Discovery of Novel Iminosugar Compounds Produced by Lactobacillus paragasseri MJM60645 and Their Anti-Biofilm Activity against Streptococcus mutans

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ABSTRACT The oral cavity contains a number of microbes. They interact with each other and play an important role in human health. Among oral cariogenic microbes, Streptococcus mutans is recognized a major etiological bacteria of dental caries. Lactobacilli strains have been promoted as possible probiotic agents against S. mutans. However, their inhibitory mechanism has not been well elucidated yet. In the present study, two new compounds with strong antibiofilm activities were purified from the culture supernatant of Lactobacillus paragasseri MJM60645, which was isolated from the human oral cavity. These compounds showed strong inhibitory activities against S. mutans biofilm formation, with IC50 (concentration at which 50% biofilm was inhibited) of 30.4 μM for compound 1 and 18.9 μM for compound 2. However, these compounds did not show bactericidal activities against S. mutans. Structure elucidation by nuclear magnetic resonance (NMR) and mass spectrometry showed that compound 1 was composed of two arabinofuranose iminosugars jointed with one glycerol and oleic acid, and compound 2 was composed of two arabinofuranose iminosugars jointed with one glycerol and nervonic acid. To the best of our knowledge, these structures were discovered for the first time in this study. Treatment of S. mutans with compound 1 strongly downregulated expression levels of genes related to biofilm formation, including gtfB, gtfC, gtfD, gbpB, brpA, spaP, ftf, and smu0630 without affecting the expression of comDE or relA. This study provides new insights into novel molecules produced by Lactobacillus to regulate the pathogenesis of S. mutans, facilitating a better understanding of the mechanism for interactions between Lactobacillus and S. mutans.

IMPORTANCE In this study, we isolated lactic acid bacteria that inhibit streptococcal biofilm from the oral cavity of infants and identified two novel compounds from the supernatant of their culture broth. The two compounds are structurally similar, and both consist of iminosugars, glycerol, and unsaturated fatty acid. A search of the SciFinder database revealed that these structures are novel and were discovered for the first time in this study. Mechanism studies have shown that these compounds can inhibit the expression of biofilm synthesis-related genes. This is the first report that lactic acid bacteria inhibit streptococcal biofilms by small molecules with new chemical structures. This study not only expands the understanding of natural products derived from lactic acid bacteria but also provides a new paradigm for the understanding of the interaction of bacteria in the oral microbiota.

KEYWORDS Lactobacillus paragasseri, iminosugar, Streptococcus mutans, biofilm, oral microbiota
Dental caries is one of the most common infectious diseases in humans and is closely related to human cardiovascular disease (1). It is a result of the homeostatic imbalance of oral microbiota. Although different bacteria are associated with the pathogenesis of dental caries, the mutans streptococcal group represented by Streptococcus mutans is considered to be a major cariogenic bacteria in oral cavity (2). S. mutans can colonize the hard tissues of tooth surface and mediate cariogenic biofilm formation, which enables them to survive in harsh environments.

Biofilm is a complex structure of bacteria consortium and extracellular polymeric matrix, consisting mainly of polysaccharides, proteins, and nucleic acids produced by bacteria or fungi (3). The so-called pathogenic biofilm is considered one of the most important and difficult to cure forms of microbial pathogenicity (4–6). Biofilm was formed via several complex processes, and many virulence factors are involved in bacterial adhesion, exopolysaccharide formation, sugar uptake, and acid tolerance (7). In S. mutans, water-insoluble (α-1,3-linked) and soluble (α-1,6-linked) glucans play an important role in the biofilm formation. They were synthesized by glucosyltransferases (GTFs) from sucrose (8). Glucans not only constitute the major polymeric matrix of biofilm, which can support the structural integrity and stability of the biofilm, but also mediate S. mutans to adhere to the tooth surfaces and enhance the aggregation and coaggregation of the bacterial cells (9). Currently, dental plaque is mainly controlled by the treatment of broad-spectrum antibiotics or mechanical removal, but the effect is limited (10). Although many strategies have been settled to control biofilms, the pursuit of natural, safe, and effective antibiofilm agents continues (11, 12).

Lactobacillus species as probiotic bacteria are being used in many dairy products to provide health benefits (13). One of the beneficial effects of Lactobacillus species as probiotics is the inhibition of pathogen infection. The mechanism includes the production of antimicrobial compounds such as organic acids, H2O2, bacteriocins, and adhesive inhibitors; improvement of epithelial barrier function; and inhibition of the adhesive ability of pathogens to epithelial cells (14, 15). Lactobacillus also has been reported that exerts healthy effects on the oral cavity. For example, the culture supernatant of Lactobacillus acidophilus can attenuate gingivitis and alleviate periodontitis (16). In addition, Lactobacillus rhamnosus GG, Lactobacillus plantarum, and Lactobacillus reuteri were reported to inhibit biofilm formation of S. mutans (17). Coculture of Lactobacillus salivarius with S. mutans inhibited the biofilm formation by regulating the glucosyltransferase (gtf) genes expression. However, most studies focused on the inhibition of biofilm formation by using cells, and the mechanisms by which lactic acid bacteria (LAB) inhibits the formation of biofilm have not been well understood. Recently, Ahn et al. (18) reported that lipoteichoic acid, the cell wall component, isolated from L. plantarum KCTC10887BP can reduce biofilm formation of S. mutans without bactericidal activity. The structure of lipoteichoic acid was crucial for inhibitory activity.

However, few studies have investigated the mechanism of supernatants of Lactobacillus on biofilm formation by S. mutans. Although substances such as organic acids and hydrogen peroxide in the supernatant contributed to a large extent to the inhibitory effect against biofilm formation, some studies have shown that even neutralized and catalase-treated supernatant still showed inhibitory activity (19). This shows the possibility that some compounds other than acid and hydrogen peroxide may be present in the supernatant. To investigate the exometabolites produced by LAB and explore the application of exometabolites on dental caries, this study focused on the exploration of the antibiofilm compounds from the supernatant of LAB. For this aim, we isolated lactic acid bacteria from the saliva of infants and screened the ethyl acetate extract of supernatant for antibiofilm activity. As a result, two single compounds with strong antibiofilm activities were purified, and the structure analysis showed that these compounds are iminosugar compounds, the structures of which are discovered for the first time in this study. Furthermore, the inhibitory mechanism was studied in the gene levels.
RESULTS

Isolation and characterization of isolates from an infant’s oral cavity. A total of 11 LAB strains were isolated from the oral cavity of infants. 16S rRNA gene sequences showed that four strains were *Streptococcus rubneri*, three strains were *Lactobacillus paragasseri*, two strains were *Streptococcus salivarius*, and two strains were *Leuconostoc mesenteroides*. Among these isolates, strain MJM60645 exhibited the highest antagonistic activity, and its extract showed the strongest antibiofilm activity (Table 1). Phylogenetic analysis based on the 16S rRNA sequence exhibited that MJM60645 was more closely related to *L. paragasseri* JCM 5343 (99%) than to *Lactobacillus gasseri* ATCC 33323 (98.3%) (Fig. 1).

**Antagonistic activity of the isolates and antibiofilm activity of the LAB crude ethyl acetate (EtOAc) extracts.** The antagonistic test showed that the *L. paragasseri* MJM60645 strain showed the strongest antagonistic activity against *S. mutans*. *S. rubneri, L. mesenteroides, and S. salivarius* strains showed relatively weak or moderate activity, and the inhibition zone ranged from 15 to 18 mm. However, one of the *S. salivarius* and one of the *L. mesenteroides* showed strong activities with inhibition zones greater than 25 mm in diameter.

Crude EtOAc extracts (1 mg/mL) from different LAB strains were treated with *S. mutans* KCTC3065, and the antibiofilm activities were assessed. Crude extract from *L. paragasseri* MJM60645 showed strong antibiofilm activity, extracts from *L. mesenteroides* showed moderate activity, and extracts from *S. salivarius* and *S. rubneri* showed moderate or low activity (Table 1).

**Stability of crude BuOH extract of *L. paragasseri* MJM60645.** To assess the pH and thermal stability of crude BuOH extract, samples were exposed to different pH levels (pH 4, 6, 8, 10, and 12) and different temperatures (50, 80, 100, and 121°C). It was found that the crude extract could strongly inhibit biofilm formation when treated with acidic conditions, and the activity was stronger than control. In neutral or alkali conditions, the extract of *L. paragasseri* MJM60645 lost activity to a certain extent but still showed activity compared with nontreated control (Fig. 2A). Interestingly, the crude extract showed significant thermal stability, and the inhibitory activity remained unchanged after treatment at different temperatures for different times, even at 121°C for 15 min (Fig. 2B).

**Effect of crude BuOH extract on the viable cell number of planktonic and biofilm.** As shown in Fig. 3A, the effect of crude BuOH extract on the cell population was determined by the CFU of *S. mutans*. In the control group, the log CFU of total bacteria (planktonic + biofilm cell) is 9.1. In the treatment group, the log CFU were 8.84, 8.75, and 8.72 at the concentration of 0.25, 0.5, and 1 mg/mL, respectively. There was a 0.2 to 0.3 log unit CFU of decrease in the treatment groups compared with the control.

*S. mutans* embedded in the biofilm were compared to the total bacteria (planktonic + biofilm cells) and is expressed as a percentage. The crude extract inhibited the percentage

| Strain no. | Strain namea | Antagonistic activity (mm) | Antibiofilm activityb (%) |
|-----------|--------------|---------------------------|----------------------------|
| MJM60645  | *Lactobacillus paragasseri* | 30 | 48 |
| MJM60646  | *Lactobacillus paragasseri* | 28 | 42 |
| MJM60648  | *Lactobacillus paragasseri* | 27 | 40 |
| MJM60649  | *Streptococcus rubneri* | 15 | 2 |
| MJM60651  | *Leuconostoc mesenteroides* | 26 | 34 |
| MJM60654  | *Streptococcus salivarius* | 25 | 21 |
| MJM60655  | *Streptococcus salivarius* | 15 | 15 |
| MJM60657  | *Leuconostoc mesenteroides* | 18 | 31 |
| MJM60658  | *Streptococcus rubneri* | 15 | 8 |
| MJM60659  | *Streptococcus rubneri* | 16 | 2 |
| MJM60660  | *Streptococcus rubneri* | 15 | 6 |

aThe highest similarity based on the 16S rRNA gene sequence.
bAntibiofilm activity of crude EtOAc extract at the concentration of 1 mg/mL.
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of *S. mutans* embedded in the biofilm in a dose-dependent manner. The percentage of cell number in biofilm decreased with the increase of the concentration treated, while the percentage of the planktonic cell increased. At a concentration of 1 mg/mL, less than 20% of *S. mutans* were embedded in the biofilm (Fig. 3B).

**Isolation and structure identification of the active compounds.** To isolate the active compounds, organic solvent extraction and chromatography were applied. Among the 16 fractions collected from reverse-phase chromatography, fractions LPE-B-SEC6-RP7 (compound 1) and LPE-B-SEC6-RP10 (compound 2) showed a single spot in the thin-layer chromatography (TLC) analysis and strong activity in the antibiofilm assay. These two compounds have no UV absorption at 254 nm nor 365 nm but can be visualized by 10% H₂SO₄ staining. The structures were identified primarily via one- and two-dimensional NMR spectroscopy and high-resolution electrospray ionization-time of flight mass spectrometry (HR-ESI-TOF-MS).

Compound 1 was isolated as a yellowish oil, and its molecular formula was established as C₃₁H₅₉N₂O₁₀ by HR-ESI-TOF-MS (m/z 619.4218 [M+H]+; calculated, 619.4164; error, 8.7 ppm). Analysis of H and C NMR spectra of compound 1 indicated that it was an iminosugar compound. In H NMR, the presence of the olefin methane proton signal was confirmed at δₙ (2H, t-like, J = 4.8 Hz, H-9',10'). The presence of two oxygenated methylene signals (δₙ, 4.22 [2H, dd, J = 7.2, 7.2 Hz, H-1], 3.66 [2H, dd, J = 7.8, 7.8 Hz, H-3]) and one oxygenated methane proton signal δₙ, 3.51 (1H, overlapped, H-2) indicated the presence of a molecule of glycerol. The proton at δₙ, 4.22 (H-1) showed a signal at a lower field than the other oxygenated methylene proton signal due to the esterification effect caused by the combination of oleic acid. In addition, the presence of two pentoses was confirmed by the two hemiacetal proton signals (δₙ, 4.47 [1H, d, J = 4.2 Hz, H-1”]), 3.97 [1H, d, J = 3.5 Hz, H-1”]).

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**FIG 1** Neighbor-joining tree based on the 16S rRNA gene sequences showing the phylogenetic relationship of strain MJM60645 and its related taxa. The accession numbers in parentheses were obtained from NCBI. The numbers at nodes indicate percentage levels of bootstrap support based on a neighbor-joining method of 1,000 replications. The evolutionary distances were computed using the Kimura two-parameter method. The scale bar indicates 0.005 substitutions per nucleotide position.

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Lactobacillus paragasseri JCM 5343T (AP018549)
Lactobacillus gasseri ATCC 33323T (CP000413)
Lactobacillus taiwanensis DSM 21401T (AYZG01000031)
Lactobacillus johnsonii ATCC 33200T (ACGR01000047)
Lactobacillus hominis CRBIP 24.179T (CAKE01000027)
Lactobacillus rodentium MYMRS TLU1T (HQ851022)
Lactobacillus coli1111A-L1T (KU161105)
Lactobacillus iners DSM 13335T (ACLNO1000018)
[\text{H-1}^+]) was observed in a high field, indicating that the carbon in position 1 or 4 of the pentose was replaced with nitrogen. Based on the molecular weight, molecular formula, and $^1$H NMR data, compound 1 was a glycoside containing a oleic acid, a glycerol, and two nitrogen-containing arabinofuranoses (Fig. 4A).

![FIG 2](image2.png)

**FIG 2** pH and thermal stability of crude extract of *L. paragasser* MJM60645. (A) Biofilm formation in the presence of the crude extract of different pH values. Control was treated with water of different pH levels. (B) Biofilm formation after treated with crude extracts that were heated at different temperatures for 30 or 60 min or 121°C for 15 min. Control was not treated with the crude extract. The data are expressed as the means ± standard deviations from three biologically independent experiments. ****, $P < 0.0001$, indicating a significant difference compared with the control groups. Statistical analysis was performed with one-way analysis of variance (ANOVA) with Dunnett’s multiple-comparison test.

![FIG 3](image3.png)

**FIG 3** Effect of crude extract on the number of viable *S. mutans* in planktonic and biofilm cells. (A) The total viable *S. mutans* (planktonic + biofilm) after treated with the crude extracts at different concentrations. *, $P < 0.05$ by Student’s two-tailed t test. (B) The cells embedded in the biofilm were compared to the total cells. The CFU values represent the means ± standard deviations from three independent experiments. The asterisks indicate the significant difference between control and extract-treated groups. *, $P < 0.05$; **, $P < 0.01$. Statistical analysis was performed with one-way ANOVA with Dunnett’s multiple-comparison test.
In $^{13}$C NMR (150 MHz, CD$_3$OD, $\delta_C$) spectrum, one ester carbon signal $\delta_C$ 175.3 (C-1'), two olefin methane carbon signals ($\delta_C$ 130.9 [d, C-9'], 130.8 [d, C-10']), fourteen methylene carbon signals ($\delta_C$ 35.0 [C-2'], 33.1 [C-16'], 30.8 to 30.2 [C-4'-7',12'-15'], 28.1 [C-8'], 28.1 [C-11'], 26.0 [C-3'], and 23.7 [C-17']), and one terminal methyl carbon signal $\delta_C$ 14.5 (C-18') were observed in a low magnetic field. Compared with the reference, these signals represented the presence of oleic acid. The signal of one oxygenated methane $\delta_C$ 71.3 (C-2) and two oxygenated methylene carbon signals ($\delta_C$ 70.1 [C-3] and 64.6 [C-1]) indicated the presence of glycerol. The third carbon signal of glycerol was observed in $\delta_C$ 70.1, showing the shift to the low field due to the glycosidation effect of the combination of glycerol with a glycoside. In addition, the presence of two hemiacetal ($\delta_C$ 87.3 [C-1'], 86.0 [C-1'']), six oxygenated methine ($\delta_C$ 81.9 [C-2''], 81.8 [C-2'], 74.4 [C-3''], 73.6 [C-3''], 71.1 [C-4''], and 70.9 [C-4'']), and two oxygenated methylene carbon signals ($\delta_C$ 70.0 [C-5''] and 62.2 [C-5'']) indicated the presence of two pentoses. Due to the signal for hemiacetal carbon $\delta_C$ 87.3 [C-1'] and 86.0 [C-1'']) being observed in Highfield, we predicted that the carbon in one or four positions in pentose was replaced with nitrogen. If the carbon in position 1 was replaced with nitrogen, there should be a peak at 45 to 55 ppm according to reference 20. The absence of the peak at 45 to 55 ppm confirmed that the carbon in position 4 was replaced with nitrogen as $\beta$-$\alpha$-4-amino-4-deoxy-arabinofuranose. Moreover, the shift of the carbon signal of C-5'' ($\delta_C$ 70.0) to the low field due to the glycosidation effect indicated that the two pentoses were connected as $\beta$-$\alpha$-4-amino-4-deoxy-arabinofuranosyl-(1'\rightarrow5')-$\beta$-$\alpha$-4-amino-4-deoxy-arabinofuranose.

In the gradient-selected heteronuclear multiple-bond correlation (gHMBC) spectrum, the cross-peak between the H-1 proton ($\delta_H$ 4.22) of glycerol and ester carbon (C-1', $\delta_C$ 175.3) of oleic acid indicated the linkage of oleic acid to the oxygenated methylene of...
glycerol C-1. The cross-peaks between δ_H 4.47 (H-1”) and δ_C 70.1 (C-3), between δ_H 3.97 (H-1”) and δ_C 70.0 (C-5”), and between δ_H 3.60 (H-5”) and δ_C 86.0 (C-1”) indicated that β-D-arabinofuranose was linked to the C-3 of glycerol, and another β-D-arabinofuranose linked to the oxygenated methylene C-5 of β-D-arabinofuranose. Combined with the 2D NMR data and mass spectrum, compound 1 was finally identified as 1-O-oleoyl-3-O-[β-D-4-amino-4-deoxy-arabinofuranosyl-(1→5)-O-β-D-4-amino-4-deoxy-rabinofuranosyl]-glycerol, a new iminosugar compound (Fig. 4A).

Compound 2 was obtained as yellowish oil. Its molecular formula was deduced as C_{37}H_{70}N_{2}O_{10} from NMR and positive-ion HR-ESI-QTOF-MS data (m/z 747.4981 [M+FA-H−]; calculated, 747.5013; error, 4.2 ppm). A comparison of the 1D NMR data (Table 2) of compound 2 with those of compound 1 revealed that they shared the same sugar and glycerol moiety, and the only obvious difference between them was that the fatty acid connected to the glycerol moiety is nervonic acid in compound 2, compared with oleic acid in compound 1. Compound 2 was finally identified as 1-O-nervonoyl-3-O-[β-D-4-amino-4-deoxy-arabinofuranosyl-(1→5)-O-β-D-4-amino-4-deoxy-rabinofuranosyl]-glycerol (Fig. 4B). The key NMR and mass spectrum are presented in

### TABLE 2

| No. | Compound 1 | Compound 2 |
|-----|------------|------------|
| 1   | 4.22 (dd, 7.2, 7.2) | 4.17 (d, 4.8) |
| 2   | 3.51 (overlapped) | 3.51 (overlapped) |
| 3   | 3.66 (dd, 7.8, 7.8) | 3.66 (dd, 5.6, 5.6) |
| 4'  | 1.58 (t-like, 7.2) | 1.58 (t-like, 7.2) |
| 5   | 1.27 (overlapped) | 1.26 (overlapped) |
| 6'  | 1.27 (overlapped) | 1.26 (overlapped) |
| 7   | 1.27 (overlapped) | 1.26 (overlapped) |
| 8   | 2.00 (overlapped) | 1.26 (overlapped) |
| 9   | 5.13 (t-like, 4.8) | 1.26 (overlapped) |
| 10' | 5.13 (t-like, 4.8) | 1.26 (overlapped) |
| 11' | 2.00 (overlapped) | 1.26 (overlapped) |
| 12' | 1.27 (overlapped) | 1.26 (overlapped) |
| 13' | 1.27 (overlapped) | 1.26 (overlapped) |
| 14' | 1.27 (overlapped) | 2.00 (overlapped) |
| 15' | 1.27 (overlapped) | 5.31 (m) |
| 16' | 1.27 (overlapped) | 5.31 (m) |
| 17' | 1.27 (overlapped) | 2.00 (overlapped) |
| 18' | 0.87 (t, 7.2) | 14.5 |
| 19' | 1.26 (overlapped) | 30.8-30.1 |
| 20' | 1.26 (overlapped) | 30.8-30.1 |
| 21' | 1.26 (overlapped) | 30.8-30.1 |
| 22' | 1.26 (overlapped) | 33.1 |
| 23' | 1.26 (overlapped) | 23.7 |
| 24' | 0.87 (t, 6.6) | 14.5 |
| 1   | 4.47 (d, 4.2) | 87.3 |
| 2'  | 4.60 (dd, 4.8, 4.2) | 81.8 |
| 3'  | 3.90 (overlapped) | 74.4 |
| 4'  | 3.89 (overlapped) | 71.1 |
| 5'  | 3.6 (overlapped) | 70.0 |
| 1'' | 3.97 (d, 3.5) | 86.0 |
| 2'' | 4.07 (m) | 81.9 |
| 3'' | 3.52 (dd, 4.8, 4.8) | 73.6 |
| 4'' | 3.76 (m) | 70.9 |
| 5'' | 3.60 (overlapped) | 62.2 |

*aNMR, nuclear magnetic resonance.
supplementary data (Fig. S2 to S13), and the detailed $^1$H and $^{13}$C assignments are given in Table 2.

**Determination of IC$_{50}$ values of compounds 1 and 2.** The concentration that inhibits *S. mutans* biofilm formation by 50% (IC$_{50}$) for compounds 1 and 2 were determined by serial dilution. *S. mutans* were treated with compound 1 or 2 at a concentration ranging from 1 to 160 μM. It was found that biofilm formation was dose-dependently inhibited by either compound 1 or 2. The IC$_{50}$ values were determined to be 30.42 and 18.95 μM for compounds 1 and 2, respectively (Fig. 5A and B).

**Effect of compounds 1 and 2 on early and mature biofilm formation of *S. mutans.*** When the active compounds were treated at the beginning of the biofilm assay, they showed significant inhibitory activities to the biofilm formation of *S. mutans*. Both compound 1 (treated at 60 μM) and compound 2 (treated at 38 μM) inhibited more than 90% of biofilm (Fig. 6A). However, there was no significant difference between the control and treated groups when the active compounds were treated to the preformed biofilm and incubated for a further 24 h (Fig. 6B).

**Expression of the biofilm-associated gene of *S. mutans* after treatment with compound 1.** Since compound 2 was purified in trace amounts, we evaluated the
effect of compound 1 on the expression of biofilm-associated genes. Relative expression levels of selected genes were examined by real-time quantitative PCR (qRT-PCR). Compared with the control group, the expression levels of eight biofilm-associated genes (gtfB, gtfC, gtfD, gbpB, brpA, spaP, ftf, and smu0630) were significantly downregulated by compound 1. However, expression levels of comDE and relA genes in S. mutans showed no significant difference between the treatment group and the control group (Fig. 7).

**DISCUSSION**

Oral microbiota is constituted of a large number of microorganisms; dominant among them are Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, Fusobacteria, etc. (21). These bacteria are closely related to oral health. Among them, S. mutans is considered a primary causative agent of dental caries, and one of the virulence properties of this bacteria was to form a biofilm known as dental plaque on the tooth surface (22). On the other hand, one of the beneficial bacteria that live in the oral cavity is lactic acid bacteria, like S. salivarius and Lactobacillus spp. LAB was reported to inhibit the biofilm formation of S. mutans either by coculture (23) or by treatment of the culture supernatant to the S. mutans (24). Even though the use of Lactobacillus strains for probiotic applications has been carried out recently (25), the mechanism for the interaction between Lactobacillus and S. mutans remains largely unknown. In this study, we screened the EtOAc extract of different LAB strains for the antibiofilm activity and isolated two iminosugar compounds from the culture supernatant of L. paragasseri MJM60645. To the best of our knowledge, this is the first report on the small molecule with strong antibiofilm activity isolated from the supernatant of Lactobacillus spp. Furthermore, the structures of these compounds were discovered for the first time through this study.

It was reported that Lactobacillus species, especially L. gasseri, were isolated more frequently from the saliva of breastfed infants than from formula-fed infants and displayed probiotic traits in vitro (26). Furthermore, Lactobacillus strains from caries-free subjects possess antimicrobial activity against the cariogenic species of S. mutans and Streptococcus sobrinus, and the activity was more efficient than those from caries-active subjects (27). Therefore, this study was initiated based on the hypothesis that Lactobacillus from caries-free infants may play a beneficial effect on oral health. In this study, four species of LAB strains were isolated from the infant’s saliva in the MRS medium, including S. salivarius and S. rubneri from a 30-month-old child, L. mesenteroides from a 10-month-old baby, and

**FIG 7** Effect of compound 1 on the expression of biofilm-associated genes in S. mutans. S. mutans was treated with compound 1 at the concentration of 60 μM in a BHI medium containing 0.2% of sucrose and incubated anaerobically at 37°C for 24 h. Biofilm cells were harvested, and the expression of biofilm-associated genes was assessed by real-time quantitative PCR (qRT-PCR). The mRNA expression levels were calibrated using 16S rRNA. The data are expressed as the means ± standard deviations from three biologically independent experiments. The asterisks indicate that the gene expression between the control group (white bars) and the compound 1-treated group (black bars) was significantly different. *, *P < 0.05; **, *P < 0.01; ***, *P < 0.001. Statistical analysis was performed with one-way ANOVA with Dunnett’s multiple-comparison test.
*L. paragasseri* from a 1-month-old infant. *S. salivarius* and *S. rubneri* are commonly isolated from the human cavity and upper respiratory tract (28, 29), and some strains of *S. salivarius* were used as a probiotic for improving oral health (28). *L. mesenteroides* is typically found on the surface of many fresh fruits and vegetables and is associated with fermentation. Intake of fermentative food, fruits, and vegetables by a 10-month-old baby may be the reason for the existence of *L. mesenteroides* in the oral cavity. *L. gasseri* is a widely studied probiotic species with various biological functions, including oral probiotic characteristics (30). *L. gasseri* is known as a part of the vaginal flora, and some researchers isolated *L. gasseri* from breast milk (31) or saliva (27). Recently, many of *L. gasseri* were reclassified to *L. paragasseri* due to the distinct difference based on whole-genome relatedness, phylogenetic analyses of the marker gene, and phenotypic and chemotaxonomic characteristics (32).

According to the phylogenetic analysis of the 16S rRNA gene sequence, strain MJM60645, along with *L. paragasseri* JCM4353, did form a clade distinct from *L. gasseri* ATCC 33323\textsuperscript{T} (Fig. 1). Thus, strain MJM60645 was finally designated *L. paragasseri* MJM60645.

The first screening was carried out by evaluation of their potential antagonistic activities against *S. mutans*. Antagonistic activity against pathogens is an important characteristic in the selection of potential probiotic strains for maintaining a healthy microbial balance in the oro-gastrointestinal tract. In the present study, all isolates tested showed antagonistic activities against *S. mutans*, but the activities are various. Strains in the same species showed different activities, indicating that antagonistic activity is strain-specific, not species-specific. Among these strains, *L. paragasseri* MJM60645 showed the highest antagonistic effect. To investigate the extracellular metabolite produced by LAB, the supernatant of the culture supernatant from each LAB was extracted with ethyl acetate, a widely used organic solvent for extraction of biological compounds with various polarities from water solution. All of these extracts did not show the antimicrobial activity by the paper disc agar diffusion assay (data not shown). However, in the test for antibiofilm activity, these extracts showed inhibitory activity to the biofilm formation of *S. mutans* at different levels. Among them, the extract from *L. paragasseri* strains showed stronger activity than other extracts. Since these *L. paragasseri* strains were isolated from the same donor and showed similar activities, we speculated that these isolates were the same or were genetically related. Thus, one of the *L. paragasseri*, MJM60645, was selected for further study.

Before purification, the stability of crude extract from *L. paragasseri* MJM60645 was investigated. The crude extract treated in alkali and neutral conditions showed decreased activities, but extracts in acidic conditions showed strong activity. We later found that the pH of compound 1 in the water solution was acidic, which is why the crude extract lose most of its activity in neutral or alkali conditions. Although the control (water in pH 4) also showed inhibitory activity, the activity was weaker than the extract-treated group. This indicated that acidity was not the only reason for the inhibitory activity. The extract works well in acidic conditions, which will help to inhibit *S. mutans* because *S. mutans* make an acidic environment on the tooth surface to erode the tooth through acid production. In addition, the extract showed strong thermal stability under different temperatures, even under 121°C, indicating that the active compound is probably a small molecule rather than a macromolecular protein.

The total number of planktonic and biofilm cells was compared between the crude BuOH extract-treated and untreated groups. There was a 0.2 to 0.3 log CFU reduction in the total number of bacteria after being treated with BuOH extract, suggesting that the extract affected *S. mutans* but not a lot. This may be due to the growth rate or biofilm formation because the BuOH extract did not show any bactericidal activity by agar diffusion assay (data not shown). Interestingly, the ratio of bacteria embedded in biofilm was dose-dependently decreased by the treatment of crude BuOH extract, indicating that the extract influenced the formation of the biofilm, allowing more planktonic bacteria.

To purify the active compounds, the ethyl acetate extract of *L. paragasseri* supernatant was partitioned with several organic solvents and applied to size exclusion chromatography and open column reverse-phase chromatography (Fig. S1). The activity of...
these subfractions was monitored by an antibiofilm assay. Finally, two single compounds (compounds 1 and 2) were obtained. The structures were chemically characterized by \(^1\)H NMR (one- and two-dimensional) and \(^13\)C NMR, and the molecular weights were analyzed by high-resolution mass spectrometry (Fig. S2 to S13). The structure of compound 1 is composed of two iminosugars (arabinofuranose in which the cyclic oxygen is replaced with nitrogen), one molecule of glycerol, and one molecule of oleic acid, which were linearly connected by a hydrogen bond. Compound 2 is a structural analog of compound 1, in which oleic acid was replaced by nervonic acid (Fig. 4A and B). The novelty of the structures was confirmed on the SciFinder database.

Iminosugars are structural analogs of carbohydrates in which the oxygen atom is replaced by nitrogen. The first iminosugar, 1-deoxynojirimycin (DNJ), was isolated from mulberry in 1976, and a few others were discovered recently (33). Due to the structural similarity to the sugar, iminosugar compounds showed strong catalytic properties to ubiquitous carbohydrate processing enzymes, such as glycosidases and glycosyltransferase, exhibiting broad-spectrum therapeutic potential in the treatment of type II diabetes, lysosomal storage disorders, etc. Furthermore, the iminosugar class of compounds was known to show antiviral activity by inhibiting the endoplasmic reticulum (ER) \(\alpha\)-glucosidase activity due to the structural similarity to sugar molecules. Moreover, some researchers have investigated the effect of iminosugars on oral biofilms. It was reported that iminosugars can affect the biosynthesis of glucan; it might be good for caries prevention (34). Another recent study has showed that 1-deoxynojirimycin (from mulberry leaves) significantly inhibited the biofilm formation of \(S.\mutans\) (4, 35). We also found that compounds 1 and 2 could significantly inhibit biofilm formation when treated at an early stage but cannot inhibit mature biofilm (Fig. 6). This indicates that their antibiofilm mechanism might be based on the reduction of exopolysaccharides synthesis in the early stage of biofilm formation. Similarly, some iminosugars can also decrease the biofilm formation of \(P.\aeruginosa\) at an early stage of biofilm development but not in the mature biofilm stage (36). Our results were consistent with the previous report about iminosugars, meaning that the antibiofilm effect of the two new compounds might be due to the presence of iminosugar moieties in their structures.

Compounds 1 and 2 have structural differences only in the moiety of fatty acids, like oleic acid in compound 1 and nervonic acid in compound 2. Although the structure of iminosugar may play a major role in the activity, the difference in MIC suggests that fatty acid moiety may also affect the magnitude of the activity. Recent studies have shown that fatty acids (FAs) possess good antibiofilm activities. Some FAs were reported to inhibit biofilm formation of various pathogens (37, 38). Oleic acid (cis-9-octadecenoic acid) can inhibit initial adhesion and biofilm development by some \(S.\aureus\) strains (39, 40). However, it has been reported that treatment of oleic acid did not inhibit the biofilm formation of \(S.\mutans\) (41). When the fatty acids (saturated and unsaturated) were incorporated into \(S.\aureus\) through a FAs kinase-dependent pathway, the membrane fluidity will be changed (42, 43). Even though the fatty acid may not inhibit biofilm directly, it may affect the efficacy of the incorporation of compounds 1 and 2 into the cell, hence affecting the activities.

To adapt to a biofilm lifestyle, biofilm cells of \(S.\mutans\) experience phenotypic changes, accompanied by a series of genes that are up- or downregulated. Our studies revealed that compound 1 downregulated eight biofilm-associated genes in \(S.\mutans\). These genes included glucosyltransferase genes \(gtfb\), \(gtfc\), and \(gtfd\) (44, 45), adherence-associated gene \(spaf\), biofilm regulatory protein \(brpA\), glucan-binding protein \(gbpB\) (46), fructosyltransferase \(ff\) gene, and hypothetical protein \(smua630\). Glucosyltransferases \(gtfB\) and \(gtfc\) can utilize sucrose as a substrate to synthesize water-insoluble glucan, which is necessary for backbone construction and structural integrity of biofilms. Gene \(gtfd\) synthesizes \(\alpha\)-1,6-linked soluble glucan. It is reported that the alkali-soluble glucans are relatively more important than water-soluble glucan in bacterial adhesion (47). 1-Deoxynojirimycin (DNJ) was reported to inhibit glucosyltransferases B rather than glucosyltransferases C and D (48). Our result also showed that compound 1 inhibited \(gtfb\) more strongly than \(gtfc\)
and *gtfd* (Fig. 7). The *ttf* gene encodes fructosyltransferase that can synthesize fructan polymers, which provides binding sites for *S. mutans*, thus enhancing its biofilm formation (49). The suppression of *gtf* genes may be associated with reduced biofilm formation. In addition, some other genes involved in bacterial attachment to the tooth surface and biofilm structure integrity were also inhibited. Glucan-binding protein (coded by *gpbB*) has an affinity for glucans, which resulted in bacterial adhesion (46). Gene *spaP* (*Ag I/II or P1*) encodes a surface protein of the antigen, which is important for *S. mutans* to initially adhere to the tooth surface (50). Gene *brpA* (biofilm regulatory protein) is involved in biofilm development and its structural integrity. *Smu630*, a hypothetical protein, also contributes to biofilm formation (51). Downregulation of the expression of these genes can suppress glucan formation and cell adhesion, thus reducing biofilm formation. However, the expression of other biofilm-associated genes is not affected, including the *relA* gene that encodes guanosine tetra-(penta)-phosphate synthetase, which is involved in the mechanisms of acid and oxidative stress tolerance in *S. mutans* (52). The regulatory gene *comDE*, involving the quorum-sensing cascade of *S. mutans* (53), was also not affected by the treatment of compound 1 (Fig. 7). These results demonstrated that compound 1 inhibited biofilm mainly by downregulation of the expression of genes that are associated with cell adherence and biofilm development.

Currently, the selection of natural compounds seems to bring a new perspective to the development of antibiofilm compounds that can effectively inhibit the formation of exopolysaccharide chains, therefore increasing the permeability of biofilms to antibiotics, disinfectants, or nano compounds. In this study, we discovered two iminosugar compounds with novel structures from *L. paragasser* MJM60645. These compounds showed strong antibiofilm activity but not antimicrobial activity. This could facilitate the use of these compounds in removing biofilms without worrying about antibiotic resistance. Undoubtedly, more studies are needed to demonstrate the molecular mechanism of the inhibitory activity of active compounds and define the specificity and antibiofilm spectrum of these compounds against various bacterial species. It is also necessary for further study to investigate the synergic effect of these compounds when combined with antibiotics for the treatment of biofilm-forming clinical pathogens.

Another significance of this study is that we provide new clues about the interactions of oral microbes. It is necessary to investigate the production of these compounds or their analogs by other microbes in future work, and the inhibitory mechanism should be deeply elucidated. Furthermore, a study on the biosynthetic pathway of this novel iminosugar compounds will facilitate the discovery and development of more derivatives, which can be widely used for research and the medical field.

**MATERIALS AND METHODS**

**Sample collection and bacteria isolation.** Saliva samples were collected from three children aged from 1 month to 30 months. The number of erupted teeth per donor was 0 for a 1-month-old baby, 8 for a 10-month-old baby, and 20 for a 30-month-old child. All three children were caries-free. They had not taken any antibiotics in the previous 14 days. We explained the purpose of the study to the children’s parents, and they signed a consent form prior to saliva collection. The protocol for saliva collection was approved by the Institutional Review Board of Kyung Hee University (institutional review board approval KHSIRB-17-010). Briefly, a sterile cotton swab was put into the mouth of the child and rubbed two or three times until the cotton head was soaked with saliva. The cotton head was then suspended in a Falcon tube containing 3 mL of sterile saline (0.85% NaCl), stored at 4°C, and immediately taken to the laboratory.

The sample was serially diluted with sterile saline and plated onto de Man-Rogosa-Sharpe (MRS) agar plates. These plates were incubated at 37°C for 48 h under anaerobic conditions in GasPak containing an anaerobe gas-generating pouch system (BD). After incubation, colonies with different morphologies were picked for further characterization.

**Screening of antagonistic isolates.** Antagonistic activities of isolates against *S. mutans* KCTC3065 were assessed using an agar diffusion assay by Cadirci and Citak (17). *S. mutans* KCTC3065 was incubated in brain heart infusion (BHI) broth at 37°C for 24 h. The suspension of *S. mutans* was diluted with BHI broth medium to a turbidity equivalent to optical density at 600 nm (OD600) of 1. The melted BHI soft agar medium (0.7% agar) held at 45°C was then mixed with 0.1% (vol/vol) of *S. mutans* suspension, and 2 mL of the mixture was poured onto an MRS agar (1.5% agar) plate to make a top-covered test plate. A small hole was made on the top-covered test plate, and 5 μL of bacterial suspension of each
isolate (OD\textsubscript{600} = 1) was loaded into the hole. Inhibition zones were measured in millimeters after incubation at 37°C for 24 h.

**Phylogenetic analysis.** Genomic DNA was extracted and purified using the Exgene cell SV kit (GeneAll, South Korea). 16S rRNA gene was amplified using the universal primer set 27F/1492R (54) and sequenced. The resulting 16S rRNA gene sequence (1.5 kb) was blasted with available 16S rRNA gene sequences in the Ezbiocloud server (ChunLab Inc., South Korea). 16S rRNA gene sequences of closely related type strains were multiple aligned using CLUSTAL_X program (55). The phylogenetic tree was constructed with a neighbor-joining method and the Kimura two-parameter model using the MEGA 6 program (56). The robustness of individual branches was evaluated by bootstrapping of 1,000 replications.

**Preparation of crude extracts.** The isolates were inoculated in 50 mL of MRS medium and cultured at 37°C for 24 h. The culture broth was harvested and centrifuged at 7,000 rpm for 15 min. The cell-free supernatant was extracted with the same volume of ethyl acetate (EtOAc), and the ethyl acetate layer was concentrated under a vacuum to yield brownish-yellow extracts. For further purification, the EtOAc extract was dissolved in \textit{H\textsubscript{2}}O and partitioned with hexane and then with butanol (BuOH) to get a crude BuOH extract (Fig. S1).

**Biofilm inhibitory assay.** Biofilm formation was assessed using the protocol as described previously (57) with a few modifications. Briefly, \textit{S. mutans} KCTC3065 was cultured in BHI medium overnight and adjusted to an OD\textsubscript{600} of approximately 1 followed by a 100-fold dilution using BHI medium supplemented with 0.2% sucrose. Then 20 \muL of \textit{S. mutans} suspension, 160 \muL of BHI with 0.2% sucrose, and 20 \muL of the crude EtOAc extract (10 mg/mL in sterile distilled water) were added to a polystyrene 96-well culture plate and incubated at 37°C for 24 h. MRS broth or phosphate-buffered saline (PBS) only served as controls. After the incubation, media and unattached cells were decanted from the microtiter plate. The remaining planktonic cells were removed by gentle rinsing with sterile distilled water. To determine the mass of biofilm, wells with adhered biofilm were stained with 125 \muL of 0.1% safranin for 30 min at room temperature, washed three times with 200 \muL of distilled water, and air-dried. The dye was extracted in 125 \muL of 33% acetic acid, and the absorbance was measured at 492 nm by a microtiter plate reader (Tecan, Infinite M200PRO, Austria).

**\textit{pH} and thermal stability of crude extract.** The crude BuOH extract was dissolved in water at a concentration of 10 mg/mL. The samples were adjusted to different pH levels (pH 4, 6, 8, 10, and 12) using HCl or NaOH and used for the inhibition of biofilm of \textit{S. mutans} as described above. The distilled water was also adjusted to the same pH (4, 6, 8, 10, or 12) and served as a control group.

To evaluate the thermal stability, the crude BuOH extract was treated with different temperatures at 50, 60, 70, 80, and 100°C for 30 or 60 min and 121°C for 15 min. For samples were cooled down to room temperature and used for antibiofilm activity as described above.

**Effect of crude extract on the viable cell number of planktonic and biofilm.** The viable cell number of \textit{S. mutans} in planktonic and biofilm were determined by serial dilution and counting of the CFU on BHI agar. Briefly, \textit{S. mutans} was grown at 37°C for 24 h in glass tubes at an angle of 30° with the 3 mL of BHI broth containing 1% sucrose and various concentrations of crude BuOH extract. After incubation, the planktonic cell suspension was taken, and the cell number was determined by serial dilution and spread on the BHI agar plate. To determine the cell number in the biofilm, biofilm was scraped from the bottom of the tube and mechanically disrupted by vigorous pipetting and vortexing. These cells were serially diluted and streaked onto BHI plates. The number of colonies was then counted to determine the CFU. The percentage of \textit{S. mutans} embedded in biofilm was calculated as follows: number of biofilm cells/number of planktonic cells × 100%.

**Fermentation, purification, and identification of active compounds from the supernatant of \textit{L. paragasseri} MJM6064S.** \textit{L. paragasseri} MJM6064S was grown in 10 mL MRS broth for 18 h at 37°C. For fermentation, 5 mL of \textit{L. paragasseri} MJM6064S suspension was inoculated to each sterile flask containing 500 mL MRS broth and incubated at 37°C for 24 h. After incubation, the supernatant of \textit{L. paragasseri} MJM6064S were obtained by centrifugation at 7,000 rpm for 15 min.

An activity-guided fractionation was carried out to isolate the active compounds. Each fraction was subjected to antibiofilm bioassay and thin-layer chromatography (TLC) stained with \textit{H\textsubscript{2}}SO\textsubscript{4} (10%). Briefly, a total of 23 L of cell-free supernatant was extracted with ethyl acetate (1:1, vol/vol) and concentrated under a vacuum at 35°C. The \textit{L. paragasseri} ETOAc extract (LPE) was partitioned by hexane and butanol step by step and concentrated under a vacuum. The active fraction from the butanol layer (LPE-B) was subjected to size exclusion chromatography (SEC) packed with Sephadex LH-20 (GE Healthcare, Uppsala, Sweden) and eluted with methanol. Among 30 fractions obtained, the most active fraction, LPE-B-SEC6 (335 mg), was subjected to reverse-phase open column chromatography (φ = 2.5 × 5 cm) packed with Lichroprep RP-18 (40 to 63 μm; Merck, Darmstadt, Germany) and eluted with a stepwise gradient of acetone/water (65:35, 80:20, and 100:0) to obtain 16 subfractions (LPE-B-SEC6-RP1 to RP16). After bioassay, fractions LPE-B-SEC6-RP7 (compound 1) and LPE-B-SEC6-RP10 (compound 2) showing the strongest antibiofilm activity were finally used for the structure identification.

Chemical structures of purified compounds were analyzed by nuclear magnetic resonance (NMR, one- or two-dimensional) and mass spectrometry. NMR data were acquired with a Bruker Avance III-600 MHz recorded in CD\textsubscript{3}OD. HR-ESI-QTOF-MS spectra were obtained on a Bruker MaXis quadrupole time of flight (QTOF) mass spectrometer.

**Determination of IC\textsubscript{50} values of compounds 1 and 2.** The IC\textsubscript{50} (concentration at which 50% biofilm was inhibited) values of compounds 1 and 2 were evaluated as described previously (58). Briefly, 180 \muL of BHI medium containing 0.2% sucrose was treated with serially diluted compound 1 or 2, inoculated with 20 \muL of \textit{S. mutans} (OD\textsubscript{600} = 0.01), and incubated at 37°C for 24 h. The final concentration of each
tested compound ranged from 1 to 160 μM. Biofilm assessment was carried out as described above. Bacteria treated with 20 μL of sterile distilled water were used as control.

**Effect of compounds 1 and 2 on early and mature biofilm formation of S. mutans.** To determine the effect of compounds 1 and 2 on the early biofilm formation, compounds 1 and 2 were treated to S. mutans at the concentration of 60 and 38 μM in a BHI medium containing 0.2% sucrose in a 96-well plate as described above. The plates were incubated at 37°C for 24 h, and the biofilm was quantified. To determine whether the active compounds could disperse preformed biofilms, bacterial biofilms were established for 24 h in a 96-well plate and then treated with compounds 1 and 2 at the concentration of 60 and 38 μM. The plate was incubated for an additional 24 h, and the biofilm was quantified. S. mutans treated with PBS served as the control.

The effect of compound 1 on biofilm-associated gene expression. The effect of compound 1 on gene expression was determined as described (49) with slight modification. The overnight culture of S. mutans was diluted to OD600 of 0.01 with BHI medium supplemented with 0.2% sucrose. S. mutans suspension was transferred to a 6-well plate and treated with compound 1 at the concentration of 60 μM. Only PBS served as control. The 6-well plate was incubated anaerobically at 37°C for 24 h. After incubation, the culture suspension was discarded. The cells adhering to the plate wells were gently rinsed twice with PBS, then dislodged, and suspended in saline by scraping and pipetting. Total RNA was isolated with a TaKaRa MiniBEST universal RNA extraction kit (TaKaRa Bio, Shiga, Japan). RNA concentration and purity were detected by the Infinite F200 microplate reader (Tecan, Austria). RNA (1 μg) was used to synthesize cDNA after removing the remaining genomic DNA in RNA samples by using PrimeScript RT reagent kit with gDNA eraser (TaKaRa Bio Inc., Shiga, Japan).

Quantitative real-time reverse transcription-PCR (qRT-PCR) was performed using a LightCycler 96 system (Roche, Basel, Switzerland). Each reaction mixture (20 μL) contained 100 ng of cDNA, 0.5 μM each primer, and 10 μL of SYBR Premix Ex Taq (TaKaRa, Japan). The qRT-PCR conditions were as follows: one cycle with 95°C for 2 min and then 40 cycles of denaturation at 95°C for 10 s, annealing, and extension at 57°C for 60 s. In all qRT-PCR runs, the negative controls without templates were run in parallel. The 16S rRNA gene, a housekeeping gene, was used as an internal control. The relative mRNA levels of genes of interest were determined and normalized to the expression of the housekeeping gene using the 2−ΔΔCt method (59). Sequences of primers for qRT-PCR are listed in Table 3.

**Statistical analysis.** The experimental results were analyzed for statistical significance using GraphPad Prism (GraphPad, San Diego, CA). The results of biofilm formation quantification in the presence of crude extract, cell growth, IC50, and gene expression were subjected to a one-way analysis of variance (ANOVA). Data comparisons were performed using Dunnett’s multiple-comparison test. P values < 0.05 were considered statistically significant.

**Data availability.** The raw data used to support the findings of this study will be made available by the authors, without undue reservation, to any qualified researcher.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.**
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M.G. and J.C. designed the research study, conducted experiments, acquired the data, analyzed the data, and wrote the manuscript. Y.L. elucidated the structure of the compounds. J.C. and J.S. contributed to the conception of the study, analyzed the data, and obtained the funding. All authors have read and agreed to the published version of the manuscript.

We declare no conflict of interest.

REFERENCES

1. Lamont RJ, Egland PG. 2015. Dental caries, p 945–955. In Tang YW, Sussman M, Liu D, Poixton I, Schwartzman J (ed), Molecular medical microbiology, 2nd ed. Academic Press, Boston, MA.

2. Forresten SD, Björklund M, Ouwehand AC. 2010. Streptococcus mutans, caries and simulation models. Nutrients 2:290–298. https://doi.org/10.3390/nu2030290

3. Matsumoto-Nakano M. 2018. Role of Streptococcus mutans surface proteins for biofilm formation. Jpn Dent Sci Rev 54:22–29. https://doi.org/10.1016/j.jdsr.2017.08.002

4. Suci PA, Mittelman MW, Yu FP, Geesey GG. 1994. Investigation of ciprofloxacin penetration into Pseudomonas aeruginosa biofilms. Antimicrob Agents Chemother 38:2125–2133. https://doi.org/10.1128/AAC.38.9.2135

5. Fazli M, Bjarnsholt T, Kirketerp-Møller K, Jørgensen B, Anderson AS, Krogfelt KA, Givskov M, Tolker-Nielsen Y. 2009. Nonrandom distribution of Pseudomonas aeruginosa and Staphylococcus aureus in chronic wounds. J Clin Microbiol 47:4084–4089. https://doi.org/10.1128/JCM.01395-09

6. Ryder C, Byrd M, Wozniak DJ. 2007. Role of polysaccharides in Pseudomonas aeruginosa biofilm development. Curr Opin Microbiol 10:644–648. https://doi.org/10.1016/j.mib.2007.09.010

7. Limoli DH, Jones CJ, Wozniak DJ. 2015. Bacterial extracellular polysaccharides in biofilm formation and function. Microbiol Spectr 3. https://doi.org/10.1128/microbiolspec.MB-0011-2014

8. Lemos JA, Palmer SR, Zeng L, Wen ZT, Kajfasz JK, Freires IA, Abranches J, Brady LJ. 2019. The biology of Streptococcus mutans. Microbiol Spectr 7. https://doi.org/10.1128/microbiolspec.GPP3-0051-2018

9. Lynch DJ, Michalek SM, Zhu M, Drake D, Qian F, Banas JA. 2013. Cariogenic dental biofilm formation and function. Microbiol Spectr 3. https://doi.org/10.1128/microbiolspec.MB-0011-2014

10. Otten MPT, Busscher HJ, van Hoogmoed CG, Abbas F, van der Mei HC. 2016. Haukioja A. 2010. Probiotics and oral health. Eur J Dent 4:348–355. https://doi.org/10.1177/154405911008501002

11. Fusetani N. 2004. Biofouling and antifouling. Nat Prod Rep 21:94

12. Tahmourespour A, Salehi R, Kermanshahi RK, Eslami G. 2011. The antibiofilm effect of Lactobacillus fermentum against Pseudomonas aeruginosa. BMC Res Notes 6:135. https://doi.org/10.1186/1756-0500-6-135

13. A

14. Resta-Lenert S, Barrett KE. 2003. Live probiotics protect intestinal epithelial cells from the effects of infection with enteroinvasive Escherichia coli (EIEC). Gut 52:988–997. https://doi.org/10.1136/gut.52.7.988

15. Wong S-S, Quan Toh Z, Dunne EM, Mulholland EK, Tang MLK, Robins-Browne RM, Lucciardi PV, Satzke C. 2013. Inhibition of Streptococcus pneumoniae adherence to human epithelial cells in vitro by the probiotic Lactobacillus rhamnosus GG. BMC Res Notes 6:135. https://doi.org/10.1186/1756-0500-6-135

16. Haukioja A. 2010. Probiotics and oral health. Eur J Dent 4:348–355.

17. Süderling EM, Martinen AM, Haukioja AL. 2011. Probiotic lactobacilli interfere with Streptococcus mutans biofilm formation in vitro. Curr Microbiol 62:618–622. https://doi.org/10.1007/s00284-010-9752-9

18. Ahn KB, Baik JE, Park OJ, Yun CH, Han SH. 2018. Lactobacillus plantarum lipoteichoic acid inhibits biofilm formation. PLoS One 13:e0192694. https://doi.org/10.1371/journal.pone.0192694

19. Wasfi R, Abd El-Rahman OA, Zafer MM, Ashour HM. 2018. Probiotic Lactobacillus sp. inhibit growth, biofilm formation and gene expression of caries-inducing Streptococcus mutans. J Cell Mol Med 22:1972–1983. https://doi.org/10.1111/jcmm.13496

20. Metlitskikh SV, Koroteev MP, Shaskhov AS, Karyyukov AA, Antipin MY, Shashkov AS, Stash AL, Nilantiev EE. 2005. Synthesis of bis (glycolaminalkanes and bis (glycosylamin) areses. Russian Chemical Bulletin 54: 2890–2898.

21. Sarmah R, Khan RA, Devi KR. 2021. Microbes in human oral cavity: a review. Rev and Res in Medical Microbiology 32:75–82. https://doi.org/10.1097/MRM.0000000000000227

22. Burton JP, Wescombe PA, Moore CJ, Chilcott CN, Tagg JR. 2006. Safety and dietary potential for probiotics. J Appl Microbiol 100:622. https://doi.org/10.1111/j.1365-2672.2006.02898.x

23. Zhang G, Lu M, Liu R, Tian Y, Yu VH, Li Y, Liu B, Kushmaro A, Li Y, Sun Q. 2020. Inhibition of Streptococcus mutans biofilm formation and virulence by Lactobacillus plantarum K41 isolated from traditional Sicilian. Front Microbiol 11:774. https://doi.org/10.3389/fmicb.2020.00774

24. Rossoni RD, Velloso M, S. de Barros PP, de Alvarenga JA, Santos JDd, Santos Prado ACC, Ribeiro Fdc, Anbinder AL, Junqueira JC. 2018. Inhibitory effect of probiotic Lactobacillus strains on mutans streptococci in vitro. Microb Pathog 123:361–367. https://doi.org/10.1016/j.micpath.2018.07.032

25. Hezam AM. 2020. Evaluate the efficacy of probiotic lactobacilli on growth and biofilm formation in Streptococcus mutans isolated from gingivitis. J Phys Conf Ser 1664:012111. https://doi.org/10.1088/1742-6596/1664/1/012111

26. Vestman NR, Timby N, Holgerson PL, Kressier CA, Claesson R, Domellöf M, Öhman C, Tanner ACR, Hernell O, Johannson I. 2013. Characterization and in vitro properties of oral lactobacilli in breastfed infants. BMC Microbiol 13:193. https://doi.org/10.1186/1471-2180-13-193

27. Simark-Mattsson C, Emilsson CG, Häkansson EG, Jacobsson C, Roos K, Holm S. 2017. Lactobacillus-mediated interference of mutants streptococci in caries-free vs. caries-active subjects. Eur J Oral Sci 115:308–314. https://doi.org/10.1111/eos.12202.2007.00458.x

28. Burton JP, Wescombe PA, Moore CJ, Chilcott CN, Tagg JR. 2006. Safety and dietary potential for probiotics. J Appl Microbiol 100:622. https://doi.org/10.1111/j.1365-2672.2006.02898.x

29. Huch M, De Bryune K, Cleenwerck I, Bub A, Cho G-S, Watzl B, Swaenaert I, Franz CMP, Vandamme P. 2013. Streptococcus rubneri sp. nov., isolated from the human throat. Int J Syst Evol Microbiol 63:4026–4032. https://doi.org/10.1099/ijs.0.05833-0

30. Mann S, Park MS, Johnston TV, Ji GE, Hwang KT, Ku S. 2021. Oral probiotic activities and biosafety of Lactobacillus gasseri HHuMIN D. Microb Cell Fact 20:75. https://doi.org/10.1186/s12934-021-01565-w
31. Rosenfeld WD, Clark J. 1989. Vulvovaginitis and cervicitis. Pediatr Clin North Am 36:489–511. https://doi.org/10.1016/s0031-3955(16)36682-2.

32. Tanizawa Y, Tada I, Kobayashi H, Endo A, Maeno S, Toyoda A, Arita M, Nakamura Y, Sakamoto M, Ohkuma M, Tohno M. 2018. Lactobacillus para- gasseri sp. nov., a sister taxon of Lactobacillus gasseri, based on whole-genome sequence analyses. Int J Syst Evol Microbiol 68:3512–3517. https://doi.org/10.1099/ijsem.0.003020.

33. Nash RJ, Kato A, Yu CY, Fleet GW. 2011. Lactobacillus as therapeutic agents: recent advances and promising trends. Future Med Chem 3:1513–1521. https://doi.org/10.4155/fmc.11.117.

34. Zhang R, Zhang W, Hu T. 2011. Dextran glucosidase: a potential target of iminosugar derivatives on Pseudomonas aeruginosa GtfB expression and function. FEMS Microbiol Lett 264:8–14. https://doi.org/10.1016/j.femsle.2006.00378.x.

35. Islam B, Khan SN, Haque I, Alam M, Mushiq AJ, Dakhel MA, Khan MA, Saha MD. 2011. Inhibition of Staphylococcus aureus fi lm formation in multispecies biofilms. Antimicrob Agents Chemother 55:2679–2687. https://doi.org/10.1128/AAC.01496-10.

36. Li Y-H, Tang N, Aspiras MB, Lau PCY, Lee JH, Ellen RP, Cvitkovitch DG. 2005. The role of Rpa in multispecies biofilms. J Oral Microbiol 23:393–401. https://doi.org/10.1111/j.1600-0749.2005.000040.x.

37. Davies DG, Marques CNH. 2009. A fatty acid messenger is responsible for inducing dispersion in microbial biofilms. J Bacteriol 191:1393–1403. https://doi.org/10.1128/JB.01214-08.

38. Kim YG, Lee JH, Raorane CJ, Oh ST, Park JG, Lee J. 2018. Herring oil and omega-3 fatty acids control biofilm formation of Streptococcus mutans. J Biotechnol 258:1–10. https://doi.org/10.1016/j.jbiotec.2018.01.024.

39. Nakamura Y, Sakamoto M, Ohkuma M, Tohno M. 2018. Fatty acid composition of Lactobacillus gasseri and its genome sequence analyses. Int J Syst Evol Microbiol 68:3512–3517. https://doi.org/10.1099/ijsem.0.003020.

40. Stenz L, François P, Fischer A, Huyghe A, Hernandez D, Gallienne E, Nicolas C, Martin OR, Kyzioł A, Machul A, Heczko PB, Chronowska A, Stochel G, Lajczyk D, Łączyński D, Łyczak A. 2016. Effects of the selected iminosugar derivatives on Pseudomonas aeruginosa. Antimicrob Agents Chemother 60:559–569. https://doi.org/10.1128/AAC.02880-X.

41. Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods 25:402–408. https://doi.org/10.1016/s1046-2023.2001.1262.