of baseline values were compared between both treatments for each age group using the Kruskal-Wallis test and compared between both age groups using the Wilcoxon signed-rank tests and paired Student's *t*-tests. Intra-group comparisons (before and after treatment) were performed using Friedman test. Inter-group comparisons (change ratios by test vs. change ratios by placebo treatment) were performed using the Wilcoxon signed-rank test. The level for statistical significance was $p < 0.05$ for all analyses.

## 3. Results

### 3.1. Effect of actinidin in a tablet on tongue coating removal

To evaluate the effects of actinidin on tongue coating removal, we conducted a double-blind, crossover, placebo-controlled study involving 20 healthy young adults and 20 elderly adults receiving nursing care. There was no significant difference in the baseline value of the TCI between the test and placebo in each of the age groups ($p > 0.05$). The average TCI value in the young subjects was significantly lower than that in the elderly subject ($p < 0.01$; Table 1 and Fig. 1), which is consistent with previous reports (Ralph, 1987). Although TCI values decreased after taking the tablets in both age groups, there was a greater decrease in the TCI for subjects taking the test tablets containing kiwi fruit extract, which contains substantial amounts of the cysteine protease actinidin, compared with taking placebo in both young and elderly subjects. There was a statistically significant difference in the TCI in the elderly group but not in the young group compared to the baseline value. These results suggest that actinidin in tablet formulations facilitates the removal of the tongue coating in this experimental condition.

### 3.2. In vitro biofilm removal by papain, trypsin and actinidin

We further investigated the *in vitro* effects of proteases on oral bacterial biofilm to elucidate the mechanism of tongue coating detachment. To clarify the ability of proteases to detach biofilms, we used a mono-species biofilm formed by *A. oris*, which is an important initial oral biofilm colonizer, and purified two types of proteases: the cysteine protease, papain, and the well-characterized serine protease, trypsin. Concentration- and time-dependent biofilm detachment was observed

**Table 1**
Tongue coating index at baseline and after treatment in test and placebo groups.

| | Actinidin (Young: n = 20, Elderly: n = 20) | | | | | | | Placebo (Young: n = 20, Elderly: n = 20) | | | | | | | Treatment difference | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Baseline | | Endpoint | | Mean change ratio | | p-intra-group comparison | Baseline | | Endpoint | | Mean change ratio | | p-intra-group comparison | p-inter-group comparison | |
| | Mean | SD | Mean | SD | Mean | SD | | Mean | SD | Mean | SD | Mean | SD | | Mean | SD |
| Young | 8.47 | 4.66 | 3.65 | 2.85 | 47.46 | 29.71 | < 0.01 | 7.83 | 5.38 | 4.75 | 3.02 | 27.29 | 35.64 | < 0.05 | 0.64 | 20.17 | 48.09 |
| Elderly | 13.5 | 8.95 | 6.68 | 6.18 | 55.96 | 18.67 | < 0.01 | 11.87 | 8.49 | 8.87 | 7.07 | 25.64 | 16.34 | < 0.01 | < 0.01 | 30.33 | 21.85 |

n: number of patients; SD: standard deviation.

**Table 2**
Oligonucleotide primers used.

| Primer | Sequence[a] |
|---|---|
| PFP1 | CCATCACGCTGACCCGAACGGCTCCACCAT |
| PFP2 | CGGATCCTTAGCGGAAGCCCGCGTTCTTCGG |
| PFP3 | GGGTCAGCGTGATGGTGATGGTGATGCGAT |
| PFP4 | GCTTCCGCTAAGGATCCGCATGCGCGAGCTCG |
| PFA1 | CCATCACACGGAAACGCCTAACTACGGCAA |
| PFA2 | CGGATCCTTAGGCCACGAGGAGGACGGAGCCGA |
| PFA3 | GTTTCCGTGTGATGGTGATGGTGATGCGAT |
| PFA4 | TCGTGGCCTAAGGATCCGCATGCGAGCTCG |

[a] Sequence is written in the 5′–3′ direction. The underlined nucleotides are the substitution sites used in the oligonucleotide-directed mutagenesis.

**Table 3**
Bacterial strains and plasmids used.

| Bacterial strains and plasmids | Relevant characterstics | References |
|---|---|---|
| Strains | | |
| A. oris MG-1 | Wild-type strain | |
| Plasmids | | |
| pNMP1 | pQE80 containing wild-type *fimP* from MG-1, Kan$^r$ | This study |
| pNMA1 | pQE80 containing wild-type *fimA* from MG-1, Kan$^r$ | This study |

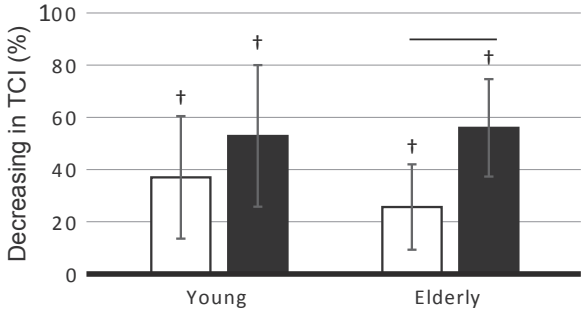


**Fig. 1.** Decrease in the tongue coating index (TCI) scores after taking the tablets.
The results show a decrease in TCI scores after taking placebo (□) or test (■) tablets. Values are presented as the mean and standard deviation, represented by vertical bars. †The mean value was significantly different from baseline ($p < 0.05$; intra-group comparison). *Change during actinidin treatment was significantly different from the change during placebo treatment for elderly people ($P < 0.01$; inter-group comparison). TCI score at baseline did not differ in the young and elderly groups (Wilcoxon signed-rank tests).

in wells with papain added (Fig. 2). Papain reduced *Actinomyces* biofilms almost completely at 10 mg/mL with a 10-min incubation and at 1 mg/mL with a 30-min incubation. Trypsin can also reduce biofilm, but we did not observe a clear correlation in wells containing trypsin, as there was for papain. In this experimental condition, papain seems to have larger effect on removing the mono-species *Actinomyces* biofilm than trypsin. Because purified actinidin was not available, we investigated detachment of biofilms using a water-soluble fraction of kiwi fruit extract containing actinidin. After a 30-min incubation, kiwi fruit extract removed biofilm in a concentration-dependent manner, which was similar to papain. Because addition of the cysteine protease-specific inhibitor E-64 fully suppressed the effect of kiwi fruit extract, actinidin in kiwi fruit extract may be involved in removal of *Actinomyces* biofilm.

### 3.3. No antimicrobial effects of proteases on Actinomyces

To investigate the mechanism of *Actinomyces* biofilm detachment by proteases, we performed two different experiments. First, we

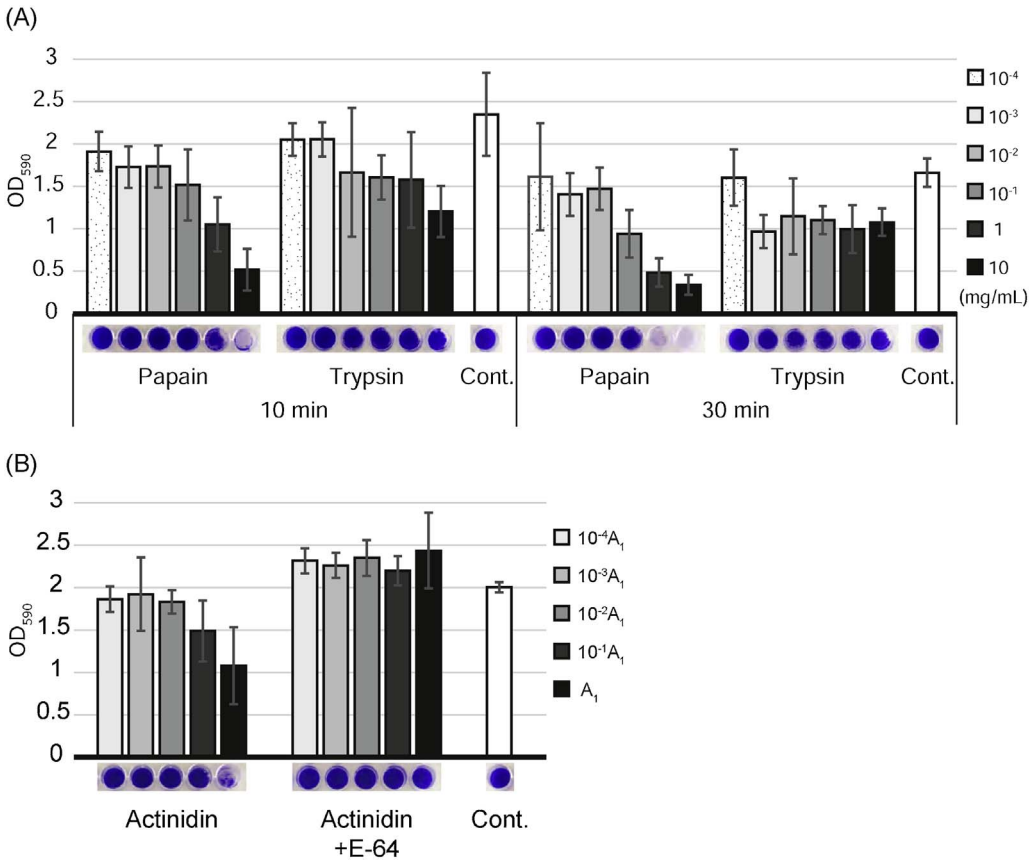


**Fig. 2.** Biofilm detachment is affected by papain, trypsin or actinidin.
Detachment assay results show increased biofilm detachment with papain or trypsin treatment (A) and actinidin treatment (B). Bacterial biofilms were stained with crystal violet and quantification was performed by determining the absorbance at 590 nm. The values presented here are the means of at least four independent experiments and the error bars represent the standard deviation. The image is representative of four independent experiments.

investigated whether proteases 234 cause potentially lethal damage to *Actinomyces* cells. *Actinomyces* cells were incubated with a final concentration of 10 mg/mL papain or trypsin for 60 min. Papain or trypsin treatments showed no decrease in the amount of viable *Actinomyces* cells compared to control (Fig. S3). This result suggest that papain and trypsin have no antibacterial effect on *A. oris*.

### 3.4. Limited proteolysis of purified FimP and FimA

We also investigated whether papain and trypsin digest main fimbrial subunits in *Actinomyces*, which are known to be essential for biofilm formation. To study the protease sensitivity of the main type 1 and 2 subunits FimP and FimA, we performed limited proteolysis of these overexpressed and purified proteins using the proteases papain and trypsin. The time-course of degradation, as monitored by SDS-PAGE, is shown in Fig. 3.

For both papain and trypsin, FimP and FimA were digested in a time-dependent manner. Although band patterns of limited proteolyzed products were different for each protease, intact FimP and intact FimA bands rapidly decreased when incubated with low enzyme concentrations (17.5 μg/mL; Fig. 3). Additionally, these limited proteolyzed products completely disappeared within 15 min when using proteases at a high concentration (0.1 mg/mL, data not shown).

### 3.5. Effects of proteases on an in vitro oral biofilm model

As shown above, the proteases used in this study disrupted the monospecies biofilm composed of *Actinomyces*. We next investigated whether these proteases can remove an oral multispecies biofilm made with plaque taken from the tongue dorsum. After application of the enzyme for 60 min, all proteases (actinidin, papain and trypsin) fully disrupted oral multispecies 256 biofilms at a high concentration, similar to the monospecies biofilm (Fig. 4). Although the actinidin's unit was very mild compared to papain (Fig. S4), it reduced biofilms. At lower concentrations, trypsin retained some removal activity. Because actinidin we used was unpurified product, we measured this unit test.

We also examined the inhibitory effects of the proteases on biofilm formation. All proteases used in this study prevented formation of multispecies oral biofilm in a concentration-dependent manner (Fig. 5). Papain and trypsin almost completely inhibited biofilm formation at 10 mg/mL, and actinidin also sufficiently inhibited biofilm formation at the highest concentration. Similar experimental results were obtained using a monospecies biofilm made from *Actinomyces* (data not shown). These data indicate that these proteases have the ability to inhibit formation of oral biofilm.

## 4. Discussion

The aim of this study was to demonstrate that proteases can reduce or remove tongue coating and an *in vitro* oral biofilm. We found that actinidin significantly removed tongue coating in elderly subjects, and trypsin, papain and actinidin reduced monospecies and multispecies biofilm *in vitro*. To date, much research has been performed on removal of the tongue coating and oral malodor to prevent various infectious diseases. Because the dorsal tongue surface possesses a large number of papillae, mechanical tongue cleaning with tongue brushes is troublesome, and chemical and biochemical methods for removal of the tongue coating are desired. Early studies showed the effectiveness of disinfectant treatment for the oral cavity, e.g. chlorhexidine (Winkel, Roldán, Van Winkelhoff, Herrera, & Sanz, 2003), cetylpyridinium chloride and zinc (Codipilly, Kaufman, & Kleinberg, 2004). Although the treatment was effective for reducing oral malodor, there was no statistically significant difference in the TCI score. Additionally, tongue coating removal by gargling with chemical compounds is thought to be difficult (Blom et al., 2012).

Oral biofilms are three-dimensional structured bacterial

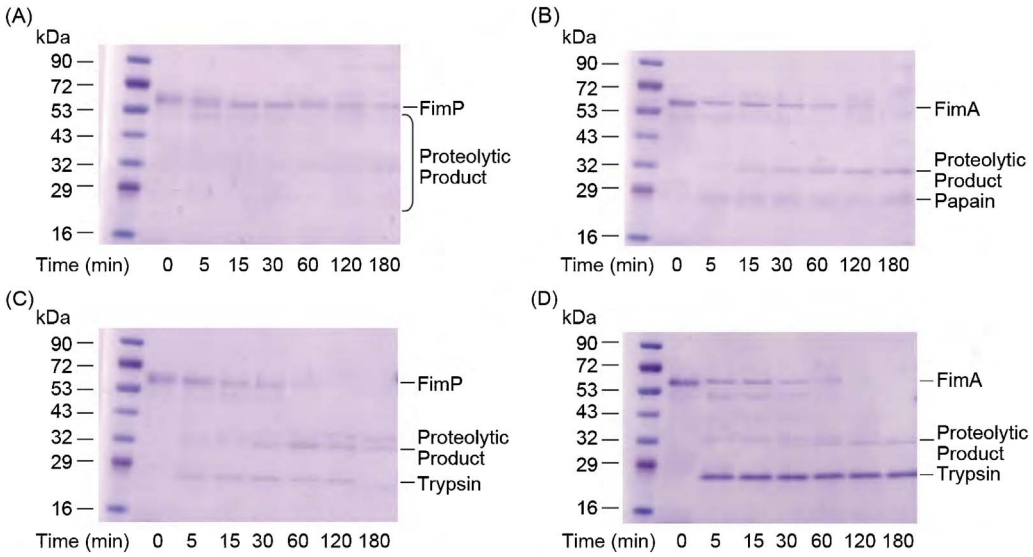


**Fig. 3.** Limited proteolysis of FimP and FimA.
Time courses for the limited papain and trypsin digestion of standard proteins using 17.5 μg/mL papain (A), 17.5 μg/mL trypsin (B), 0.1 mg/mL papain (C) and 0.1 mg/mL trypsin (D). A and B show the digestion results for 70 μg/mL FimP. C and D show the digestion results for 60 μg/mL FimA. The aliquot at time 0 min includes deactivated papain or trypsin (A–D). In each plot, the first lanes correspond to the molecular mass standards and are presented in kilodaltons on the left. Numbers reflect the time points at which the proteolysis was quenched. Both papain and trypsin decomposed FimP and FimA. Although trypsin decomposed FimP faster than papain, papain decomposed FimA as fast as trypsin.

communities attached to each other or to a surface and enclosed in structures such as EPSs, fimbriae and flagella that are produced by the bacteria. Extensive studies using glycolytic enzymes, nucleases and proteases have been conducted to remove oral biofilm. Among the proteases, synergistic cleaning effects of trypsin and proteinase K on the inside of the tooth root canal were reported (Niazi et al., 2014, 2015). Nohno et al. (2012) reported that a tablet containing kiwi extract decreased oral malodor, and Ford (2008) demonstrated that pineapple juice removed tongue coating. Kiwi and pineapple possess a large number of cysteine proteases, actinidin and bromelain. In this study, we showed that the actinidin significantly reduced the tongue coating in elderly people. However, the effect of actinidin was not markedly greater than that of physically rubbing a tablet, and for young people, physical rubbing of a tablet may be more effective than proteases. Although actinidin in the tablet did not show remarkable effects, the TCI scores in this study were obtained using only a single intake of two tablets. Therefore, eating actinidin tablets frequently may lead to more effective reduction of the tongue coating. Moreover, because the salivary flow rate is known to be related to age (Ship, Pillemer, & Baum, 2002; Fox, van der Ven, Sonies, Weiffenbach, & Baum, 1985; Osterberg, Birkhed, Johansson, & Svanborg, 1992), the difference in the concentration and retention time of proteases between the two age groups might affect the TCI results.

Carica papaya extract, which contains benzyl-isothiocyanate, papain and calpain (Adebiyi, Ganesan Adaikan, & Prasad, 2003; Nakamura et al., 2007; Da Silva et al., 2010; Julianti, Oufir, & Hamburger, 2014), was reported to have disinfectant activity (Emeruwa, 1982; Dawkins, Hewitt, Wint, Obiefuna, & Wint, 2003). Brock, Arzabe, Piñeiro, and Olivito, 1977 showed the killing effect of trypsin. Conversely, neither papain nor trypsin showed any significant antimicrobial effect under our experimental conditions. Using mouthwash with intensive antimicrobial activity may lead to the perturbation of the oral flora in the oral cavity and may increase the chance of infection by opportunistic pathogens. Therefore, our results suggest that protease treatment using the appropriate concentration and reaction time can be used as an agent in routine clinical use. Although *in vitro* biofilm removal required a relatively high concentration of proteases, there are synergistic effects between the flow of saliva and mechanical rubbing of tablets in the oral environment. Determination of the enzyme, concentration and reaction time requires further analysis.

In this study, we showed the inhibitory effect of the proteases on oral biofilm formation. To prevent infection, safe and natural inhibitory agents with an inhibitory effect on biofilm formation and without an antimicrobial effect have been desired and developed. Therefore, these proteases may provide a preventive effect against oral plaque formation for routine oral care in healthy and elderly people. A more detailed analysis is necessary to evaluate these proteases for prevention of oral plaque accumulation.

## 5. Conclusion

This study provides direct evidence that actinidin, a cysteine protease, reduces the tongue coating in elderly subjects. Oral *Actinomyces* biofilm and reconstructed biofilm taken from a tongue coating were significantly reduced by the proteases papain, actinidin and trypsin *in*

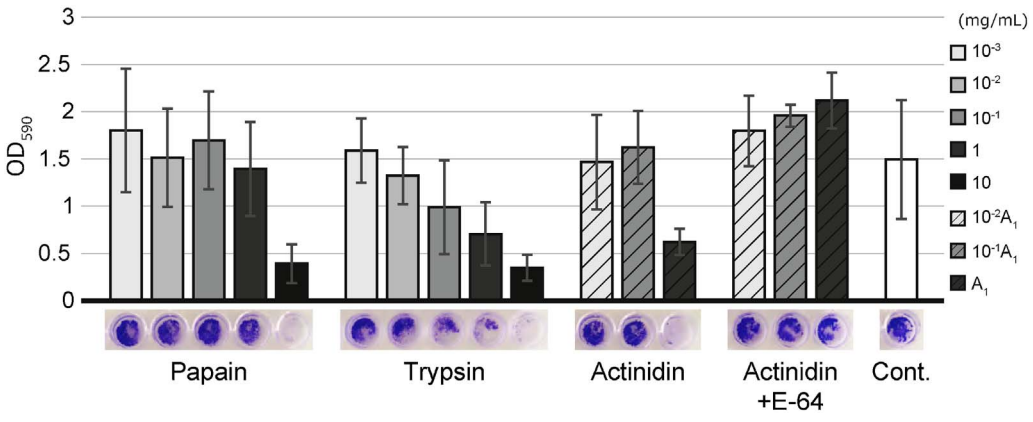


**Fig. 4.** Biofilm reconstructed from the tongue coating is affected by papain, trypsin or actinidin.
The amount of biofilm retained was determined as in Fig. 2. The values presented here are the means of at least three independent experiments and the error bars represent the standard deviation. The image is representative of three independent experiments.

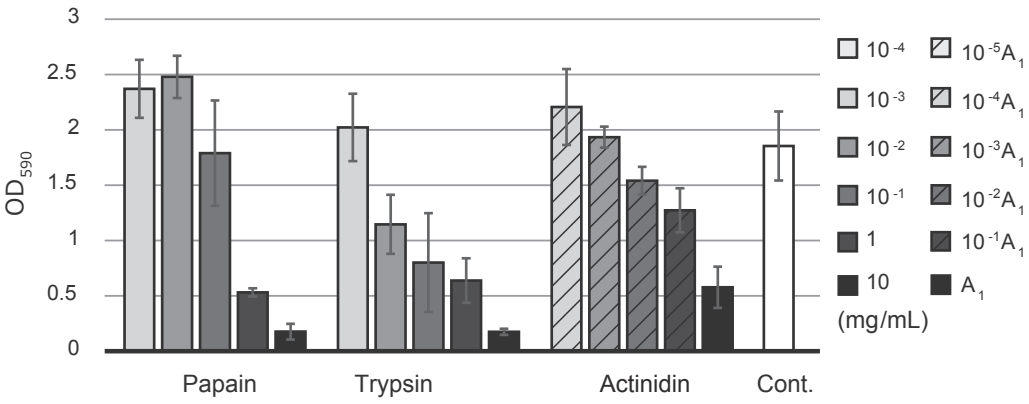


**Fig. 5.** Biofilm formation is affected by papain, trypsin and actinidin.
Growth assay results show decreased biofilm formation in the presence of papain, trypsin and actinidin. The amount of biofilm retained in the wells was determined as in Fig. 2. The values presented here are the means of at least three independent experiments and the error bars represent the standard deviation.

*vitro*. Additional results suggest that proteases digest fimbriae to disrupt the biofilm structure and also inhibit biofilm formation.

## Conflict of interest statement

This work was partially supported by Osaka Dental University (#14-2) and by JSPS KAKENHI grants (16K11469, 16K11876, and 16K11877). The authors declare that we have no conflicts of interest.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.archoralbio.2017.04.035.

## References

Abe, S., Ishihara, K., Adachi, M., & Okuda, K. (2008). 322 Tongue-coating as risk indicator for aspiration pneumonia in edentate elderly. *Archives of Gerontology and Geriatrics*, *47*, 267–275.
Adebiyi, A., Ganesan Adaikan, P., & Prasad, R. N. (2003). Tocolytic and toxic activity of papaya seed extract on isolated rat uterus. *Life Sciences*, *74*, 581–592.
Blom, T., Slot, D. E., Quirynen, M., & Van der Weijden, G. A. (2012). The effect of mouthrinses on oral malodor: A systematic review. *International Journal of Dental Hygiene*, *10*, 209–222.
Bollen, C. M., & Beikler, T. (2012). Halitosis: The multidisciplinary approach. *International Journal of Oral Science*, *4*, 55–63.
Brock, J. H., Arzabe, R., Piñeiro, A., & Olivito, A. M. (1977). The effect of trypsin and chymotrypsin on the bactericidal activity and specific antibody activity of bovine colostrum. *Immunology*, *32*, 207–213.
Cavaliere, R., Ball, J. L., Turnbull, L., & Whitchurch, C. B. (2014). The biofilm matrix destabilizers, EDTA and DNaseI: Enhance the susceptibility of nontypeable Hemophilus influenzae biofilms to treatment with ampicillin and ciprofloxacin. *Microbiology Open*, *3*, 557–567.
Chaignon, P., Sadovskaya, I., Ragunah, C., Ramasubbu, N., Kaplan, J. B., & Jabbouri, S. (2007). Susceptibility of staphylococcal biofilms to enzymatic treatments depends on their chemical composition. *Applied Microbiology and Biotechnology*, *75*, 125–132.
Christensen, G. J. (1998). Why clean your tongue? *The Journal of the American Dental Association*, *129*, 1605–1607.
Codipilly, D. P., Kaufman, H. W., & Kleinberg, I. (2004). Use of a novel group of oral malodor measurements to evaluate an anti-oral malodor mouthrinse (TriOralTM342) in humans. *The Journal of Clinical Dentistry*, *15*, 98–104.
Da Silva, C. R., Oliveira, M. B. N., Motta, E. S., De Almeida, G. S., Varanda, L. L., De Pádula, M., et al. (2010). Genotoxic and cytotoxic safety evaluation of papain (Carica papaya L.) using in vitro assays. *Journal of Biomedicine and Biotechnology*, 197898.
Dawkins, G., Hewitt, H., Wint, Y., Obiefuna, P. C. M., & Wint, B. (2003). Antibacterial effects of carica papaya fruit on common wound organisms. *The West Indian Medical Journal*, *52*, 290–292.
De Geest, S., Laleman, I., Teughels, W., Dekeyser, C., & Quirynen, M. (2000). Periodontal diseases as a source of halitosis: A review of the evidence and treatment approaches for dentists and dental hygienists. *Periodontology*, *71*, 213–227.
Di Bonaventura, G., Uriani, M., Fabbri, A., Flati, V., Martinotti, S., Pompilio, A., et al. (2012). A novel biotechnology product for the degradation of biofilm-associated polysaccharides produced by *Streptococcus mutans*. *Journal of Biological Regulators and Homeostatic Agents*, *26*, 1–7.
Emeruwa, A. C. (1982). Antibacterial substance from Carica papaya fruit extract. *Journal of Natural Products*, *45*, 123–127.
Erovic Ademovski, S., Lingstrom, P., Winkel, E., Tangerman, A., Persson, G. R., & Renvert, S. (2012). Comparison of different treatment modalities for oral halitosis. *Acta Odontologica Scandinavica*, *70*, 224–233.
Flemming, H. C., & Wingender, J. (2010). The biofilm matrix. Nature reviews. *Microbiology*, *8*, 623–633.
Ford, S. J. (2008). The importance and provision of oral hygiene in surgical patients. *International Journal of Surgery*, *6*, 418–419.
Fox, P. C., van der Ven, P. F., Sonies, B. C., Weiffenbach, J. M., & Baum, B. J. (1985). Xerostomia: Evaluation of a symptom of increasing significance. *The Journal of the American Dental Association*, *110*, 519–525.
Fredheim, E. G., Klingenberg, C., Rohde, H., Frankenberger, S., Gaustad, P., Flaegstad, T., et al. (2009). Biofilm formation by Staphylococcus haemolyticus. *Journal of Clinical Microbiology*, *47*, 1172–1180.
Gilan, I., & Sivan, A. (2013). Effect of proteases on biofilm formation of the plastic-degrading actinomycete Rhodococcus ruber C208. *FEMS Microbiology Letters*, *342*, 18–23.
Harris, L. G., Nigam, Y., Sawyer, J., Mack, D., & Pritchard, D. I. (2013). Lucilia sericata chymotrypsin disrupts protein adhesin-mediated staphylococcal biofilm formation. *Applied and Environmental Microbiology*, *79*, 1393–1395.
Howlin, R. P., Fabbri, S., Offin, D. G., Symonds, N., Kiang, K. S., Knee, R. J., et al. (2015). Removal of dental biofilms with an ultrasonically activated water stream. *Journal of Dental Research*, *94*, 1303–1309.
Julianti, T., Oufir, M., & Hamburger, M. (2014). Quantification of the antiplasmodial alkaloid carpaine in papaya (Carica papaya) leaves. *Planta Medica*, *80*, 1138–1142.
Kilian, M., Larsen, M. J., Fejerskov, O., & Thylstrup, A. (1979). Effects of fluoride on the initial colonization of teeth in vivo. *Caries Research*, *13*, 319–329.
Kumar, P. S. (2013). Oral microbiota and systemic disease. *Anaerobe*, *24*, 90–93.
Li, J., Helmerhorst, E. J., Leone, C. W., Troxler, R. F., Yaskell, T., Haffajee, A. D., et al. (2004). Identification of early microbial colonizers in human dental biofilm. *Journal of Applied Microbiology*, *97*, 1311–1318.
Meyle, E., Stroh, P., Günther, F., Hoppy-Tichy, T., Wagner, C., & Hänsch, G. M. (2010). Destruction of bacterial biofilms by polymorphonuclear neutrophils: Relative contribution of phagocytosis, DNA release: And degranulation. *The International Journal of Artificial Organs*, *33*, 608–620.
Miyazaki, H., Sakao, S., & Katoh, Y. (1995). Correlation between volatile sulphur compounds and certain oral health measurements in the general population. *Journal of Periodontology*, *66*, 679–687.
Nakamura, Y., Yoshimoto, M., Murata, Y., Shimoishi, Y., Asai, Y., Park, E. Y., et al. (2007). Papaya seed represents a rich source of biologically active isothiocyanate. *Journal of Agricultural and Food Chemistry*, *55*, 4407–4413.
Nakano, M., Shimizu, E., Wakabayashi, H., Yamauchi, K., & Abe, F. (2016). 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. *BioMed Central Oral Health*, *16*, 37.
Neeser, J. R., Chambaz, A., Del Vedovo, S., Prigent, M. J., & Guggenheim, B. (1988). Specific and nonspecific inhibition of adhesion of oral actinomyces and streptococci to erythrocytes and polystyrene by caseinoglycopeptide derivatives. *Infection and Immunity*, *56*, 3201–3208.
Niazi, S. A., Clark, D., Do, T., Gilbert, S. C., Foschi, F., Mannocci, F., et al. (2014). The effectiveness of enzymic irrigation in removing a nutrient-410 stressed endodontic multispecies biofilm. *International Endodontic Journal*, *47*, 756–768.
Niazi, S. A., Al-Ali, W. M., Patel, S., Foschi, F., & Mannocci, F. (2015). Synergistic effect of 2% chlorhexidine combined with proteolytic enzymes on biofilm disruption and killing. *International Endodontic Journal*, *48*, 1157–1167.
Nohno, K., Yamaga, T., Kaneko, N., & Miyazaki, H. (2012). Tablets containing a cysteine protease, actinidine, reduce oral malodor: A crossover study. *Journal of Breath Research*, *6*, 017107.
Nyvad, B., & Fejerskov, O. (1987). Scanning electron microscopy of early microbial colonization of human enamel and root surfaces in vivo. *Scandinavian Journal of Dental Research*, *95*, 287–296.
Osterberg, T., Birkhed, D., Johansson, C., & Svanborg, A. (1992). Longitudinal study of stimulated whole saliva in an elderly population. *Scandinavian Journal of Dental Research*, *100*, 340–345.
Pieralisi, N., de Souza Bonfim-Mendonca, P., Negri, M., Jarros, I. C., & Svidzinski, T. (2016). Tongue coating frequency and its colonization by yeasts in chronic kidney disease patients. *European Society of Clinical Microbiology*, *35*, 1455–1462.
Rahmani-Badi, A., Sepehr, S., & Babaie-Naiej, H. (2015). A combination of cis-2-decenoic acid and chlorhexidine removes dental plaque. *Archives of Oral Biology*, *60*, 1655–1661.

Ralph, W. J. (1987). Hygiene of the tongue. *Gerodontics*, *3*, 167–170.
Rice, K. C., Mann, E. E., Endres, J. L., Weiss, E. C., Cassat, J. E., Smeltzer, M. S., et al. (2007). The cidA murein hydrolase regulator contributes to DNA release and biofilm development in Staphylococcus aureus. *Proceedings of the National Academy of Sciences of the United States of America*, *104*, 8113–8118.
Rowley, E. J., Schuchman, L. C., Tishk, M. N., & Carlson, H. C. (1987). Tongue brushing versus tongue scraping. *Clinical Preventive Dentistry*, *9*, 13–16.
Shimizu, T., Ueda, T., & Sakurai, K. (2007). New method for evaluation of tongue-coating status. *Journal of Oral Rehabilitation*, *34*, 442–447.
Ship, J. A., Pillemer, S. R., & Baum, B. J. (2002). Xerostomia and the geriatric patient. *Journal of the American Geriatrics Society*, *50*, 535–543.
Singh, P. K., Parsek, M. R., Greenberg, E. P., & Welsh, M. J. (2002). A component of innate immunity prevents bacterial biofilm development. *Nature*, *417*, 552–555.
Slot, D. E., De Geest, S., van der Weijden, F. A., & Quirynen, M. (2015). Treatment of oral malodour: Medium-term efficacy of mechanical and/or chemical agents: A systematic review. *Journal of Clinical Periodontology*, *42*, 303–316.
Suzuki, N., Fujimoto, A., Yoneda, M., Watanabe, T., Hirofuji, T., & Hanioka, T. (2016). Resting salivary flow independently associated with oral malodor. *BMC Oral Health*, *17*, 23.
Tada, A., Nakayama-Imaohji, H., Yamasaki, H., Hasibul, K., Yoneda, S., Uchida, K., et al. (2016). Cleansing effect of acidic L-arginine on human oral biofilm. *BioMed Central Oral Health*, *16*, 40.
Takeshita, T., Tomioka, M., Shimazaki, Y., Matsuyama, M., Koyano, K., Matsuda, K., et al. (2010). Microfloral characterization of the tongue coating and associated risk for pneumonia-related health problems in institutionalized older adults. *Journal of the American Geriatrics Society*, *58*, 1050–1057.
Tonzetich, J., & Ng, S. K. (1976). Reduction of malodor by oral cleansing procedures. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics*, *42*, 172–181.
Tonzetich, J. (1977). Production and origin of oral malodor: A review of mechanisms and methods of analysis. *Journal of Periodontology*, *48*, 13–20.
Ueno, M., Takeuchi, S., Takehara, S., & Kawaguchi, Y. (2014). Saliva viscosity as a potential risk factor for oral malodor. *Acta Odontologica Scandinavica*, *72*, 1005–1009.
Van Tornout, M., Dadamio, J., Coucke, W., & Quirynen, M. (2013). Tongue coating: Related factors. *Journal of Clinical Periodontology*, *40*, 180–185.
Winkel, E. G., Roldán, S., Van Winkelhoff, A. J., Herrera, D., & Sanz, M. (2003). Clinical effects of a new mouthrinse containing chlorhexidine, cetylpyridinium chloride and zinc-lactate on oral halitosis. A dual-center: Double-blind placebo-controlled study. *Journal of Clinical Periodontology*, *30*, 300–306.
Yaegaki, K., & Sanada, K. (1992). Volatile sulfur compounds in mouth air from clinically healthy subjects and patients with periodontal disease. *Journal of Periodontal Research*, *27*, 233–238.