

# 学位論文

D-Tagatose Effectively Reduces the Number of Streptococcus mutans and Oral Bacteria in Healthy Adult Subjects: a Chewing gum Pilot Study and Randomized Clinical Trial

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Original Article

## D-Tagatose Effectively Reduces the Number of *Streptococcus mutans* and Oral Bacteria in Healthy Adult Subjects: A Chewing Gum Pilot Study and Randomized Clinical Trial

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We examined the effect of D-Tagatose on the growth of oral bacteria including *Streptococcus mutans* (*S. mutans*). Saliva collected from 10 healthy volunteers was plated on BHI medium (to culture total oral bacteria) and MBS medium (to culture *S. mutans*, specifically). Agar plates of BHI or MBS containing xylitol or D-Tagatose were cultured under aerobic or anaerobic conditions. We then counted the number of colonies. In BHI plates containing D-Tagatose, a complete and significant reduction of bacteria occurred under both aerobic and anaerobic conditions. In MSB medium, significant reduction of *S. mutans* was also observed. We then performed a double-blind parallel randomized trial with 19 healthy volunteers. They chewed gum containing xylitol, D-Tagatose, or both for 4 weeks, and their saliva was collected weekly and plated on BHI and MSB media. These plates were cultured under anaerobic conditions. Total bacteria and *S. mutans* were not effectively reduced in either the D-Tagatose or xylitol gum group. However, *S. mutans* was significantly reduced in volunteers chewing gum containing both D-Tagatose and xylitol. Thus, D-Tagatose inhibited the growth of *S. mutans* and many types of oral bacteria, indicating that D-Tagatose intake may help prevent dental caries, periodontitis, and many oral diseases.

**Key words:** D-Tagatose, xylitol, *Streptococcus mutans*, oral bacteria, chewing gum

A 2012 study estimated that approx. 35% of permanent teeth and 9% of primary teeth have dental caries [1]. The main risk factor for dental caries is our daily dietary intake of sugars, generally called “free sugars”—especially sucrose. The anaerobic, gram-positive coccus *Streptococcus mutans* uses sucrose as a nutrient source, in part for the production of intracel-

lular storage components, but also for assembling extracellular glucans via the glucosyltransferases (GTFs) [2]. Extracellular glucans are the main factor promoting the adhesion of *S. mutans* to the surface of teeth, and they form an oral biofilm commonly known as dental plaque. This production of an oral biofilm is an important step in the initiation of dental caries. Non-mutans streptococci are the first actor in the early stage of the colonization associated with other species of bacteria,

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including *Actinomyces* [2]; they attach to the tooth surface with salivary pellicles and form an aggregated mass. The acid production at this stage may be neutralized by equilibrium of the demineralization/remineralization balance [3].

"Rare sugars" are defined as monosaccharides and their derivatives, which exist in only very small amounts in the natural world. A technique to produce rare sugars in large quantities was developed at Kagawa University, Japan. One of the rare sugars, xylitol, has been considered an effective dental caries inhibitory agent since the Turku sugar studies in the mid-1970s [4, 5]. Xylitol has been shown to decrease salivary mutans as a long-term effect [6-8], but a study of xylitol in a short-term trial did not identify any beneficial effects [6]. There are few reports about anti-caries factors of rare sugars other than xylitol.

Our *in vitro* study revealed that the rare natural hexo-*ketose* D-Tagatose significantly inhibited the growth of the GS5 strain of *S. mutans*, and we observed potent inhibitory effects of D-Tagatose on the acid production and water-insoluble glucan synthesis of *S. mutans* GS5 [2]. D-Tagatose also inhibits biofilm formation. The inhibitory effects of D-Tagatose on the growth of *S. mutans* strains other than GS5 and other oral bacteria have not been determined. Figure 1 shows the chemical structures of D-Tagatose and xylitol.

Many oral hygiene agents have been discussed in the field of dental science. Some of these agents can prevent

tooth decay, either by preventing biofilm formation or by exerting cleansing effects on oral biofilms [3]. Antibacterial agents are also an option. Despite the use of such tools, there is always a chance of the development of bacterial resistance. We focused on *S. mutans* activity in the present study because this bacterium plays a vital role not only in dental caries but also in periodontitis and other oral diseases that start with oral biofilm formation.

The use of chewing gum containing different compounds has become a frequent topic of study in preventive and therapeutic oral science. The wide range of chewing-gum interventions includes the use of different sugars, sugar alcohols, teas, polyphenols, volatile sulphur compounds (VSCs), cranberry extract, and herbal compounds [9-11]. Chewing gums containing test sugars have been the most widely accepted among trial subjects. Gums that contain xylitol have been studied under different conditions for varying durations, and the findings have been diverse. The effects of xylitol chewing gum have been examined in a wide range of contexts, including for patients with dental caries, acute otitis media, and type 2 diabetes, or as a component of postoperative therapy after bowel surgery [12].

In the short-term study cited above, the use of xylitol chewing gum did not change the salivary mutans level [6]. A systematic review also found that there was insufficient evidence of direct therapeutic effects from xylitol chewing gum [13]. As the lack of a supply of sucrose results in an inability to produce acid, chewing gum containing polyols has been suggested as a potential source of non-cariogenic sugars [14]. Moreover, the roles of xylitol-containing products or xylitol alone in the prevention of dental caries have been quite clear and established [15].

In previous *in vitro* studies, the potential, feasibility, and intervention implementation of chewing gum containing these sugars were assessed. We conducted the present double-blind randomized pilot study to observe oral microbial changes after 4 weeks of using gum with different compounds. Chewing gum was chosen because of its convenience and extensive exposure in the oral cavity.

## Materials and Methods

**Sugars.** Xylitol is already widely consumed as an oral health care product. Structurally, D-Tagatose is a

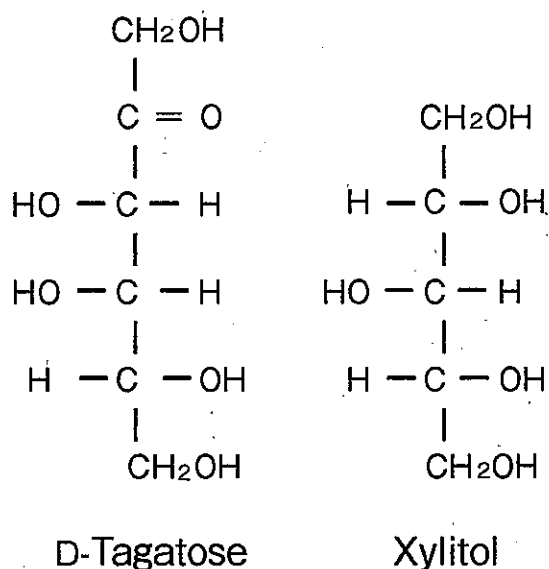


Fig. 1 Chemical structures of D-Tagatose and xylitol.

keto-hexose that is very different from xylitol (Fig. 1). D-Tagatose has been confirmed as a safe food ingredient for human health [16]. The US Food and Drug Administration (FDA) approved D-Tagatose as a food additive in October 2003, and designated it as generally recognized as safe (GRAS). Xylitol and D-Tagatose were supplied by Matsutani Chemical Co. (Itami, Japan).

**Culturing oral bacteria of saliva on BHI and MSB media.** Preparation of the agar plate media with added D-Tagatose and xylitol

For our investigation of the changes in the growth of oral bacteria in saliva, we used brain heart infusion (BHI; Eiken Chemical Co., Tokyo) agar plate medium for the cultures of total bacteria. For the selective cultures of *S. mutans*, we used Mitis Salivarius Agar plate medium (Becton Dickinson, Lincoln Park, NJ, USA) containing 100 µg/ml of bacitracin (MSB). D-Tagatose or xylitol were added to these media before they solidified. We prepared 4 types of media in both BHI and MSB: (1) control medium, with no added xylitol or D-Tagatose; and media with (2) 5.0% D-Tagatose added; (3) 5.0% xylitol added; and (4) 2.5% D-Tagatose and 2.5% xylitol added.

**Saliva collection from subjects and oral bacteria cultures.**

#### 1. Subjects

A random set of healthy volunteers was recruited from among the Faculty of Medicine, Kagawa University, Japan (2014-2016). The volunteers who met eligibility criteria and passed an oral bacterial screening to confirm moderate oral hygiene status were accepted. Some volunteers were excluded during the eligibility screening due to their low number of salivary *S. mutans*. The minimum detection limit for salivary *S. mutans* was set at above  $2 \times \log$  CFU/ml after plate culture. Volunteers with salivary samples below that number were excluded. The final group of subjects was 10 healthy adult volunteers (3 males, 7 females, age range 22-61 years, mean 35.4 years) from Kagawa University.

#### 2. Culturing and counting

We counted the number of total bacteria colonies and number of *S. mutans* colonies in saliva samples in standard agar plate cultures using BHI or MSB medium, respectively. To facilitate bacterial growth, the plates were pre-incubated anaerobically at 37°C for 24 h before the seeding of saliva. Anaerobic cultivation was performed with an Anaeropack anaerobic cultivation system (Mitsubishi Gas Chemical Co., Tokyo).

The saliva of each subject was collected as follows: the subject chewed paraffin gum for 5 min and then spat saliva into a sterile cup. The saliva sample was then immediately diluted with phosphate-buffered saline (PBS), divided for addition to each diluting pattern, plated on BHI and MSB agar plate media, and then incubated under anaerobic or aerobic conditions. We prepared diluting patterns to count the number of colonies, because the number of bacteria in the saliva of each volunteer differed. We incubated the BHI medium for 48 h at 37°C and the MSB medium for 72 h at 37°C.

After incubation, each plate of diluting patterns was checked, and the number of total bacteria colonies or *S. mutans* colonies on each plate was counted. Colonies were counted as colony forming units (CFU) of log phase bacteria. The characteristics of *S. mutans* colonies on MSB agar plates were identified and confirmed under a stereomicroscope. To obtain consistent colony data, the same researcher counted and identified all of the plates' colonies. After the counting was completed, the numbers of colonies were converted into CFU/ml, and compared with the corresponding numbers in control cultures.

**The double-blind, parallel randomized controlled trial.**

We also conducted a double-blind, parallel randomized controlled trial, which is presented as a flowchart in Fig. 2. A total of 19 healthy adult volunteers completed this study; their ages ranged from 21-49 years (mean, 30.0 years). We divided the subjects into three different gum groups: gum containing D-Tagatose (n=6), gum containing xylitol (n=7), and gum containing D-Tagatose and xylitol (1:1) (n=6). The individuals were asked to chew two tablets of the gum three times a day (for  $\geq 10$  min each after breakfast, lunch, and dinner) for 4 weeks. This study was conducted randomly among healthy volunteers from the Faculty of Medicine, Kagawa University, Japan (from 2014-2016). Ethical approval for the study was provided by the university's institutional review board (IRB), approval no. H24-085. This study has been registered in the Clinical Trial Registry (UMIN-CTR) (UMIN000026233).

#### 1. Subjects

Volunteers who fulfilled the eligibility criteria and passed an oral bacterial screening confirming their moderate oral hygiene status were examined. Forty-seven volunteers were screened. Some subjects were excluded during eligibility checking due to low numbers

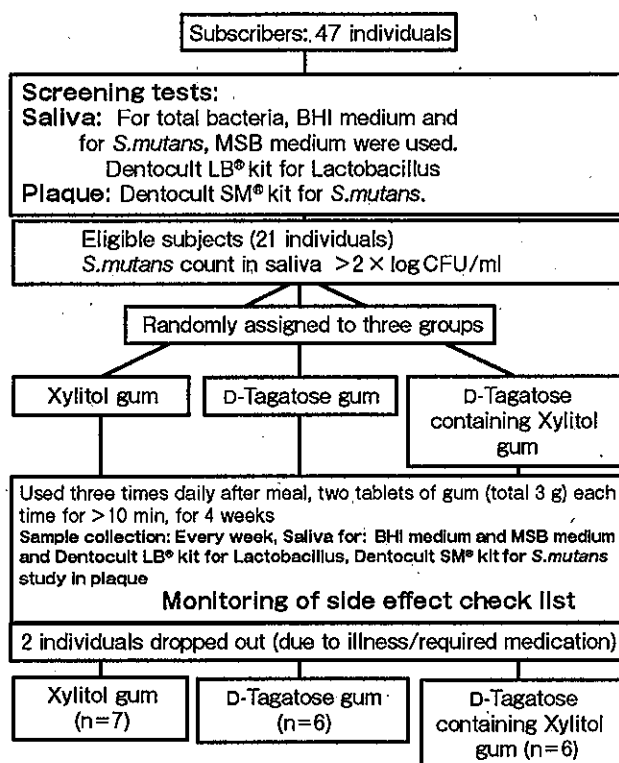


Fig. 2 Study flow chart.

of salivary *S. mutans*; the minimum detection limit for salivary *S. mutans* was set at above  $2 \times \log$  CFU/ml after plate culture, and subjects with salivary samples below that number were excluded. A final total of 21 subjects was randomized into 3 different gum groups for the investigation. Two subjects did not complete the trial due to illness or a medication requirement, and they were deemed dropouts. The inclusion criteria were absence of (i) any underlying illness, (ii) any antibiotic or immunosuppressive drug treatment in the past 3 months, (iii) increased body temperature, (iv) edentulous jaw, and (v) use of any dentures.

## 2. Screening and eligibility checking

Total bacteria and *S. mutans* in saliva samples were counted by standard agar plate cultures using BHI and MSB medium, respectively. The plates were pre-incubated anaerobically at 37°C for 24 h before the seeding of saliva to facilitate the bacterial growth. Anaerobic cultivation was performed with an Anaeropack system. Dentocult® LB and Dentocult® SM (Orion Diagnostica, Espoo, Finland) test kits were used to examine the subjects' saliva and plaque, respectively.

## 3. Randomization and blinding

A staff member who was not the examiner supplied each subject with one of the 3 types of gum. The gum was discriminated by three different color marks, and only the staff member (not the examiner or the subject) knew which gum preparation was provided to the subject. Subjects were asked not to discuss the gum with the examiner or others. The color code was only disclosed after the evaluation measures were completed. To ensure good comprehension, all subjects were given a demonstration regarding the trial details including the gums, the study background and materials, and the study plan and procedures. In addition, a set of self-assessment questionnaires was given to each subject for his or her assessment of the gum-chewing experience. The questionnaires assessed 18 different parameters, including circulation, hypersensitivity, digestive problems, skin problems, and others. Written consent to participate in the study was also obtained at this stage. To obtain unbiased results, all data were summarized after the completed trial, although saliva and plaque samples were collected and processed weekly.

## 4. Preparation of chewing gum containing D-Tagatose and/or xylitol

Three different types of gum were prepared in tablet form: one containing 1.5 g of D-Tagatose, one with 1.5 g of xylitol, and one with 0.75 g of D-Tagatose + 0.75 g of xylitol. A total of 9 g of sugar-containing chewing gum was packed for a single day (3 g for each of the three post-meal times). Table 1 lists the chewing gum ingredients. All of the chewing gum was produced, packed, and supplied by Matsutani Chemical Co. (Itami, Japan). These chewing gums were specially prepared for this study, and are not commercially available.

## 5. Main outcome measures: Sample collection

Approximately 10 ml of saliva was collected at week 0 (baseline) and weeks 1, 2, 3, and 4; thus, a total of five samples were collected from each individual during the 4-week trial. Before the saliva collection, a paraffin gum base was given to the subject to chew in order to stimulate salivary secretion. Although the salivary flow differed among the subjects, no significant reduction of salivary secretion was noted. The saliva was collected in Iwaki centrifuge tubes with triple-seal caps (Asahi Glass Co., Tokyo). All of the subjects needed approx. 15-25 min to accumulate 10 ml of saliva. Collected saliva (100 µl) was diluted immediately for seeding on BHI and MSB agar plates. A volume ( $\leq 3$  ml) of saliva

Table 1 Composition of the three types of chewing gum, in percentages

Material	Weight ratio (%)	Gum ingredients
Ketohexose/Rare sugar	61.5	1. Xylitol or 2. D-Tagatose or 3. D-Tagatose (50%) + Xylitol (50%)
Gum base	30.0	Regular type
Syrup	5.0	Water, maltitol, sorbitol et al.
Glycerin	1.0	Softener
Aspartame	0.5	Taste persistent material
Peppermint oil	1.0	Flavoring agent
Menthol powder	1.0	Flavoring agent
Total	100.0	

was also used for the Dentocult LB kit.

On the same days as the saliva collection, dental plaque was collected from the cervical area-buccal surface of each subject's mandibular right first molar. If notable plaque was absent in any case, an additional collection was made from the proximal area of that molar. Plaque was collected from the mandibular left first molar if the mandibular right first molar was absent. None of the subjects were missing both molars. The subjects maintained their normal daily oral hygiene routines throughout the 4-week trial.

The majority of the saliva samples were collected in the morning to ensure an adequate salivary flow. Discrepancies in the sample collection were limited by having only one blinded researcher collect all of the samples.

#### 6. Sample processing

After the collection of baseline samples, the saliva was diluted with normal saline (0.85%) by serial dilution up to  $1 \times 10^{-6}$ . Subsequently, the appropriate dilutions of saliva (0.1 ml each) were seeded on BHI and MSB agar plates. For total bacteria, three different dilutions of saliva were seeded on BHI agar plates, which were then incubated for 24 h anaerobically at 37°C. For *S. mutans*, two different dilutions of saliva were seeded on MSB agar plates, which were incubated for 96 h anaerobically at 37°C.

After incubation, the numbers of colonies that had grown on the plates were counted as colony forming units (CFU) of log phase bacteria. The characteristics of *S. mutans* colonies on the MSB agar plates were identified and confirmed under a stereomicroscope. To obtain consistent colony data, the same blinded researcher

identified and counted the plate colonies. After the 4-week trial was completed, the colony numbers were converted into CFU/ml.

The Dentocult LB<sup>®</sup> kit was used to determine the number of Lactobacillus in saliva as CFU/ml, according to the manufacturer's instructions. After a 96-h incubation, the Lactobacillus level in saliva was scored using the standard chart provided by the manufacturer: -, 1,000 CFU/ml; +, 10,000 CFU/ml; ++, 100,000 CFU/ml; and +++, 1,000,000 CFU/ml (Table 2).

A Dentocult SM<sup>®</sup> strip was used to score the *S. mutans* detected in subjects' plaque samples. Bacitracin tablets were added to SM<sup>®</sup> kit media 15 min before the plaque collection. SM<sup>®</sup> media with strips were incubated anaerobically for  $\geq 48$  h at 37°C. After incubation, the plaque-generating scores were obtained (Table 3). The *S. mutans* colonies grown from the plaque sample of each subject were also scored as -, +, ++ or +++.

#### 7. Statistical methods

All data calculations were performed using StatFlex software (version 6.0; Artech Co., Tokyo). Continuous variables were shown as average  $\pm$  standard deviation (SD), and differences between 2 groups were analyzed with Student's *t*-test.

Analysis of the double-blind parallel randomized trial was used to compare the means of each of the 2 groups. Dunnett's test was applied to compare the test groups with the control group. Dentocult<sup>®</sup> LB and SM scoring between 2 groups were compared using the chi-squared test.

Values of  $p < 0.05$  were considered to indicate a statistically significant difference.

**Ethical approval.** All procedures performed in

studies involving human subjects were in accordance with the ethical standards of the institutional review committee and the Helsinki Declaration as revised in 2013. The experimental protocol for this study was approved by the ethics committee of the Faculty of Medicine, Kagawa University (IRB ID: H24-085). CONSORT statement extension 2016 was applied, and written informed consent to participate was obtained from each subject in this study.

## Results

**Culturing oral bacteria of saliva on BHI and MSB media.** In the BHI medium, we observed significant reductions in the total bacterium count in the xylitol, 5.0% D-Tagatose, and 2.5% D-Tagatose+2.5% xylitol cultures (hereafter, “xylitol,” “D-Tagatose”, and “D-Tagatose+xylitol”) compared to the control cultures under both aerobic and anaerobic conditions (Fig. 3). Notably, the suppression of the total bacterium count provided by D-Tagatose or by D-Tagatose+xylitol was significantly stronger than that afforded by xylitol ( $p < 0.001$ ) under both aerobic and anaerobic conditions.

In the MSB medium, a significant reduction of *S. mutans* was produced by xylitol, D-Tagatose, and D-Tagatose+xylitol under both aerobic and anaerobic conditions compared to the control (Fig. 4). The sup-

pressions of *S. mutans* by D-Tagatose and by D-Tagatose+xylitol were significantly stronger than that shown by xylitol ( $p < 0.05$ ) under both aerobic and anaerobic conditions.

**The double-blind parallel randomized trial.** The presence/absence of side effects among the subjects was evaluated by a set of self-assessment questionnaires that was given at the beginning and end of the gum-chewing trial. According to the 19 subjects' completed questionnaires, no side effects occurred. There were no complaints of diarrhea or abdominal upset. Moreover, the subjects' body weights did not change from the baseline to the end of the 4-week trial, confirming that the subjects' appetite for food was not altered by the tested sugars.

The total salivary bacterial count was compared between week 0 and after 4 weeks of chewing the gum. There were no significant changes in the count across time in any group (Fig. 5), but a nonsignificant tendency of a reduction in the total salivary bacterial count was observed in each of the individual gum groups. Among the three groups, the D-Tagatose group showed the greatest response; from week 0 to week 4, the count difference was approx.  $0.5 \times \log$  CFU/ml. The other two gum groups maintained the same approximate total count, which was  $\sim 8 \times \log$  CFU/ml.

We then checked the total salivary *S. mutans* count in these groups. In the xylitol group, the salivary *S. mutans*

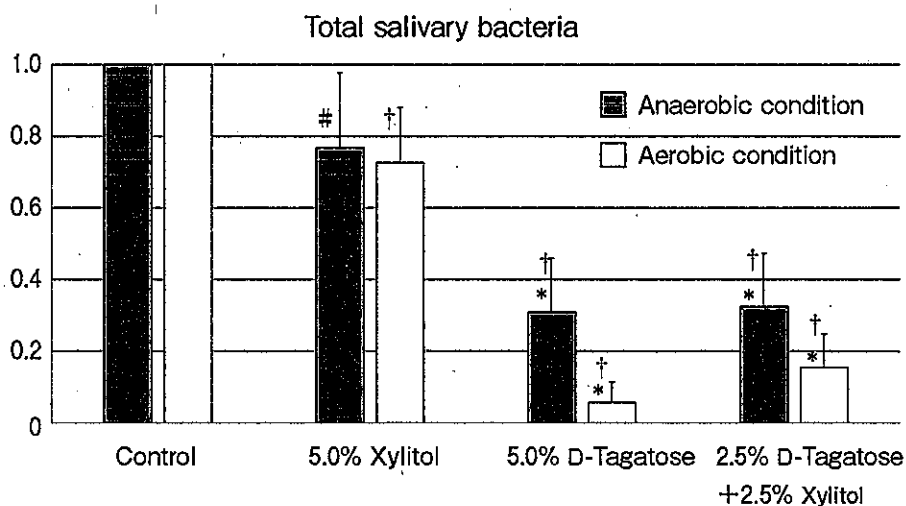


Fig. 3 Salivary total bacterial numbers compared to controls after culture in BHI medium. Total bacteria in the saliva of 10 healthy adult volunteers were cultured in BHI medium with added D-Tagatose, xylitol, or D-Tagatose+xylitol under anaerobic or aerobic conditions. The number of colonies was counted 48 h later. The number of colonies in each condition compared to the Control (1.0) is presented. † $p < 0.001$ , # $p < 0.05$  vs. Control. \* $p < 0.001$  vs. the xylitol group, by Student's *t*-test.

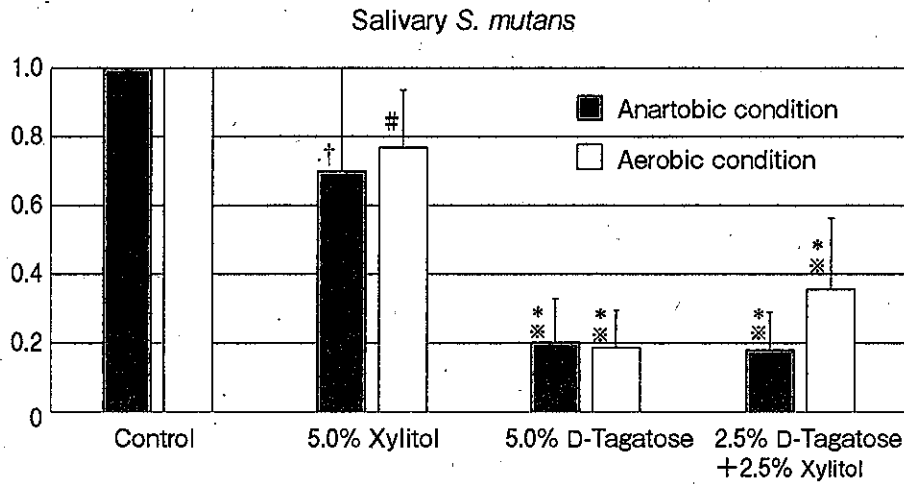


Fig. 4 Ratio of salivary *S. mutans* number compared to the control after culture in MSB medium. *S. mutans* in the saliva of 10 healthy adults were cultured in MSB medium to which D-Tagatose, xylitol, or D-Tagatose+xylitol was added. MSB medium was used because it specifically cultures *S. mutans* under both anaerobic and aerobic conditions. The number of colonies was counted at 72 h of culture. Data are the number of colonies compared to Control (1.0). † $p < 0.05$ , # $p < 0.005$ , \* $p < 0.001$  vs. Control. \* $p < 0.05$  vs. the xylitol group. Student's *t*-test was used for all comparisons.

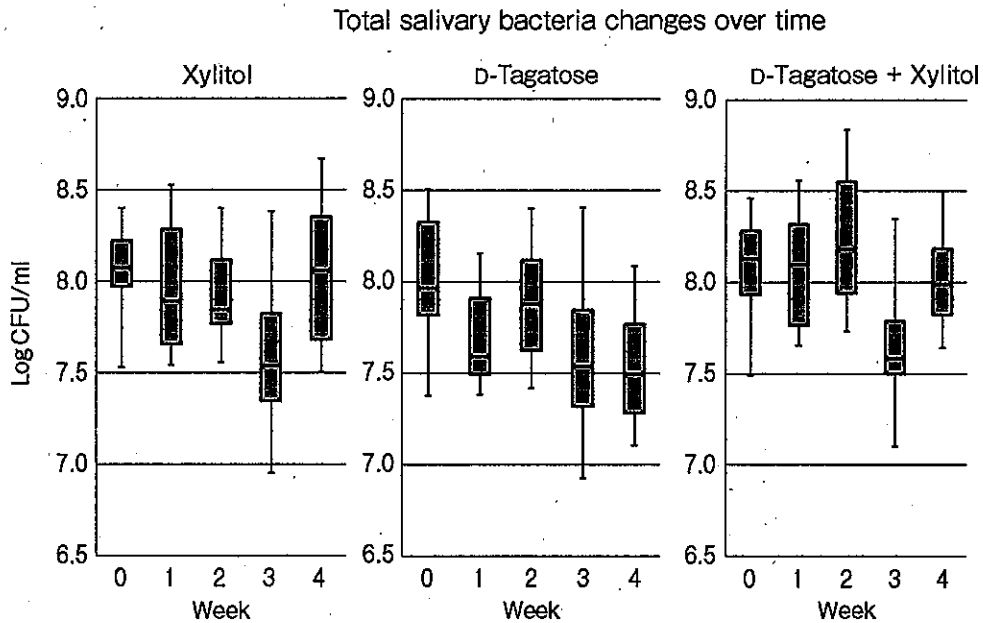


Fig. 5 Periodic changes of the salivary total bacterial number after chewing the gum containing the indicated sugars. There was a downward trend in total bacterial number for each type of gum. Differences were analyzed by two-way repeated ANOVA.

count changed from  $2 \times \log$  CFU/ml at week 0 to  $6 \times \log$  CFU/ml at week 4. In the D-Tagatose group, the salivary *S. mutans* count changed from  $2 \times \log$  CFU/ml to  $5 \times \log$  CFU/ml. Interestingly, in the D-Tagatose+xylitol gum group, the salivary *S. mutans* count declined

from  $6 \times \log$  CFU/ml to  $3 \times \log$  CFU/ml (maximum response). Even 3 weeks' use of the D-Tagatose+xylitol chewing gum resulted in a significant reduction of the salivary *S. mutans* count. The weekly increments in *S. mutans* counts are shown in Fig. 6. The reduction of *S.*



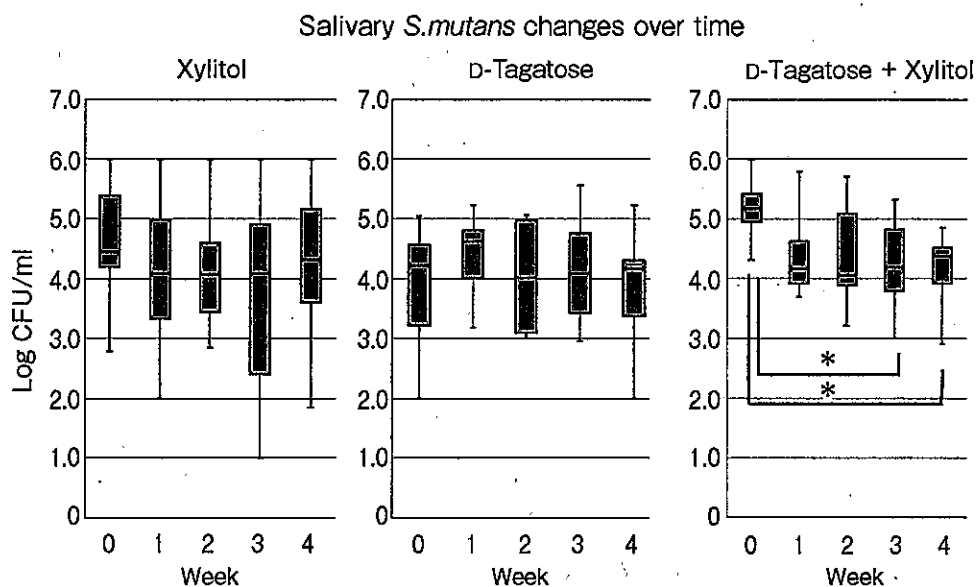


Fig. 6 Periodic changes of salivary *S. mutans* number after culture in MSB medium. We observed a tendency of *S. mutans* reduction after week 1. A significant difference was observed at weeks 3 and 4 of gum chewing. Differences were analyzed by two-way repeated ANOVA followed by Dunnett's test. \* $p < 0.05$  vs. baseline.

Table 2 Dentocult® LB scoring representing the Lactobacillus amount in subjects' saliva

Score	Week 0			Week 1			Week 2			Week 3			Week 4		
	Xylitol	D-Tagatose	D-Tagatose + Xylitol	Xylitol	D-Tagatose	D-Tagatose + Xylitol	Xylitol	D-Tagatose	D-Tagatose + Xylitol	Xylitol	D-Tagatose	D-Tagatose + Xylitol	Xylitol	D-Tagatose	D-Tagatose + Xylitol
-	1	3	3	3	4	3	1	3	2	3	5	2	3	3	4
+	2	0	0	2	2	0	4	2	1	1	0	2	2	3	0
++	4	2	3	2	0	3	2	1	2	3	1	2	2	0	2
+++	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0

Comparison of the three gum group scores (total count of each score) at different time points. The dark-colored scores show the incremental reduction at ++ and +++ in all groups from week 0 to weeks 3 and 4 of gum use. -,  $\leq 10^3$ /ml; +,  $\leq 10^4$ /ml; ++,  $\leq 10^5$ /ml; and +++,  $\leq 10^6$ /ml. Up to + is considered low-risk, and more than ++ is considered high-risk.

Chi-square test ( $\chi^2$ ): NS

*mutans* was first noted at the completion of the first week of gum use. The maximum reduction was also observed at that time point; the reduction was approx.  $1 \times \log$  CFU/ml. Therefore, a significant reduction in *S. mutans* was established by D-Tagatose + xylitol chewing gum after 3 and 4 weeks of use ( $p < 0.05$ ).

The Dentocult LB scoring for Lactobacillus revealed no notable changes for the 3 gum groups separately (Table 2), but it showed a gradual reduction of the total scores. At weeks 3 and 4, the Lactobacillus scores for each of the 3 gum groups were the lowest; this result

was in accord with the plate culture data. The Dentocult LB kit also identified some individuals who were highly responsive to the D-Tagatose + xylitol gum.

Regarding plaque formation, the Dentocult SM scores revealed that the 4-week use of the mixed D-Tagatose + xylitol gum produced a static and unchanged score for the detection of *S. mutans* (Table 3). This finding is important for plaque control. Many individual subjects achieved a reduction of plaque scores. The high scores such as ++ and +++ declined over weeks 2-4. These plaque data reflected the MSB

Table 3 Dentocult® SM scoring representing the *S. mutans* amount in plaque

Score	Week 0			Week 1			Week 2			Week 3			Week 4		
	Xylitol	D-Tagatose	D-Tagatose + Xylitol	Xylitol	D-Tagatose	D-Tagatose + Xylitol	Xylitol	D-Tagatose	D-Tagatose + Xylitol	Xylitol	D-Tagatose	D-Tagatose + Xylitol	Xylitol	D-Tagatose	D-Tagatose + Xylitol
-	1	2	4	1	2	4	1	1	2	1	0	3	1	2	2
+	2	2	1	5	4	1	6	5	2	6	6	3	6	4	3
++	3	2	1	0	0	1	0	0	2	0	0	0	0	0	1
+++	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0

At weeks 2, 3 and 4 (dark color) of D-Tagatose, xylitol and D-Tagatose + xylitol gum use, scores ++ and +++ were eliminated; this did not result in any significant differences among the three gum groups. The scoring value interpretations are the same as in Table 2.

Chi-square test ( $\chi^2$ ): NS

culture data. However, there were no significant differences in scores among the 3 gum groups.

### Discussion

The collection of saliva is noninvasive and easy, providing a sample that contains different species of bacteria, some of which can cause chronic infectious disease in the oral cavity. Tolerant bacteria and acid production are the most important factors for the development of dental caries. *S. mutans* is the strongest acid producer in the oral cavity, followed by *Lactobacillus*. A biofilm acts to form and sustain a more acidic environment, which can result not only in adult caries but also in severe early childhood caries in the primary dentition. Oral biofilms that include different species lead to periodontitis later in life [17], and persistent biofilm or plaque formation aggravates this process.

One of our earlier studies demonstrated that D-Tagatose inhibits *S. mutans* biofilm formation in the presence of sucrose [2]. The oral biofilms (also known as dental plaque) produced by these bacteria along with other factors cause caries and periodontitis. Sucrose and other monosaccharides enhance *S. mutans* growth [18]. Xylitol has already become a popular sugar substitute, and xylitol chewing gum has shown some therapeutic effect in oral disease prevention [7,8,16,19]. The mechanism of D-Tagatose metabolism in *S. mutans* differs from that of xylitol, and it is unique. *S. mutans* can metabolize D-Tagatose more easily than xylitol. D-Tagatose inhibits the glucosyltransferase B enzyme, which is the factor most responsible for dental plaque production. Plaque can also provide a more acidic environment for other bacterial species [20].

*In vitro*, the additions of D-Tagatose and D-Tagatose + xylitol resulted in significant reductions in bacterial colonies compared to the control and xylitol-only cultures. This result might indicate that the mechanisms underlying both the inhibition of *S. mutans* and the oral bacteria growth by D-Tagatose differ from those of xylitol [20]. In the BHI medium to which D-Tagatose was added, the numbers of colonies of oral bacteria were significantly reduced compared to the xylitol-only and control cultures. This revealed that D-Tagatose inhibits many types of oral bacteria both anaerobically and aerobically. The formation of an oral biofilm involves a series of steps starting with the initial colonization of the pellicle, and ending with the complex formation of the mature biofilm [4,5]. In the first colonization, the proportion of aerobic bacteria is high, but in the mature biofilm, anaerobic bacteria grow and the oral bacterial composition in the biofilm demineralizes the tooth surface or induces inflammation of the periodontal tissue.

It is necessary to elucidate the compositional changes and the mechanism of D-Tagatose's inhibition of oral bacteria. The short-term use of xylitol is not very effective against oral bacteria [6,21], and this finding caused some confusion about the preventive role of xylitol. However, it has been confirmed that xylitol is non-cariogenic rather than anti-cariogenic. We obtained similar results in our present trial, but interestingly, when xylitol and D-Tagatose were combined, the result was significantly different. These results suggest that xylitol and D-Tagatose each have some distinct properties that yield additive effects when the 2 compounds are mixed together. We also performed a primary preliminary study for 3 days with the same gum preparations and the same doses of xylitol and D-Tagatose (data

not shown). Even in that short-term use, the D-Tagatose + xylitol gum was significantly more effective than a gum containing Maltitol (another sugar alcohol) alone. Scoring by a Dentocult LB kit for Lactobacillus also showed a significant difference in oral bacteria eradication between before and after 3 days of gum chewing.

Although many studies with designs similar to the present investigation have been reported, none of them included chewing gums containing combined sugars. This study is the first to identify a potential effect of such a gum. Further studies are needed to examine the role of other ketohexoses mixed with xylitol. There is a complex relationship between different sugars and microbes, and it is necessary to determine the optimal concentrations of various sugars. There may be doses other than those used herein that are effective. In addition, Haghgoo *et al.* stated that the efficacy of chewing gum on the salivary *S. mutans* level depends on the frequency of gum use rather than the sugar concentration [22]. Some studies of the long-term use of xylitol have found it effective, but the short-term use (~6 months) has no clear effect on salivary mutans. Our present analyses revealed new findings about D-Tagatose + xylitol chewing gum.

This study has some limitations. The small number of subjects was always a concern, but as a pilot study for future investigations assessing the design and feasibility of gum-containing sugars, the sample size was considered adequate. A minimum level of *S. mutans* in saliva was used as a subject-inclusion criterion, and many volunteers were excluded for not meeting that criterion. Frequent sample collection was a difficult task for the subjects. The production of test sugar-containing gum was also a time-consuming task. A crossover study remains to be performed. Finally, while only chewing gum was used, sugar-containing mouthwash, toothpaste, or candies are also potential vehicles.

This pilot trial of chewing gum was conducted to test our hypothesis following *in vitro* studies [10]. It was necessary to conduct this trial both to answer our research questions and to assess a protocol for a longer version of the trial. Another trial that is  $\geq 6$  months with a larger number of subjects is needed.

In conclusions, *in vitro*, D-Tagatose had potent inhibitory effects on the growth of *S. mutans* and many other types of oral bacteria cultured under both aerobic and anaerobic conditions. *In vivo*, gum containing only

D-Tagatose or only xylitol did not significantly decrease the number of total bacteria or *S. mutans*, but D-Tagatose + xylitol chewing gum significantly decreased *S. mutans* numbers. The results of these *in vivo* and *in vitro* trials indicate that D-Tagatose is a strong candidate sugar for preventing dental caries, periodontitis, and many other oral diseases. Comprehensive studies are thus needed to identify the optimized effects of D-Tagatose on oral bacteria and oral diseases, dental caries, and periodontitis.

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