Effect of Rubusoside, a Natural Sucrose Substitute, on *Streptococcus mutans* Biofilm Cariogenic Potential and Virulence Gene Expression *In Vitro*

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ABSTRACT Dental caries is a biofilm-mediated disease in which *Streptococcus mutans* is the main pathogenic microorganism, and its incidence is closely related to sucrose. Rubusoside is a natural nonnutritive sweetener isolated from *Rubus suavissimus* S. Lee. This study was designed to determine the effect of this sucrose substitute on the cariogenic properties and virulence gene expression of *S. mutans* biofilms. *S. mutans* was exposed to brain heart infusion (BHI) medium (as a control), 1% sucrose-supplemented medium, 1% rubusoside-supplemented medium, and 1% xylitol-supplemented medium. The growth curve of the biofilm was monitored by crystal violet staining, and the pH was measured every 24 h. After 5 days, the biofilms formed on the glass coverslips were recovered to determine the biomass (dry weight and total amount of soluble proteins), numbers of CFU, and amounts of intra- and extracellular polysaccharides. Biofilm structural imaging was performed using a scanning electron microscope (SEM). Virulence gene expression (*gtfB*, *gtfC*, *gtfD*, *ftf*, *spaP*, *gbpB*, *ldh*, *atpF*, *vicR*, and *comD*) was determined by reverse transcription-quantitative PCR. Growth in rubusoside resulted in lower levels of acid production than observed during growth in sucrose, xylitol, and the control, while it also reduced the level of biofilm accumulation and bacterial viability and even reduced the level of production of extracellular polysaccharides. By SEM, the levels of biofilm formation and extracellular matrix during growth in rubusoside were lower than these levels during growth in sucrose and xylitol. From the perspective of virulence genes, growth in rubusoside and xylitol significantly inhibited the expression of virulence genes compared with their levels of expression after growth in sucrose. Among these genes, *gtfB*, *gtfC*, *gbpB*, *ldh*, and *comD* downregulation was found with growth in rubusoside compared with their expression with growth in xylitol. Therefore, rubusoside appears to be less potentially cariogenic than sucrose and xylitol and may become an effective sucrose substitute for caries prevention. Further studies are needed to deepen these findings.

IMPORTANCE Dental caries is a major public health challenge and places heavy biological, social, and financial burdens on individuals and health care systems. To palliate the deleterious effect of sucrose on the virulence factors of *S. mutans*, massive commercial efforts have been oriented toward developing products that may act as sucrose substitutes. Rubusoside, a natural sucrose substitute, is a plant extract with a high level of sweetness. Although some studies have shown that rubusoside does not produce acids or inhibit the growth of *S. mutans*, little attention has been paid to its effect on dental biofilm and the underlying mechanisms. Our study focuses on the effect of rubusoside on the formation and structure of biofilms and the expression of virulence genes. The results confirm that rubusoside can inhibit accumulation, bacterial viability, polysaccharide production by the biofilm, and related gene

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expression. These results provide further insight into the cariogenicity of *S. mutans* biofilms and demonstrate a new perspective for studying the impact of sucrose substitutes on caries.

KEYWORDS rubusoside, *Streptococcus mutans*, biofilm formation, dental caries, virulence gene

Dental caries, one of the most prevalent diseases among civilized human populations, is a biofilm-mediated chronic progressive disease. Despite the significant advancement of technology and the increasing awareness of oral health care, the prevalence rate of dental caries is still, alarmingly, on the rise, which makes this disease of great medical, social, and economic importance (1).

Both culture-based and DNA/RNA-based studies have confirmed that *Streptococcus mutans* is the prime pathogenic microorganism responsible for the occurrence and development of dental caries (2). When dietary carbohydrates are excessively and frequently consumed, they can be metabolized by cariogenic bacteria to synthesize a series of organic acids diffusing through the biofilm. Gradually, the net loss of tooth mineral structure begins to appear on the surface of the tooth. With subsequent attenuation, the weakened dental hard tissue is then degraded to form a cavity and the teeth are progressively destroyed (3).

The cariogenic properties of *S. mutans* biofilms are regulated by a variety of genes, which are mainly involved in several essential aspects. Research has demonstrated that *S. mutans* adheres to dental surfaces mainly through two mechanisms: sucrose-dependent adhesion and sucrose-independent adherence. The latter is also called nonspecific adhesion or initial adherence. In the absence of sucrose, the sucrose-independent mechanism is achieved by specific substrate recognition and the adherence of surface adhesins, such as SpaP (4). In the presence of sucrose, glucosyltransferases (GTF) GtfB, GtfC, and GtfD (encoded by the *gtfB*, *gtfC*, and *gtfD* genes, respectively) synthesize the water-insoluble or -soluble glucans (5), adsorbed to saliva-coated dentine and bacterial surfaces (4, 6). Fructosyltransferase (FTF), the product of the *ftf* gene, is capable of catalyzing the formation of fructans from sucrose. Studies have verified that the fructan polymers produced by *S. mutans* function principally for the storage of extracellular nutrients, which may be utilized during periods of nutrient deprivation (7). Glucan-binding proteins (GbpB), encoded by the *gbpB* gene, are cell-associated nonenzymatic proteins in the absence of cell wall anchors (8). They mediate the bacterial interaction with extracellular glucans and play a significant role in sucrose-dependent adhesion and biofilm formation, contributing to the maintenance of a stable, symbiotic microbial population in the oral cavity (9).

Lactate dehydrogenase (LDH), encoded by the *ldh* gene, is an important intrinsic enzyme and catalyzes the conversion of pyruvate to lactic acid (10), which is the predominant cause of the decrease in pH of the plaque biofilm after the bacterial metabolism of carbohydrates. The acid tolerance of biofilm primarily depends on the activities of the membrane-bound F-ATPase (H^+ -translocating ATPase), encoded by the *atpF* gene. It allows the bacteria to keep cytoplasmic pH homeostasis by transporting protons out of the cells and making the internal pH more alkaline than the pH of the environment, stabilizing the change in the pH across the cell biofilm (11).

Two-component signal transduction systems (TCSTS) are regulatory networks crucial for the adaptation, survival, and virulence of *S. mutans*, and of these, the VicRK signal transduction system functions as a molecular switch to sense changes in the environment and triggers the differential expression of genes in response to external continuous dynamic challenges (12). *vicR* is the intracellular response regulator gene of the VicRK system, which has been proven to control the expression of several virulence-related genes, including *gtfBCD*, *ftf*, and *gbpB* (13). Cariogenic microorganisms use a quorum-sensing (QS) system, which interacts with various oral environmental conditions and which acts as a mechanism to signal between bacterial cells to control the expression of genes in response to population density. In *S. mutans*, the most common

QS system is the Com-dependent QS system, which is stimulated by the competence-stimulating peptide under conditions of high cell density and which, when expressed, results in genetic transformation, bacteriocin production, and acid resistance (14). ComD, an autophosphorylating histidine kinase encoded by *comD*, is an environmental sensor whose activity is regulated to induce and regulate bacterial populations at the critical extracellular concentration (15).

In order to avoid the effect of sucrose on the virulence factors of *S. mutans*, sucrose substitutes are generally widely used. Rubusoside is a diterpene glycoside mainly extracted from the leaves of *Rubus suavis* S. Lee (known as Chinese sweet tea), which is widely distributed in the Guangxi and Guizhou Provinces of China. Rubusoside not only has low toxicity and few side effects but also has the pharmacological effects of anti-inflammatory, antiallergic, and antiangiogenic activities (16), having been made into traditional Chinese herbal medicine by local people for a long time (17). In terms of sweetness and calories, it is about 143-fold sweeter than sucrose at a concentration of 0.025% (18), while it has just 1% of the calories of sucrose (19, 20).

Recent experiments have shown that rubusoside, like other sucrose substitutes, has the ability to inhibit the growth of *S. mutans*. Our previous study showed that a rubusoside supplement can reduce the acid production and adherence of *S. mutans* to glass rods (19). Furthermore, studies have proven that rubusoside can inhibit the activity of glucosyltransferase (GTF) of *S. mutans* (21, 22).

However, current researchers are mainly focused on the single-cell state (planktonic-state bacteria), which simplifies the influence of the external environment to the greatest extent. Bacterial cells in biofilms exhibit biology and phenotypic traits extraordinarily distinct from those of their planktonic counterparts (5), and these are accompanied by significant changes in the bacterial gene expression profile.

In the present study, the effect of rubusoside on biofilm formation and polysaccharide production by *S. mutans* biofilm was evaluated. By comparing the expression of caries-associated virulence factors (*spaP*, *gbpB*, *gtfB*, *gtfC*, *gtfD*, *ftf*, *ldh*, *atpF*, *comD*, *vicR*) in each treatment group, the underlying mechanism of this effect was preliminarily explored. The findings will help to provide insight into the pathogenesis of dental caries caused by *S. mutans* and facilitate subsequent research and the promotion of rubusoside as a sucrose substitute.

RESULTS

Rubusoside inhibits *S. mutans* biofilm accumulation. The growth curve of the *S. mutans* biofilm made from the data is shown in Fig. 1A. The level of formation of biofilms grown in sucrose was higher than that of biofilms grown in rubusoside and xylitol at each time point ($P < 0.05$), and biofilm accumulation reached its peak at 96 h. The accumulation of biofilm grown in xylitol was significantly less than that for the blank control after 12 h ($P < 0.05$), and the maximum value was obtained in 48 h. In the presence of rubusoside, biofilm accumulation reached a peak at 24 h, and *S. mutans* grown in rubusoside showed a significantly lower level of biofilm accumulation than *S. mutans* grown in sucrose and xylitol ($P < 0.05$).

Rubusoside leads to decreased acid production of *S. mutans* biofilm. The initial pH of each group was adjusted to the same value (pH 7.04). As can be seen from Table 1, at 24 h after exposure to sucrose, the pH had rapidly decreased to below 5.5 (the enamel demineralization critical pH). In addition, the pH of the medium of the sucrose group was lower than that of the other groups ($P < 0.05$) throughout the experiment, and the pH gradually decreased over time ($P < 0.05$). There was no significant difference in pH between the blank control and the xylitol groups at 48 h, 72 h, and 96 h ($P > 0.05$). At 24 h, the pH of the xylitol group was higher than that of the control group, while at 120 h, xylitol induced a significantly higher acidogenicity ($P < 0.05$). The pH remained at a higher level (close to 7) in the presence of rubusoside than in the presence of the other treatments ($P < 0.05$), but the pH did not show a significant change over time ($P > 0.05$).

FIG 1 (A) Growth curve for the *S. mutans* biofilm. (B) Dry weight and amounts of soluble proteins (in milligrams) of the *S. mutans* biofilm. (C) Viability of the *S. mutans* biofilm. (D) Production of soluble extracellular polysaccharides (SEPS) by the *S. mutans* biofilm. (E) Production of insoluble extracellular polysaccharides (IEPS) by the *S. mutans* biofilm. (F) Production of intracellular polysaccharide (IPS) by the *S. mutans* biofilm. Control, *S. mutans* growth in BHI medium; sucrose, *S. mutans* growth in BHI medium supplemented with 1% sucrose; rubusoside, *S. mutans* growth in BHI medium supplemented with 1% rubusoside; xylitol, *S. mutans* growth in BHI medium supplemented with 1% xylitol. The results are expressed as the mean $\pm$ SD. Error bars indicate SD. Different letters represent significant differences among treatments. Statistical significance is marked with asterisks: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Rubusoside causes reductions in biomass, bacterial viability, and the amount of polysaccharide of *S. mutans* biofilms. (i) Biomass growth.

The biomass growth of the bacterial biofilm was measured from the dry weight and the amount of soluble proteins, which are shown in Fig. 1B. The dry weight of the biofilm grown in sucrose was higher than that of the biofilms grown in the other treatments ($P < 0.001$). The biofilms grown in xylitol showed a dry weight similar to that of the biofilms of the control group ($P > 0.05$), and the dry weight of the bacteria was the lowest in the presence of rubusoside ($P < 0.01$). The total amount of soluble proteins in *S. mutans* biofilms grown in sucrose was significantly greater than that of biofilms grown in rubusoside, xylitol, and the control treatment ($P < 0.001$), but there was no significant difference in the amount of proteins produced by *S. mutans* biofilms in the last three groups ($P > 0.05$).

TABLE 1 Effects of rubusoside on acid production of *S. mutans*

Time (h)	pH of spent medium at the end of each time point ^a			
	Control	Sucrose	Rubusoside	Xylitol
24	5.71 $\pm$ 0.02A	3.95 $\pm$ 0.09B	6.84 $\pm$ 0.07C	5.86 $\pm$ 0.03D
48	5.78 $\pm$ 0.02A	3.96 $\pm$ 0.10B	6.74 $\pm$ 0.04C	5.79 $\pm$ 0.05A
72	5.74 $\pm$ 0.08A	3.93 $\pm$ 0.08B	6.67 $\pm$ 0.06C	5.75 $\pm$ 0.05A
96	5.74 $\pm$ 0.10A	3.89 $\pm$ 0.02B	6.62 $\pm$ 0.10C	5.69 $\pm$ 0.04A
120	5.74 $\pm$ 0.05A	3.80 $\pm$ 0.02B	6.55 $\pm$ 0.03C	5.58 $\pm$ 0.05D

^aData are the mean $\pm$ SD. The pH in the sucrose and xylitol groups changed significantly over time, based on linear regression ($P < 0.05$). The difference for data with the same letter was not significant ($P > 0.05$) at the same time point, based on repeated ANOVAs and Tukey's test. Control, *S. mutans* growth in BHI medium; sucrose, *S. mutans* growth in BHI medium supplemented with 1% sucrose; rubusoside, *S. mutans* growth in BHI medium supplemented with 1% rubusoside; xylitol, *S. mutans* growth in BHI medium supplemented with 1% xylitol.

FIG 2 Architecture of *S. mutans* biofilms and distribution of bacteria determined using SEM. Specimens were examined at magnifications of $\times 10,000$ and $\times 30,000$. Control, *S. mutans* growth in BHI medium; sucrose, *S. mutans* growth in BHI medium supplemented with 1% sucrose; rubusoside, *S. mutans* growth in BHI medium supplemented with 1% rubusoside; xylitol, *S. mutans* growth in BHI medium supplemented with 1% xylitol.

(ii) Bacterial viability. The population of viable bacteria recovered from the biofilms is shown in Fig. 1C. The number of viable bacteria was significantly higher ($P < 0.001$) in the presence of sucrose than in the presence of rubusoside, xylitol, and the control treatment. Meanwhile, the CFU count of the biofilm grown in xylitol was slightly lower than that of the biofilm grown in the control treatment ($P < 0.05$). The relative numbers of recoverable viable cells remained lower for the rubusoside treatment than for the other treatments ($P < 0.001$).

(iii) Amounts of polysaccharides. The amounts of soluble extracellular polysaccharide (SEPS) and intracellular polysaccharide (IPS) (Fig. 1D and F) were significantly greater in the biofilms grown in sucrose than in the biofilms receiving the other three treatments ($P < 0.001$), but no difference was observed between the control and the xylitol groups ($P > 0.05$). The relative amount of SEPS produced by the rubusoside group was smaller than that produced by the control and xylitol groups ($P < 0.05$), but there was no significant difference in the amount of IPS produced between the rubusoside group and the other two groups ($P > 0.05$). Regarding insoluble extracellular polysaccharides (IEPS) (Fig. 1E), only the biofilms grown in sucrose showed a significant increase in the amount of IEPS ($P < 0.001$). The amount of IEPS produced by the biofilms in the control and xylitol groups was in the middle position, and the amount of IEPS produced by the xylitol group was slightly smaller than the amount produced by the control group ($P < 0.05$). In the presence of rubusoside, the amount of IEPS produced was significantly less than that produced in the presence of the other treatments ($P < 0.05$).

Inhibitory effect of rubusoside on architecture of *S. mutans* biofilms. Micrographs obtained by scanning electron microscopy (SEM) at $\times 10,000$ and $\times 30,000$ magnifications (Fig. 2) showed that there was particularly little extracellular matrix produced by bacterial metabolism in the control group. The bacterial cells showed a scattered short-chain distribution without an obvious cluster-like network distribution. In the presence of sucrose, *S. mutans* decomposed and produced large amounts of extracellular substances, and the bacterial cells were encapsulated in the three-dimensional structure formed by these substances. The number of *S. mutans* cells in the rubusoside and xylitol groups was significantly reduced compared with that in the other groups. Secretion on the surface of the cells was also greatly reduced, the three-dimensional structure was not obvious, and the bacteria were scattered and distributed in short chains. Rubusoside significantly reduced the formation of bacterial biofilm, but the difference from the xylitol group was not obvious.

Effects of rubusoside on expression of cariogenicity-related virulence factors. The presence of sucrose, rubusoside, and xylitol profoundly influenced the expression

FIG 3 Expression of cariogenicity-related virulence factors of *S. mutans* biofilms by real-time reverse transcription-quantitative PCR. (A) Results for *gtfB*, *gtfC*, *gtfD*, and *ftf*. (B) Results for *spaP* and *gbpB*. (C) Results for *ldh* and *atpF*. (D) Results for *vicR* and *comD*. The level of expression of each gene was normalized to the level of 16S rRNA expression, and the fold change relative to the findings for the control was calculated using the $2^{-\Delta\Delta CT}$ method. Control, *S. mutans* growth in BHI medium; sucrose, *S. mutans* growth in BHI medium supplemented with 1% sucrose; rubusoside, *S. mutans* growth in BHI medium supplemented with 1% rubusoside; xylitol, *S. mutans* growth in BHI medium supplemented with 1% xylitol. The results were expressed as the mean $\pm$ SD, and error bars indicate SD. Different letters represent significant differences among treatments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

of the caries-related virulence factors of *S. mutans*. The genes that are associated with polysaccharide synthesis and sucrose-dependent adhesion (*gtfB*, *gtfC*, *gtfD*, *ftf*) (Fig. 3A) were significantly upregulated in the presence of sucrose and were expressed at the highest level in the sucrose group ($P < 0.001$). The upregulation of *gtfB* ($P < 0.05$) and *gtfC* ($P < 0.001$) in response to rubusoside was lower than that in response to xylitol ($P < 0.05$). For *gtfD*, there was no statistically significant difference in expression among the rubusoside, xylitol, and control groups ($P > 0.05$), and *ftf* was upregulated at a higher slightly level in the rubusoside-supplemented medium than in the xylitol-supplemented medium ($P < 0.01$).

In contrast, the expression of *spaP* (Fig. 3B), which is related to sucrose-independent adhesion, in the rubusoside and xylitol groups was not significantly different from that in the control group ($P > 0.05$), but its expression in all three of these groups was far lower than that in the sucrose group ($P < 0.001$). The expression of *gbpB* (Fig. 3B) was upregulated in the rubusoside group compared with the level of expression in the control group ($P < 0.05$), but the level of expression in the rubusoside-supplemented medium was lower than that in the xylitol-supplemented medium ($P < 0.05$). The highest level of upregulation of *gbpB* was observed in the presence of sucrose ($P < 0.001$).

Among the genes related to acid production and acid resistance (Fig. 3C), the level of the transcripts of *ldh* in the rubusoside group was similar to that in the control group ($P > 0.05$), and the levels in both of those groups were significantly lower than those in the sucrose ($P < 0.001$) and xylitol ($P < 0.01$) groups. The expression of *atpF* was upregulated in the rubusoside-supplemented medium compared with the level of expression in the control medium ($P < 0.001$), while the difference in *atpF* expression between the xylitol and control groups was not significant ($P > 0.05$). Sucrose induced the highest level of upregulation of expression of *ldh* and *atpF* ($P < 0.001$).

The regulated gene *vicR* (Fig. 3D) was downregulated only in the presence of rubusoside and xylitol ($P < 0.05$), but no difference was observed between these two groups ($P > 0.05$). Nevertheless, the expression of the *vicR* gene was upregulated in

sucrose-supplemented medium ($P < 0.001$). The expression of a member of two-component systems related to acid tolerance (Fig. 3D), *comD*, was assessed. Although it was slightly upregulated, the difference between the rubusoside and the control groups was not significant ($P > 0.05$). The increase in *comD* expression in the xylitol group was higher than that in the rubusoside group ($P < 0.05$), while its expression was undoubtedly the greatest in the sucrose group ($P < 0.05$). A significant upregulation of *comD* was observed in the sucrose- and xylitol-supplemented media ($P < 0.001$), but the transcript levels in the sucrose group were higher than those in the xylitol group ($P < 0.001$).

DISCUSSION

Dental biofilm is a community of cariogenic microorganisms that steadily adhere to the surface of organic or inorganic materials. Bacteria are encapsulated in the extracellular polymeric substance matrix (consisting of polysaccharides, proteins, extracellular DNA, and other kinds of microbes) (23), protecting themselves from desiccation, host defenses, and predators (24). *S. mutans* has been regarded as the main pathogenic microorganism of humans and animals involved in the development of dental caries (25).

The excessive consumption of fermentable carbohydrates, especially sucrose, has been shown to be the main cause for the prevalence of caries. As can be seen from our results, whether they are observed from the perspective of bacterial accumulation, biofilm formation, acid production, extracellular polysaccharide (EPS) production, biofilm structure, or the expression of related virulence factors, sucrose has an extraordinarily strong cariogenic ability, which is consistent with many previous reports (26).

Many synthetic sucrose substitutes have been extensively used in food and drugs, and several types of these, such as aspartame, saccharin, and cyclamate, are deemed to be friendly for teeth (27). However, they have some disadvantages, such as delayed sweetness, a bitter aftertaste, and persistent sweetness, which can hinder consumers from accepting them (28). Sugar alcohol sweeteners have been widely used recently, and of these, the most popular are xylitol and sorbitol (5). One of the drawbacks, however, is that they are not easily digested and absorbed from the gastrointestinal tract. When consumed in large quantities, they may induce osmotic diarrhea (29). Nowadays, researchers are oriented toward discovering and evaluating new noncariogenic sucrose substitutes from nature.

Rubusoside is a naturally sweet substance with a low calorific value, high sweetness, and high temperature resistance and is extremely safe to consume (30). A considerable number of experiments have proven that rubusoside has certain auxiliary effects on obesity, diabetes, cardiovascular disease, hypertension, arteriosclerosis, and other diseases (31, 32). Rubusoside shows good economic value in various industries, such as the food, beverage, cold food product, condiment, pharmaceutical, and beauty cosmetics industries. In Japan, rubusoside has been officially certified as a sweetener or health food supplement, and its use has expanded in recent years. However, few studies have reported on the effect of rubusoside on the cariogenicity of *S. mutans* biofilms and its potential mechanism.

The attachment and accumulation of *S. mutans* on tooth surfaces are critical stages in the initial formation and the further development of pathogenic plaque biofilms (33). In this study, the biofilm growth curve showed that rubusoside had an inhibitory effect on cariogenic biofilm accumulation. The results of studies of dry weight and the total amount of proteins showed that the biomass in the rubusoside group remained at the lowest level compared with that in the other groups, indicating that the use of rubusoside can significantly inhibit increases in bacterial biomass. The number of viable cells in the *S. mutans* biofilm was the lowest in the presence of rubusoside, which indicated that rubusoside may dramatically reduce the viability of *S. mutans* biofilms.

The metabolic activity of acid production is the factor initiating caries. The mineral content in tooth tissue is extremely sensitive to increases in the amounts of organic acid produced by carbohydrate metabolism. When the pH drops below 5.5, the

demineralization process is faster than the remineralization process. The addition of sucrose results in the lowest pH (a pH far below 5.5) compared with the pHs achieved with the other treatments. This proves again that *S. mutans* has the strongest ability to produce acid by sucrose metabolism, which probably leads to the demineralization of tooth hard tissue. Xylitol induced a slightly higher acidogenicity than the control in the later period of the experiment. This may have been due to the fact that the concentration of xylitol did not reach the effective minimum acid inhibition concentration. With a minimum pH drop, the acid production of the bacteria in the rubusoside group was fairly low; therefore, the ability of *S. mutans* to utilize rubusoside to produce acid was weak.

It could be seen from SEM images that sucrose was metabolized by *S. mutans* to produce a large quantity of extracellular polysaccharides, forming a viscous three-dimensional biofilm scaffold, which would lead to the difficulty of antibacterial drugs to penetrate into the interior of the biofilm. Nevertheless, the number of bacterial cells growing in the presence of rubusoside was obviously lower than the number growing in the presence of sucrose, and the biofilm formed was relatively thin. The bacteria were distributed in a scattered way, and the amount of polysaccharide was also significantly reduced.

Studies have shown that the extracellular matrix is the main scaffold of a biofilm, accounting for 85% of the total volume of the biofilm, and that extracellular polysaccharides (EPS) are the main component of the extracellular matrix (34). EPS, particularly glucans synthesized by surface-adsorbed glucosyltransferases, facilitate the development of dental caries by at least 6 different routes: (i) supplying specific binding sites for bacterial colonization, enhancing firm adherence and the further accumulation of cariogenic bacteria on the surface; (ii) increasing the structural integrity, stability, and bulk of the biofilms and allowing the microorganisms to form microcolonies; (iii) changing the porosity of the dental biofilm and restricting the diffusion of substances through the biofilm; (iv) acting as a reserve energy source; (v) protecting microbes from the harmful effects of antimicrobials and other environmental pressures; and (vi) contributing to the concentration of metal ions or other physiological nutrients in a microecological environment (33).

Regarding the production of three kinds of polysaccharides, the biofilms grown in rubusoside produced smaller amounts of SEPS, IPS, and IEPS than the biofilms grown in sucrose. The inhibitory effect of rubusoside on the production of SEPS and IEPS was better than that of xylitol. IEPS, the most crucial virulence factor of biofilms, have the capability to enhance the pathogenic potential of the biofilm by increasing the adherence and accumulation of cariogenic microorganisms on the tooth surface and boosting the bulk and structural integrity of dental plaque (35). Therefore, it can be concluded that rubusoside can reduce the levels of synthesis of polysaccharides synthesized in the biofilm state, which contributes to inhibition of the occurrence of dental caries.

Our findings from the present study show that rubusoside can inhibit the adhesion, accumulation, and acid production of *S. mutans*. Moreover, it can inhibit the increase in biofilm mass (dry weight and the total amount of soluble proteins) and reduce the levels of production of extracellular polysaccharides, decreasing the formation of biofilms, and this inhibition was stronger than that of xylitol.

Xylitol, a naturally occurring sugar alcohol, has been approved for use in food by the U.S. Food and Drug Administration since 1996 (36). Xylitol can be transported into the cells of *S. mutans* through the formation of xylitol-5-phosphate by the main route of sugar metabolism: the phosphoenolpyruvate phosphotransferase (PEP-PTS) system (37). Xylitol-5-phosphate inhibits the activity of glycolytic enzymes, inhibiting the growth and acid production of bacteria. Furthermore, the so-called futile xylitol-5-phosphate cycle, which provides no energy, can reduce the growth of *S. mutans* (38). In addition, chewing xylitol gum can significantly increase saliva flow and, thus, the amount of rapidly flowing saliva with a high pH and high concentrations of calcium and phosphate, which is conducive to the remineralization of enamel and resistance to the development of dental caries (39).

Considering the mechanism behind the noncariogenic effect of rubusoside, we studied the expression of several cariogenicity-related virulence factors. The surface-associated protein P1 (SpaP), anchored on the surface of *S. mutans*, works as a multifunctional adhesin and is of great importance in initial adherence and microbial community development (5). It has the ability to facilitate binding to salivary agglutinin glycoprotein and proline-rich proteins present in the pellicle acquired by enamel to form surface fibrillar adhesion on the tooth surface (40). Our real-time reverse transcription (RT)-quantitative PCR (qPCR) assay data indicated that, when bacterial cells were exposed to rubusoside and xylitol, *spaP* was less expressed by biofilm cells than by biofilm cells exposed to sucrose, which may contribute to the lower levels of biofilm growth in cells exposed to rubusoside and xylitol.

Research on dental plaque biofilm development and the etiology of dental caries has established the central role of glucans on sucrose-dependent adhesion (8). Our data suggest that *gbpB*, *gtfB*, *gtfC*, *gtfD*, and *ftf*, involved in sucrose-dependent adhesion, are expressed at lower levels in the presence of rubusoside and xylitol than in the presence of sucrose, contributing to inhibition of the adhesion of *S. mutans* and the formation of a biofilm. Furthermore, fructans have been proven to be conducive to the accumulation of cariogenic microorganisms and plaque formation (41). GbpB, proven to be highly antigenic in humans and rodents, is expected to become a critical target for the preparation and development of caries vaccines (42).

IEPS are crucial virulence factors of *S. mutans* and are mainly produced by GtfB and GtfC (9). The expression levels of the *gtfB* and *gtfC* genes in the presence of rubusoside were lower than those in the presence of xylitol and sucrose, which could contribute to the lower levels of production of insoluble polysaccharides. The results may also explain the differences in biofilms observed by SEM. To be exact, the biofilms grown in sucrose were relatively larger, had a thicker bulk, and contained more matrix substrate surrounded by polysaccharides, while the biofilms grown in the presence of rubusoside and xylitol were extraordinarily smaller and the bacteria were more dispersed.

The finding of the lowest pH in the spent medium of the sucrose group is supported by the finding of increased expression of *ldh*. At the same time, maintenance of the highest pH in the rubusoside group was also related to the low level of *ldh* expression. LDH, one of the important virulence factors of *S. mutans*, needs to be further evaluated in subsequent enzyme activity experiments. The expression level of *atpF*, which is closely related to the vitality of bacteria under environmental pressure, in the rubusoside and xylitol groups was lower than that in the sucrose group, but the inhibitory effect of rubusoside on *atpF* was not as good as that of xylitol. Studies have suggested that the activity of F-ATPase is associated with increased transcription of the *atpF* operon, which can be transcriptionally regulated by the growth pH. Besides, the possibility that the allosteric regulation of enzyme may contribute to the activity of F-ATPase in cells adapted to acidic conditions cannot be excluded (41). In addition, some independent studies have shown that F-ATPase can act as an ATP synthase to generate ATP for growth and persistence when starving cells live in a low-pH environment (43).

As a potential target for the prevention or treatment of dental caries, indeed, VicRK have long been applied to research on the inhibition of pathogenic microorganisms (44). Our data show that rubusoside and xylitol may cause the downregulated expression of *vicR*. The expression level of the *comD* gene in the presence of rubusoside was low, showing that it can inhibit the formation of biofilm and the growth of bacteria, and its inhibitory effect was better than that of xylitol. The QS system has become an attractive target for interventions against biofilm infections because it can control biofilm formation, activate virulence factors, and withstand acidic conditions (45).

The inhibitory effect of rubusoside on *S. mutans* biofilms is obviously related to the expression of related genes, but it is worth noting that the transcription levels of different genes are not completely consistent with those of the corresponding proteins. The levels of all proteins are also determined by posttranscriptional factors, including translational efficiency, posttranslational modification, and protein degradation (46). The effect of rubusoside on related proteins is due to the disruption of the spatial

conformation of the protein. A recent study suggested that rubusoside may inhibit the mutansucrase activity of *S. mutans* by influencing hydrophobic and hydrogen bond (H bond) interactions between rubusoside and the amino acid residues at the active-site pocket of mutansucrase (47). More studies of *S. mutans* are needed, and the quantitative PCR results need to be confirmed at the protein level to verify the current results.

There are also some limitations to this experiment that cannot be ignored. It has been acknowledged that bacterial biofilms are formed on the natural tooth surface by the interaction between different kinds of bacterial species, the host, diet, and other external factors instead of by an individual bacterium (48). More than 700 bacterial species spanning more than 20 phyla have been found in the human oral cavity (49). However, in the model used in this experiment, only a single bacterial species (*S. mutans*) was applied. Although the monospecies biofilm model presented in this study does not mimic the complex microbial community of dental plaque, it is beneficial for investigating the specific roles of the test agents on the growth and metabolism of *S. mutans*, biofilm architecture, and genetics as a prelude to subsequent studies with multibacterial biofilms (50). Animal experiments, *in vivo* experiments, and large clinical trials also need to be performed to ascertain the effect of rubusoside on dental caries.

In conclusion, in contrast to the growth of *S. mutans* in the presence of sucrose, its growth in the presence of rubusoside supports the formation of much less biofilm, the production of much smaller amounts of acids, and the synthesis of much smaller amounts of polysaccharides, all of which are conducive to caries prevention. With the characteristics of being natural, nontoxic, and noncaloric, rubusoside may be an effective tool to improve oral hygiene. Given its rapidly increasing use worldwide, more in-depth research on rubusoside is required in the future.

MATERIALS AND METHODS

Bacteria and culture conditions. *Streptococcus mutans* ATCC 25175 (BeNa Culture Collection Center, China), a well-characterized international standard strain, is a proven virulent cariogenic pathogen. This strain belongs to serotype C, the most prevalent serogroup in the human oral cavity, and its genome sequence is available (51). *S. mutans* was routinely reactivated in brain heart infusion (BHI) broth (Haibo Biotechnology Co. Ltd., Qingdao, China) at 37°C under anaerobic conditions (90% N₂, 5% CO₂, 5% H₂). The methods of identification and evaluation of reactivated bacteria included observing the morphology of the bacteria under a microscope after Gram staining and observing the colony morphology after inoculation on mitis-salivarius-bacitracin (MSB) agar medium. Then, the bacteria were incubated, harvested at the mid-exponential phase by centrifugation at 12,000 × *g* and 4°C for 5 min, and resuspended in sterile phosphate-buffered saline (PBS) to an optical density (OD) at 550 nm of 1. At an absorbance of 550 nm, the concentration of bacterial cells was standardized to approximately 5 × 10⁷ cells/ml. The standard bacterial suspension obtained was used for subsequent experiments. The stock culture was mixed with 50% glycerol and the mixture was put into a cryopreservation tube, which was sealed, marked, and stored at −80°C.

Test compound. The *S. mutans* biofilms were grown in four different media: BHI medium (as a control; 0.2% endogenous glucose), 1% sucrose-supplemented BHI medium, 1% rubusoside (Chengdu MUST Bio Technology Co. Ltd.)-supplemented BHI medium, and 1% xylitol-supplemented BHI medium. The purity (98%) and authenticity of all sweeteners were verified by high-performance liquid chromatography and gas chromatography-mass spectrometry. The concentration (1%, wt/vol) of rubusoside, sucrose, and xylitol used was chosen on the basis of other previous dose-response experiments (48).

Growth curve of the *S. mutans* biofilm. The biofilm formation of *S. mutans* was detected by a crystal violet (CV) staining assay. In brief, culture medium from each group and the standard bacterial suspension were added to sterile 96-well microtiter plates, and the plates were incubated at 37°C for 12, 24, 36, 48, 60, 72, 96, and 120 h. After incubation, the planktonic bacteria and culture medium were gently removed and the wells were washed with PBS. The attached bacteria in each well were subsequently fixed with methanol for 15 min, washed again, and dried naturally. Crystal violet solution (0.1%) was added to stain the biofilm, and the plates were incubated for 15 min at room temperature. The wells were thoroughly rinsed with sterile water until the controls appeared colorless. After drying for 10 min, 33% glacial acetic acid solution was added to solubilize the crystal violet under gentle shaking in an incubator at 37°C for 30 min. Finally, the contents of the wells with acetic acid solution were transferred to a new 96-well microplate, and the absorbance at an OD of 590 nm was recorded to quantify the biofilm formation using a microplate reader. Four replicates were measured for each group.

Biofilm analyses. The standard bacterial suspension, prepared as described above, was inoculated into the solution of each medium group, and the biofilms of *S. mutans* were developed on the surface of 10-mm-diameter sterilized glass coverslips placed in sterile 24-well microtiter plates at 37°C under anaerobic conditions for 5 days. The culture medium was renewed every 24 h, while the pH of the spent medium was measured daily with a precise pH meter (Mettler-Toledo, Columbus,

OH, USA). After 120 h, the biofilms were scraped off the glass coverslips with a sterile cell scraper and suspended in PBS. After ultrasonic treatment, a uniform bacterial suspension was obtained and divided into aliquots for analysis.

(i) Biomass. For determination of the dry weight, a sonicated suspension was transferred into a new preweighed centrifugation tube. Three volumes of 100% cold ethanol (-20°C) were added, and the mixture was incubated for 15 min. The suspension was centrifuged at $12,000 \times g$ at 4°C for 10 min, and the resulting pellet was collected, washed twice with 75% cold ethanol, centrifuged again, and then lyophilized for 24 h and weighed. By subtracting the final weight from the initial weight of the empty tube, the weight of the biomass was measured, and the results are expressed in milligrams.

To obtain the total amount of soluble proteins, the cell suspension was treated for 1 h at 0°C under agitation with 2 M NaOH containing 1 mM EDTA. After being vortexed, the tubes were placed at 100°C for 15 min. They were then centrifuged at $12,000 \times g$ at 4°C for 10 min, and the concentration of total soluble protein in the supernatant was determined (bicinchoninic acid protein assay; Vazyme, Nanjing, China) (52).

(ii) Bacterial viability. An aliquot of the homogenized biofilm bacteria was serially diluted from 10^4 to 10^6 with PBS buffer and inoculated on mitis-salivarius-bacitracin (MSB) agar medium in duplicate. The plates were incubated anaerobically at 37°C for 2 days. Afterwards, the number of CFU was counted, and the results were corrected by the dilution factor and expressed as the $\log_{10}$ number of CFU per milligram of biofilm dry weight (53).

(iii) Polysaccharide determination. The bacterial suspension was centrifuged at $12,000 \times g$ at 4°C for 5 min to harvest the supernatant, which contained soluble extracellular polysaccharides (SEPS). NaOH (1 M) was added to the precipitate; the mixture was vortexed, agitated, and centrifuged; and then the supernatant was collected to obtain insoluble extracellular polysaccharides (IEPS). For intracellular polysaccharide (IPS) extraction, 1 M NaOH was added to the residual pellet. The tube was vortexed and heated at 100°C for 15 min. After centrifugation, the supernatant was transferred to a new IPS tube. Immediately afterwards, 3 volumes of cold 100% ethanol were added into each of the tubes containing the three polysaccharides and the tubes were vortexed. The tubes were incubated at -20°C for 30 min, centrifuged, and washed twice with cold 70% ethanol. The new pellet was resuspended in 1 M NaOH, and the amounts of the different polysaccharides were determined with the phenol-sulfuric acid method, using glucose as a standard (54). The data were standardized by the dry weight of biofilm, and the results are expressed as the percentage of the different components of polysaccharides by milligram of biomass (53, 55).

Biofilm structural imaging. The architecture of *S. mutans* biofilms and the distribution of bacteria were examined by scanning electron microscopy (SEM), which was performed by using a slight modification of previously described methods (56). The preparation of resin disk specimens was based on the methods described by other researchers (57). The sterilized resin disks were placed in sterile 24-well microtiter plates, and *S. mutans* was inoculated at a ratio of 1:10 (vol/vol) of the prepared standard bacterial suspension to the respective medium solution. After 48 h of anaerobic incubation, the biofilms that adhered to the resin disks were obtained.

The biofilms were gently washed with PBS to remove the unattached bacteria and the medium remaining on the specimen. The specimens were fixed with prepared 2.5% glutaraldehyde solution for 12 h at 4°C in the dark, serially dehydrated with ethanol, and then frozen at -20°C , -40°C , and -80°C for 6 h. Afterwards, the specimens were placed in a freeze dryer (model Lyovac GT2; SRK System Technology Co., Ltd., Germany) and vacuum dried for 12 h. The specimens were fixed on the stage with conductive glue, sputter coated with gold by use of an ion sputtering apparatus in a vacuum, and the specimens were observed using a scanning electron microscope (model Quanta 250 FEG; FEI, USA) at magnifications ranging from $\times 2,000$ to $\times 30,000$. Three randomly selected areas from each specimen were assessed for a total of three replicates.

Transcriptional analysis by RT-qPCR. The sterilized glass coverslips were placed in sterile 24-well plates, and *S. mutans* was inoculated at a ratio of 1:10 (vol/vol) of the prepared standard bacterial suspension to each group of medium solutions. After 5 days of anaerobic culture, the biofilm samples were obtained on the surface of glass coverslips and RNA isolation was started. Total RNA was extracted on ice using a TransZol Up Plus RNA kit (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. The concentration and purity of the total RNA obtained were determined spectrophotometrically with a NanoDrop instrument (Thermo Scientific, USA). The integrity of the RNA was assessed by agarose gel electrophoresis under UV spectroscopy.

cDNA was synthesized with a PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Dalian, China). Real-time reverse transcription (RT)-quantitative PCR (qPCR) was performed using ChamQ Universal SYBR qPCR master mix (Vazyme, Nanjing, China). The primers (Table 2) were designed and synthesized by Sunya Biotechnology Co., Ltd. (Henan, China). Real-time PCR detection with a LineGene 9600 instrument (Bioer Technology Co. Ltd., Hangzhou, China) started from an initial denaturation at 95°C for 30 s, and then 40 cycles of amplification, including denaturation at 95°C for 10 s, followed by annealing and extension at 60°C for 30 s, were performed.

The relative expression of each target gene was normalized to that of the internal control (the 16S rRNA gene of *S. mutans*). The melting curve profile was analyzed to evaluate the amplification specificity (58). The critical threshold cycle (C_T) was defined as the cycle in which fluorescence could be detected above the background fluorescence and was inversely proportional to the logarithm of the initial number of template molecules. The data were analyzed by the instrument supporting software. Each assay was performed with at least three independent RNA samples in duplicate.

TABLE 2 Nucleotide sequences of the primers

Primer name	Primer sequence (5'–3')	Gene description
16S-F	CCTACGGGAGGCAGCAGTAG	Normalizing internal standard
16S-R	CAACAGAGCTTTACGATCCGAAA	
gtfB-F	GGTCACTGGTGCTCAATCAAT	Glucosyltransferase B (GTfB)
gtfB-R	AAGCGTAAGTTCCATCTTCATTCT	
gtfC-F	TCAGACAACACCTTCCTTCCTA	Glucosyltransferase SI (GTfC)
gtfC-R	GAGCACCAGTGACCATATAACC	
gtfD-F	GCCTTTTACGCTTGTTTGT	Glucosyltransferase I (GTfD)
gtfD-R	CCATATTCATATTCTCCGCC	
ftf-F	CGAACGGCGACTTACTCTTAT	Fructosyltransferase (FTf)
ftf-R	TTACCTGCGACTTCATTACGATT	
spaP-F	TCCGTGCCGATAATCCAAGA	Surface-associated protein P1
spaP-R	CGCTGTTTGTCCCATTTGT	
gbpB-F	AGCAGCGGCAGGATATAGAG	Glucan-binding proteins (GbpB)
gbpB-R	ACCAACCACGGTAGTTACCAATA	
ldh-F	TTGCTCGTATCACTAAGGCTATTC	Lactate dehydrogenase (LDH)
ldh-R	GGGCTGACCGATAAAGACTTC	
atpF-F	TTGATAACGCTAAGGAACTGGTA	H ⁺ -translocating F-ATPase
atpF-R	AACGCTTGATAGGGCTCTCTG	
vicR-F	GCATCACTTAGCGACACACA	Two-component regulatory system regulator
vicR-R	CAGACGACGAACAGTAACATCAA	
comD-F	ATGGTCTGCTGCCTGTTG	Com-dependent QS system
comD-R	CGATCATATAGGTGGTTA	

Statistical analysis. Data are expressed as the mean values $\pm$ standard deviations (SD) and were analyzed for statistical significance using GraphPad Prism (version 8.0.2) software (GraphPad Software, San Diego, CA, USA). After one-way analysis of variance (ANOVA) was performed, the data were compared using Dunnett's multiple-comparison test or Tukey's multiple-comparison test. All experiments were performed at least in triplicate. Differences were considered significant at the 95% confidence level.

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Chunru Guan and Jinpu Chu participated in the design of this study, and they both performed the statistical analysis. Chunru Guan and Faai Che carried out the study and collected important background information. Huoxiang Zhou provided assistance with data acquisition and statistical analysis. Yiwei Li, Chunru Guan, and Yaru Li carried out a literature search, data acquisition, and manuscript editing. All authors read and approved the final manuscript.

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