Original Article

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# Maillard Reaction Product of Rare Sugar Allulose Decreases Bacteria-derived and Chemically-prepared Hydrogen Sulfide

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**Abstract**

Purpose: Oral malodor, which concerns many people, is associated with volatile sulfur compounds (VSCs) produced by periodontopathic bacteria such as *Porphyromonas gingivalis*. Maillard products are used in the food industry to decrease hydrogen sulfide ($H_2S$), which disturbs the flavor and taste of food. In the present study, we planned to apply Maillard reaction products for reducing VSCs to meet the needs demand of patients who want to rapidly decrease oral malodor.

Methods: The effect of maple sugar solution (a Maillard reaction product) on VSC decrease was examined by adding it to sonicated extract of *P. gingivalis*. Next, the rare sugar allulose was used for the Maillard reaction (to generate Maillard-allulose), because allulose has no calories and does not cause dental caries. The effect of Maillard-allulose on VSC decrease was examined by adding it to *P. gingivalis*-derived and chemically-prepared $H_2S$. After 1 min, headspace air was collected and the concentration of $H_2S$ was measured using the Oral Chroma portable gas chromatography system. Separately, headspace air from a mixture of sodium mono-hydrogen sulfide and Maillard-allulose was analyzed by a solid phase micro extraction method with gas chromatography-mass spectrometry.

Results: Both maple sugar and Maillard-allulose decreased bacteria-derived and chemically-prepared $H_2S$. New material seemed to be generated after mixing sodium mono-hydrogen sulfide and Maillard-allulose, indicating that $H_2S$ may have bound to a component of Maillard-allulose, resulting in loss of volatility of $H_2S$.

Conclusion: Both maple sugar and Maillard-allulose had $H_2S$-decreasing activity. Maillard-allulose is not associated with dental caries and obesity, so it may be a good candidate for use in foods for decreasing oral malodor.

**Key words**: oral malodor, Maillard reaction, rare sugar, allulose

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## Introduction

Many people worry about oral malodor[1,2], and the number of such patients seems to be increasing because of the wearing of masks to prevent infection[3]. Oral malodor is associated with volatile sulfur compounds (VSCs) produced by periodontopathic bacteria such as *Porphyromonas gingivalis* and *Tannerella forsythia*[4]. These bacteria, which are included among the red complex bacteria[5], have high proteinase activity and degrade proteins in the oral cavity, resulting in VSC production[6].

To decrease oral malodor, maintenance of oral health by proper mouth cleaning is important. Mechanical plaque control, such as by using a toothbrush, interdental brush, dental floss, and tongue scraper, is the first line of prevention. Chemical plaque control, such as mouth rinse and dentifrice, used in addition to mechanical oral hygiene procedures, is helpful in decreasing oral infectious diseases. Many studies have supported significant plaque decrease by use of chemical plaque control measures[7]. However, the side effects and safety of these measures are often of concern. For example, chlorhexidine, the bactericidal agent that has been most studied and is recognized as the most effective for inhibiting plaque and preventing gingivitis, periodontitis, and oral malodor, has several adverse effects, including extrinsic tooth staining, calculus build-up, transient taste disturbance, and effects on the oral mucosa[8].

Thus, we have been examining safer methods to decrease oral malodor, one of which is the use of probiotics. Probiotics have traditionally been used to treat diseases related to the gastrointestinal tract. However, recently, many studies have investigated the effects of probiotic bacteria on oral health[9]. Several clinical trials have reported that regular consumption of *Lactobacillus salivarius* WB21 decreases periodontitis and oral malodor[10–13]. The use of oil drops containing *L. salivarius* WB21 resulted in an improvement in periodontal condition and a decrease in oral malodor in a randomized clinical trial[12]. A 14-day, double-blind, placebo-controlled, randomized crossover trial of tablets containing *L. salivarius* WB21 in patients with oral malodor resulted in a significant decrease in the concentration of VSCs and the average probing pocket depth in the probiotic period compared with the placebo period[13].

However, there is a weakness associated with probiotic treatment: it aims to improve the oral microbiota, so it takes time to obtain good results and does not help patients who want to rapidly decrease oral malodor. Thus, we added green tea catechins to the probiotic tablet[14]. The probiotic tablet containing green tea catechins was effective in controlling cariogenic and periodontopathic bacteria. It also lowered the concentration of methyl mercaptan, a VSC. However, although the VSC-decreasing effect was statistically significant, the change was not so drastic and patients may not notice the effect. Therefore, we tried another strategy of decreasing the amount of hydrogen sulfide ($H_2S$), which is another major VSC in oral malodor.

The Maillard reaction is an amino-carbonyl reaction. It is a non-enzymatic browning reaction, which plays an essential role in food processing to improve the appearance and functional properties of food[15]. The Maillard reaction product is also known to decrease $H_2S$ produced in the food industry; $H_2S$ disturbs the taste and flavor of food[15]. We considered using a Maillard reaction product, maple sugar, to decrease $H_2S$ in oral malodor. However, maple sugar may cause dental caries, so instead we considered using a rare sugar to control oral malodor."Rare sugar" is a comprehensive term for monosaccharides and sugar alcohols that are rare in nature. Strategies to produce rare sugars using epimerases have recently been reported by Kagawa University[16]. Rare sugars cannot be used by organisms for their metabolism; indeed, they are known to inhibit cariogenic bacteria[17]. Allulose is a rare sugar, and as an additional advantage, it is non-calorific, which is important for people who are concerned about their visceral fat or blood sugar level[18]. Allulose can be chemically produced by epimerization of D-fructose, and its price has decreased in recent years[19]. Therefore, we used allulose for the Maillard reaction to generate Maillard-allulose, aiming to decrease levels of $H_2S$.

## Materials and Methods

### 1. Bacterial strain and culture conditions

*P. gingivalis* ATCC 33277 was maintained on CDC anaerobic blood agar (Becton Dickinson, Cockeysville, MD, USA) in an anaerobic atmosphere (80% $N_2$, 10% $H_2$, 10% $CO_2$), and inoculated into tryptic soy broth

(Difco Laboratories, Detroit, MI, USA) supplemented with hemin (5 μg/m*l*, FUJIFILM Wako Pure Chemical Co., Osaka, Japan) and menadione (1 μg/m*l*, FUJIFILM Wako Pure Chemical Co.).

**2. Preparation of sonicated extracts of *P. gingivalis* cells**

Sonicated extracts (SE) of *P. gingivalis* cells were prepared as reported previously[20]. Briefly, *P. gingivalis* cells in the late logarithmic growth stage in tryptic soy broth were harvested by centrifugation and washed with phosphate-buffered saline (PBS). Five hundred micrograms of bacterial cells were suspended in 5 m*l* of PBS, and the cells were disrupted by sonication on ice. Intact cells were removed by centrifugation.

**3. Measurement of *P. gingivalis*-derived VSCs**

One milliliter of distilled water or maple sugar was added to 1 m*l* of *P. gingivalis* SE in a plastic tube with a soft cap, and vortexed at room temperature. After 1 min, the cap of the tube was penetrated with a needle attached to a plastic syringe, and the headspace air was collected. The level of $H_2S$ was measured using a portable gas chromatography system, Oral Chroma CHM-2 (Nissha FIS, Osaka, Japan)[21].

**4. Maillard reaction of allulose**

Allulose (final conc. 0.1 M; FitLane Nutrition, Conroe, TX, USA) was mixed with various amino acids (final conc. 0.1 M; FUJIFILM Wako Pure Chemical Co.) and the pH was adjusted to 9.0 with sodium hydroxide if the pH of the solution was $<$9.0. The solution was heated in a water bath at 95℃ for 90 min[22]. Completion of the Maillard reaction was confirmed by the browning of the allulose solution. The reaction product is referred to hereafter as Maillard-allulose.

**5. Decrease of *P. gingivalis*-derived $H_2S$ by maple sugar suspension and Maillard-allulose**

Maple sugar solution (0.3 g/m*l*; Maple-Farms Japan Co., Osaka, Japan) or Maillard-allulose solution was added to *P. gingivalis* SE suspension. After 1 min, headspace air was collected with a needle and the concentration of $H_2S$ was measured using the Oral Chroma CHM-2 apparatus.

**6. Treatment of chemically-prepared $H_2S$ by Maillard-allulose**

Chemically-prepared $H_2S$ was generated by dissolving $NaHS \cdot nH_2O$ (FUJIFILM Wako Pure Chemical Co.) in distilled water. It was diluted to $10^{-5}$%.

**7. Heat-concentration and dialysis of Maillard-allulose**

Maillard-allulose was concentrated by heating until the volume became half of the original. The concentrated Maillard-allulose solution was then dialyzed against distilled water using a Spectra/Por membrane (molecular weight cutoff 6–8,000; The Spectrum Companies, Charlotte, NC, USA), and reconcentrated to the original volume by heating.

**8. Experiment to assess the possible mechanism of $H_2S$ reduction by Maillard-allulose**

Headspace air from solution containing sodium mono-hydrogen sulfide, Maillard-allulose, or a mixture of sodium mono-hydrogen sulfide and Maillard-allulose was collected and adsorbed to Stableflex Fiber Carb/PDMS 85 μm (Merck KGaA, Darmstadt, Germany) by a solid phase micro extraction (SPME) method[23]. The air component of each sample was analyzed by gas chromatography-mass spectrometry (7890A GC system, Agilent Technologies Japan, Ltd., Tokyo, Japan) connected to a VF-WAXms (Agilent CP9205) column (Agilent Technologies Japan, Ltd.).

**9. Statistical analysis**

To analyze the decrease of $H_2S$ by maple sugar, Student's *t*-test was applied. To compare the decrease of $H_2S$ by various materials and test the homogeneity of variance, the Levene test was applied. Analysis of variance was also performed. For multiple comparison, Tukey's test was applied.

## Results

**1. Decrease of *P. gingivalis*-derived VSCs by maple sugar**

Three milliliters each of distilled water and either maple sugar suspension (final conc. 0.3 g/m*l*) or glucose suspension (final conc. 0.3 g/m*l*) were added to *P. gingivalis* SE suspension. After 1 min, the headspace air was collected and the $H_2S$ concentration was measured using the Oral Chroma system (Figure 1). Maple sugar suspension significantly decreased the $H_2S$ concentration in the *P. gingivalis* SE suspension tube ($p<0.05$).

Maple sugar decreased the concentration of $H_2S$ from just after addition of the maple sugar suspension. The $H_2S$ concentration then increased slightly over 40 min. The amount of $H_2S$ in the control started to decline after 30 min (Figure 2).

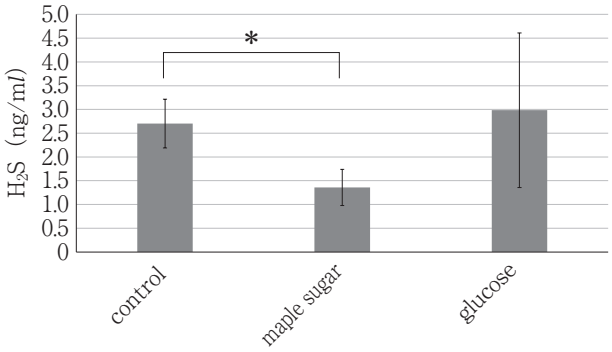


**Fig. 1** Effect of maple sugar solution on $H_2S$ concentration

An equal amount of maple sugar solution or glucose solution (0.3 g/m*l*) was added to *Porphyromonas gingivalis* sonicated extract suspension. After 1 min, headspace air was collected and the $H_2S$ concentration was measured using the Oral Chroma system. Maple sugar suspension significantly decreased the $H_2S$ concentration ($p<0.05$). Data show the means and standard deviations for three samples.

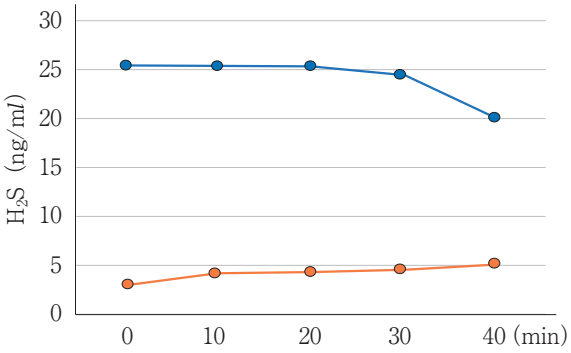


**Fig. 2** Time course of $H_2S$ concentration

An equal amount of maple sugar solution (0.3 g/m*l*) or distilled water was added to *P. gingivalis* sonicated extract suspension. At each time point, headspace air was collected and the $H_2S$ concentration was measured using the Oral Chroma system. The $H_2S$ concentration decreased just after addition of maple sugar suspension. The control $H_2S$ concentration (blue line) began to decrease after 30 min (no statistical analysis). The $H_2S$ concentration with maple sugar solution (orange line) was low but increased slightly over 40 min. The results shown are representative data for four similar sets of experimental data.

## 2. Maillard reaction of allulose

An illustration of Maillard-allulose is shown in Figure 3. A mixture of allulose and L-arginine was almost colorless. It became brown after heating at 95℃ for 90 min, and the Maillard reaction was considered to be completed.


**Fig. 3** Color change after Maillard reaction

Allulose (final conc. 0.1 M) was mixed with L-arginine (final conc. 0.1 M) and the pH was adjusted to 9.0 with sodium hydroxide. The solution was heated in a water bath at 95℃ for 90 min. The mixture of allulose and L-arginine was initially colorless (right), but after heating, it became brown (left), which indicates the completion of the Maillard reaction. The results shown are representative data for six similar sets of experimental data.

## 3. Combination with various amino acids

Various amino acids were mixed with allulose for the Maillard reaction. Among the 11 tested amino acids, the Maillard reaction product obtained using arginine had the greatest effect in decreasing the concentration of $H_2S$, and the amount of $H_2S$ became 0 in this experimental condition. L-serine, L-alanine, and L-cysteine had no $H_2S$-decreasing effect (Figure 4).

## 4. Confirmation of necessity of Maillard reaction for $H_2S$ reduction

Maillard-allulose produced with L-arginine showed a strong decreasing effect on $H_2S$, but an unheated mixture of allulose and L-arginine had no effect ($p<0.01$), indicating that the heating process (i.e., the Maillard reaction) is necessary for $H_2S$ decrease (Figure 5).

## 5. Effect of heat-concentration and dialysis on the effect of Maillard-allulose

Maillard-allulose was concentrated by heating. The concentrated Maillard-allulose showed a strong decreasing effect on the level of $H_2S$, indicating that the reagent was heat-stable. Even after dialysis against distilled water, it retained a strong effect (Figure 6).

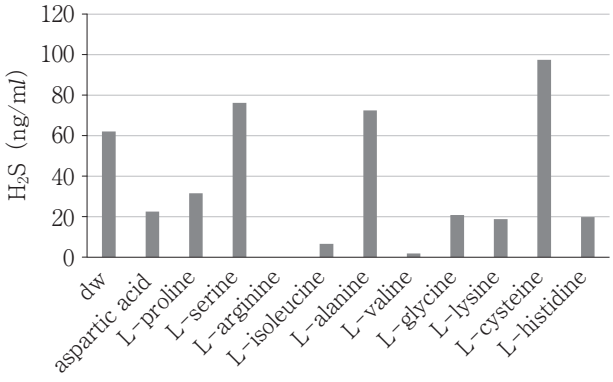


**Fig. 4** Effect of various amino acids on reduction of $H_2S$

Allulose (final conc. 0.1 M) was mixed with various amino acids (final conc. 0.1 M) and the pH was adjusted to 9.0 with sodium hydroxide if the pH of the solution was <9.0. The resulting solution was heated in a water bath at 95°C for 90 min. Among the 11 amino acids tested, the product with L-arginine showed the strongest reducing effect on $H_2S$ (no statistical analysis). The results shown are representative data for three similar sets of experimental data.

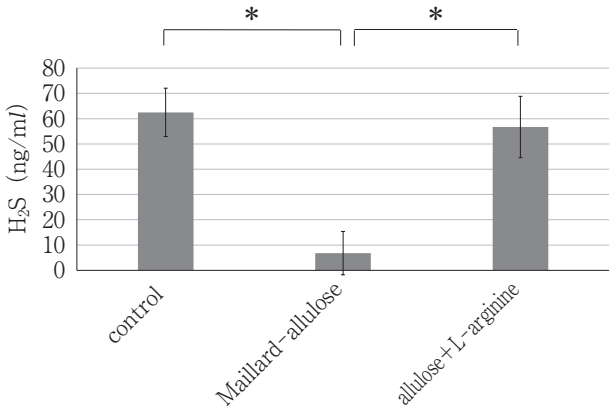


**Fig. 5** Effect of Maillard reaction on reduction of $H_2S$

Allulose (final conc. 0.1 M) was mixed with L-arginine (final conc. 0.1 M) and the pH was adjusted to 9.0 with sodium hydroxide. The solution was heated in a water bath at 95°C for 90 min. Control solution was not heated. Maillard-allulose significantly decreased the $H_2S$ concentration, but the unheated mixture of allulose and L-arginine had no effect, indicating that Maillard-allulose is necessary for the $H_2S$ reduction ($p < 0.01$). The data show the means and standard deviations for eight samples.

### 6. SPME-gas chromatography analysis

The results of SPME-gas chromatography-mass spectrometry analysis are shown in Figure 7. The peak patterns of $H_2S$ alone and Maillard-allulose were different. Then, $H_2S$ and Maillard-allulose were mixed and the headspace air was subjected to gas chromatography. The peak pattern of this mixture mostly looked like a mixture of the two individual peak patterns. However, a new big peak, which was above the scale limit, was generated at a retention time of around 21.6 min. An analysis of the retention time and a database search by Kumamoto Industrial Research Institute indicated that there was a 97% probability that the new peak was caffeine.

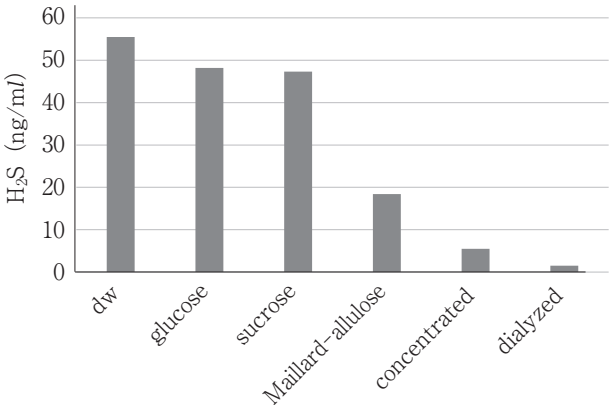


**Fig. 6** Effect of heat-concentration and dialysis of Maillard-allulose on decrease of $H_2S$

Maillard-allulose was concentrated by heating until the volume became half of the original. The concentrated Maillard-allulose was then dialyzed against distilled water, and reconcentrated to the original volume by heating. Heated Maillard-allulose decreased the concentration of $H_2S$, indicating that the effect was heat-stable. Dialysis had no effect on the activity, indicating that large molecules in the Maillard-allulose reaction mixture are responsible for $H_2S$ reduction (no statistical analysis). The results shown are representative data for four similar sets of experimental data.

## Discussion

The Maillard reaction is necessary in the food industry because it can decrease $H_2S$, which worsens the flavor of various foods. This fact prompted us to think of using a Maillard reaction product to decrease oral malodor. Maple sugar, which is a Maillard reaction product, showed a strong decreasing effect on $H_2S$, as we had expected. The effect was observed just after addition to sonicated bacterial extract. However, continuously taking maple sugar to decrease oral malodor could lead to

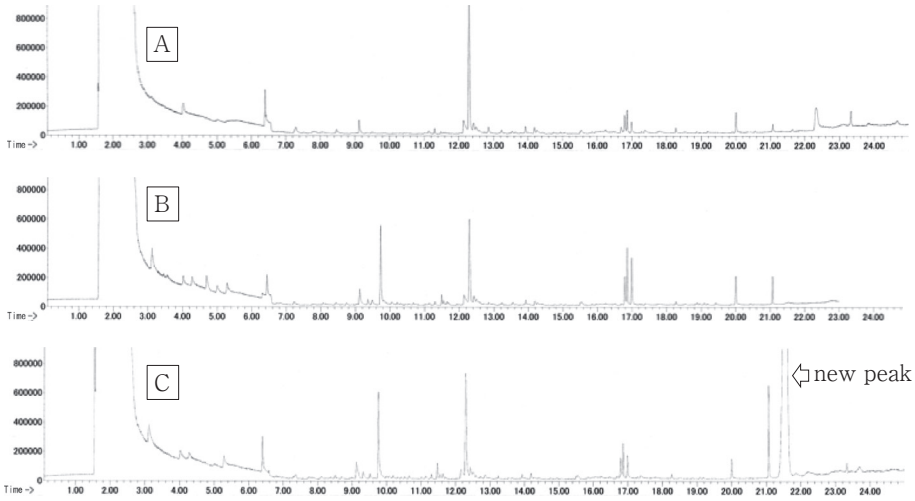


**Fig. 7** Solid phase micro extraction-gas chromatography-mass spectrometry analysis

Headspace air from solution containing sodium mono-hydrogen sulfide, Maillard-allulose, or a mixture of sodium mono-hydrogen sulfide and Maillard-allulose was collected and adsorbed to Stableflex Fiber Carb/PDMS 85 $\mu$m by a solid phase micro extraction method. The air component of each sample was analyzed by gas chromatography-mass spectrometry using a VF-WAXms column. A : Peak pattern of headspace air of solution of sodium mono-hydrogen sulfide alone. B : Peak pattern of headspace air of solution of Maillard-allulose alone. C : Peak pattern of headspace air of mixture of sodium mono-hydrogen sulfide and Maillard-allulose. The arrow indicates the new peak (above scale limit) that was generated on mixing sodium mono-hydrogen sulfide and Maillard-allulose.

dental caries and obesity, so it is not appropriate to use maple sugar for oral malodor control.

Next, we considered using a rare sugar instead of maple sugar to control oral malodor. Rare sugars are non-calorific[18] and do not cause dental caries[17]. Therefore, we used allulose for the Maillard reaction, to generate Maillard-allulose to decrease levels of $H_2S$.

The Maillard reaction is completed by mixing sugars and amino acids under alkaline conditions, followed by heating. The proportion of sugar and amino acid affects the viscosity of the reaction product, so we used conditions that did not become too sticky. Various kinds of amino acids may be used for the Maillard reaction, so we first determined the most suitable combination for decreasing $H_2S$. We tested inexpensive amino acids with a view to applying our approach clinically in the future. Among the 11 amino acids used in this experiment, the Maillard reaction product of L-arginine was found to be the most effective in decreasing the level of $H_2S$, so we used L-arginine for further experiments. The Maillard reaction was shown to be necessary, because simply mixing allulose and L-arginine without heating did not decrease $H_2S$. The $H_2S$ concentration of the *P. gingivalis* SE-maple sugar mixture was low, but it slightly increased over 40 min. The reason is not clear yet, but the binding between $H_2S$ and maple sugar may have been reversible; further study is required.

To clarify the mechanism of $H_2S$ reduction, we performed SPME-gas chromatography-mass spectrometry experiments[23]. Headspace air from samples containing $H_2S$ alone and Maillard-allulose alone showed different peak patterns. The peak pattern of the mixture of $H_2S$ and Maillard-allulose mostly looked like a mixture of those two peak patterns, but an intense new big peak was generated when $H_2S$ and Maillard-allulose were mixed. Analysis of the retention time and a database search by Kumamoto Industrial Research Institute indicated that there was a 97% probability that the new peak was caffeine. These results indicate that some molecule in Maillard-allulose combined with $H_2S$ to form a new product. By combining with this molecule, $H_2S$ is considered to have lost its volatility. To confirm this

hypothesis, identification of the putative caffeine-like reaction product is under way.

This work has some limitations. Even in the controls, the level of $H_2S$ began to decrease during the assay, due to evaporation. The Oral Chroma measurement took >8 min for each assay. Thus, we could not repeat assays many times and could not present standard deviations in some experiments. To overcome this problem, we repeated each experiment under the same conditions and confirmed reproducibility. We need to find new assay methods that allow quicker measurement.

Oral malodor sometimes affects patients' quality of life and disturbs communication with others[24,25]. Some patients use antibiotics and disinfectants to decrease oral malodor[26], and this can be effective for a time. Periodontitis is caused by infection with periodontal pathogens, but periodontal tissue damage is thought to be caused by various factors[27]. Continuous use of disinfectants may give rise to side effects such as bacterial resistance and microbial substitution[28]. Thus, we are focusing on non-disinfectant control of oral malodor. In our previous experiments, the use of probiotics was found to improve the oral microbiota and decrease oral malodor, because oral malodor is strongly associated with the oral microbiota[29,30]. Gut microbiota are also related to oral conditions, including oral malodor[31,32]. However, improvement of the oral microbiota by using probiotics is slow. Zinc ions are also known to effectively decrease oral malodor[33], but care is required to avoid excessive intake.

Maillard-allulose has a good taste and little effect on obesity[34] or caries formation. If the new method developed here using Maillard-allulose to decrease levels of $H_2S$ is successful in clinical application, oral malodor could be decreased instantly. We are also considering combining Maillard-allulose and probiotics, which would contribute to both improvement of the oral microbiota and rapid decrease of oral malodor.

## Conclusions

Maple sugar solution decreased *P. gingivalis*-derived $H_2S$. The Maillard reaction product with rare sugar allulose decreased *P. gingivalis*-derived $H_2S$ and chemically-prepared $H_2S$. Among various amino acids, the Maillard reaction product with L-arginine was most effective in decreasing the concentration of $H_2S$. The Maillard reaction was required to achieve this effect because an unheated mixture of allulose and L-arginine did not decrease $H_2S$. New material may have been generated after mixing sodium mono-hydrogen sulfide and Maillard-allulose, indicating that some molecule in Maillard-allulose combined with $H_2S$ to form a new product, which reduced the volatility of $H_2S$. From these results, Maillard-allulose may be a good candidate for use in foods for decreasing oral malodor.

### Conflict of Interest

The authors declare that there is no conflict of interest in this study.

### Ethical Statement

Not applicable.

### Acknowledgments

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