Lactobacillus reuteri AN417 cell-free culture supernatant as a novel antibacterial agent targeting oral pathogenic bacteria

Kyung Mi Yang1,3, Ji-Sun Kim2,3, Hye-Sung Kim1, Young-Youn Kim1, Jeong-Kyu Oh1, Hye-Won Jung1, Doo-Sang Park2* & Kwang-Hak Bae1*

Lactobacillus reuteri AN417 is a newly characterized probiotic strain. The activity of AN417 against oral pathogenic bacteria is unknown. We investigated the antibacterial activity of cell-free L. reuteri AN417 culture supernatant (LRS) against three oral pathogens: Porphyromonas gingivalis, Fusobacterium nucleatum, and Streptococcus mutans. P. gingivalis and F. nucleatum have been implicated in periodontal disease, whereas S. mutans causes dental caries. Exposing these oral pathogenic bacteria to LRS significantly reduced their growth rates, intracellular ATP levels, cell viability, and time-to-kill. The minimal inhibitory volume of LRS was 10% (v/v) against P. gingivalis, 20% (v/v) for F. nucleatum, and 30% (v/v) for S. mutans. LRS significantly reduced the integrity of biofilms and significantly suppressed the expression of various genes involved in P. gingivalis biofilm formation. The L. reuteri AN417 genome lacked genes encoding reuterin, reuteran, and reutericyclin, which are major antibacterial compounds produced in L. reuteri strains. LRS treated with lipase and α-amylase displayed decreased antibacterial activity against oral pathogens. These data suggest that the antibacterial substances in LRS are carbohydrates and/or fatty acid metabolites. Our results demonstrate that LRS has antimicrobial activity against dental pathogenic bacteria, highlighting its potential utility for the prevention and treatment of P. gingivalis periodontal disease.

Periodontal disease is a chronic inflammatory disease caused by the accumulation of different pathogenic biofilm-forming bacteria in dental pockets1. Biofilms that develop on tooth surfaces contain oral microbes, and the formation of bacterial plaque causes gingivitis with redness and swelling of the gingiva2. Periodontitis refers to an irreversible loss of adhesion, in which tissues such as periodontal ligaments, alveolar bone, and chalk that support teeth are absorbed as the inflammatory response of the tissues to pathogenic bacterial stimulation intensifies3. Collagen fibers in the periodontal ligament are destroyed, forming a periodontal sac between the gingiva and the teeth, deepening through the absorption of the alveolar bone4. This creates an ecological environment that favors the propagation of various anaerobic bacteria, causing periodontal disease5. Endotoxins or metabolites formed by microorganisms participate in tissue breakthrough processes by increasing the secretion of pro-inflammatory cytokines from tissues and immune cells6.

The pathogens most known to be associated with the development of oral diseases are Porphyromonas gingivalis, Fusobacterium nucleatum, and Streptococcus mutans. Among oral pathogens, P. gingivalis and F. nucleatum are Gram-negative and obligate anaerobes that cause periodontal diseases7,8. These two oral pathogens degrade collagen, induce halitosis, and produce endotoxins, such as lipopolysaccharides (LPS), which destroy alveolar bone and cause tooth loss5. S. mutans produces acidic compounds and plays an important role in the formation of biofilms on teeth, which cause dental caries9. These oral pathogens can penetrate directly into the vascular endothelial cells or enter damaged blood vessels and adhere to specific organs, ultimately leading to systemic disease10.

Substances produced by lactic acid bacteria during the metabolism of prebiotics (dietary fiber) can be beneficial to human health11. In a rat model, the administration of live probiotic Bifidobacteria was reported to protect against periodontal destruction and to decrease inflammatory intermediates in rats with ligature-induced

1Institute of Biomedical Science, Apple Tree Dental Hospital, 1450, Jungang-ro, Iisaseo-gu, Goyang-si, Gyeonggi-do 10387, Republic of Korea. 2Biological Resources Center, Korea Research Institute of Bioscience & Biotechnology (KRIBB), Jeong-up 56212, Republic of Korea. 3These authors contributed equally: Kyung Mi Yang and Ji-Sun Kim. *email: dspark@kribb.re.kr; sadent@naver.com
periodontitis. A recent review highlighted potential antimicrobial agents, including those produced by lactic acid bacteria, for the treatment of dental diseases.

Although antibiotics are still widely used to treat oral diseases, they cause side effects, such as the generation of resistant strains and microbial substitution. Thus, a variety of natural substances that have few side effects are being used proactively. Numerous ongoing studies are examining the antimicrobial activities of plant extracts that inhibit the growth of oral pathogenic bacteria. Adjuvant treatments using ozone are also being explored as a new approach for managing chronic periodontitis.

Recent data have indicated the potential value of probiotic microorganisms in oral health. For example, cell-free culture supernatants of a Weissella cibaria strain showed antibacterial activity against periodontal pathogens. The effects were dependent on either the acidity of the supernatant or the level of hydrogen peroxide produced by W. cibaria. Another study reported antimicrobial and antibiofilm activities of Lactobacillus kefiranofaciens culture supernatants against oral pathogens. Heat-killed L. reuteri and supernatants from cell-free L. reuteri cultures had antibacterial effects on P. gingivalis that were similar to those of live L. reuteri cells, although in an invertebrate model, hemocyte density was significantly increased only in the presence of live L. reuteri and not with the culture supernatant or heat-killed cells. During fermentation, lactic acid bacteria produce metabolites, such as extracellular polymeric substances, functional proteins, and peptides, which are bioactive compounds with known beneficial effects on human health. The antimicrobial effect of the supernatant of lactic acid bacteria (LAB) was not after treatment with proteinase K, pepsin, and papain.

This study evaluated the antibacterial potential of a new probiotic strain, L. reuteri AN417, to improve oral health. Our findings indicate that this strain has potential as a therapeutic agent for the treatment of chronic periodontitis and the prevention of dental caries.

Results
Isolation and characterization of L. reuteri strains. We isolated 135 L. reuteri strains from human infants and 6-month-old female swine under anaerobic conditions. Bacterial isolates were identified by 16S rRNA gene sequencing and matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) biotype (Bruker, Table 1). Primary screening for antimicrobial activity against pathogens, including Escherichia coli (KCTC 2571), Pseudomonas aeruginosa (DSM 50071), and S. mutans (KCTC 3065), was performed to select L. reuteri strains that had exhibited antimicrobial activity against periodontopathic bacteria in a disk diffusion assay (Fig. 1A). The results showed that L. reuteri AN417 displayed the strongest antibacterial activity against pathogens.

In addition, the production of 1, 3-propanediol (1, 3-PDO) by the isolated L. reuteri strains was determined using a high-performance liquid chromatography (HPLC) system (Table 1 and Supplementary Fig. S1A). The 1, 3-PDO is valuable compound used as a replacement for petroleum-based glycols, including propylene glycol, butylene glycol, and glycerin. It has been reported that L. reuteri metabolizes glycerol to reuterin (3-hydroxypropionaldehyde, 3-HPA) and then converts reuterin to 1, 3-PDO. L. reuteri cannot grow on glycerol as the sole carbon source and the conversion to 1, 3-PDO from glycerol requires NADH produced by glucose metabolism. Therefore, we tested the production level of 1, 3-PDO under co-fermentation in the presence of both glycerol and glucose in the culture medium.

Interestingly, most (but not all) L. reuteri isolates originating from swine did not produce 1, 3-PDO, whereas every strain of human origin did (Table 1). Genomic analysis showed that L. reuteri AN417 isolated from swine did not encode glycerol dehydratase (dhaB), which catalyzes 3-HPA production, and 1,3-propanediol dehydrogenases (dhaT), which catalyze 1,3-PDO production. The data implied that the L. reuteri strains exhibit host-specific characteristics in metabolite production.

Potentially important inhibitory activity of L. reuteri AN417 supernatant (LRS) against oral bacterial pathogens. When the antimicrobial activity of the newly identified L. reuteri strains was assessed, we observed that L. reuteri AN417 culture supernatant (LRS) outperformed those of our other tested strains. We first observed whether isolated L. reuteri strains affected the growth of the oral pathogens S. mutans KCTC 3065 and P. gingivalis BAA-308 (Fig. 1A, B). The clear zone was the largest when S. mutans was treated with LRS compared to the supernatants from other strains. Moreover, the growth of P. gingivalis was more highly inhibited when 10% (v/v) LRS was added to the medium than when supernatants derived from other L. reuteri strains were treated. Furthermore, growth of P. gingivalis was most inhibited when 10% (v/v) LRS was added to the medium (Fig. 1B). The findings indicated that cell-free culture supernatant derived from L. reuteri AN417 exhibited the highest activity against P. gingivalis and potentially against other oral pathogenic bacteria.

Next, we determined that the antimicrobial bioactive substances are present in the culture supernatant, not inside the bacterial cells themselves. To establish whether the active substances were present in the culture supernatant or in the bacterial cells, bacterial cell extracts (BE) were prepared using ethyl acetate. BE and LRS, which were concentrated and diluted to the desired values, were treated with P. gingivalis for 24 h. Compared with the control treated only with HEPES or de Man Rogosa and Sharpe (MRS), LRS substantially inhibited the growth of P. gingivalis. However, no effect was observed with the BE treatment. Furthermore, compared with control treatments, LRS significantly reduced P. gingivalis intracellular ATP levels, whereas the BE treatment did not (Fig. 1C). Administration of 20% or 40% (v/v) LRS for 96 h significantly reduced ATP levels and growth of the pathogenic bacteria (Fig. 1D).

LRS inhibits the growth of oral pathogenic bacteria. The inhibitory effects of 10%, 20%, 30%, and 40% (v/v) LRS on the growth of selected oral pathogenic bacteria (P. gingivalis, E. nucleatum, and S. mutans) were assessed. As the results, growth inhibition against tested pathogens was dependent on LRS concentrations. Treat-
| No | ID     | Strain              | Origin                      | Residual glucose (g/L) | Residual glycerol (g/L) | 1,3-PDO production (g/L)\(^a\) | Reuterin production\(^b\) |
|----|--------|---------------------|-----------------------------|------------------------|-------------------------|---------------------------------|-----------------------------|
| 1  | PB3    | Lactobacillus reuteri | Swine feces                | 1.96                   | 6.95                    | ++                              | +                           |
| 2  | PB6    | Lactobacillus reuteri | Swine feces                | 0.16                   | 5.51                    | +++                             | +                           |
| 3  | PF4    | Lactobacillus reuteri | Swine feces                | 1.18                   | 4.96                    | +++                             | –                           |
| 4  | PPF3   | Lactobacillus reuteri | Swine feces                | 2.76                   | 8.10                    | ++                              | –                           |
| 5  | PMA2   | Lactobacillus reuteri | Swine feces                | 0.05                   | 21.49                   | –                               | –                           |
| 6  | PMF1   | Lactobacillus reuteri | Swine feces                | 0.64                   | 20.99                   | –                               | –                           |
| 7  | PMF2   | Lactobacillus reuteri | Swine feces                | 0.57                   | 21.45                   | –                               | –                           |
| 8  | AN306  | Lactobacillus reuteri | Small intestine of swine    | 0.35                   | 21.47                   | –                               | –                           |
| 9  | AN313  | Lactobacillus reuteri | Small intestine of swine    | 0.56                   | 20.84                   | –                               | –                           |
| 10 | AN403  | Lactobacillus reuteri | Small intestine of swine    | 4.51                   | 21.47                   | –                               | –                           |
| 11 | AN413  | Lactobacillus reuteri | Small intestine of swine    | 0.05                   | 6.05                    | –                               | –                           |
| 12 | AN417  | Lactobacillus reuteri | Small intestine of swine    | 1.08                   | 20.40                   | –                               | –                           |
| 13 | AN507  | Lactobacillus reuteri | Small intestine of swine    | 0.45                   | 20.09                   | –                               | –                           |
| 14 | AN509  | Lactobacillus reuteri | Small intestine of swine    | 0.08                   | 21.43                   | –                               | –                           |
| 15 | AN510  | Lactobacillus reuteri | Small intestine of swine    | 2.21                   | 21.50                   | –                               | –                           |
| 16 | AN511  | Lactobacillus reuteri | Small intestine of swine    | 0.28                   | 21.42                   | –                               | –                           |
| 17 | AN513  | Lactobacillus reuteri | Small intestine of swine    | 5.19                   | 21.26                   | –                               | –                           |
| 18 | AN516  | Lactobacillus reuteri | Small intestine of swine    | 1.39                   | 21.48                   | –                               | –                           |
| 19 | AN519  | Lactobacillus reuteri | Small intestine of swine    | 0.20                   | 21.30                   | –                               | –                           |
| 20 | AN521  | Lactobacillus reuteri | Small intestine of swine    | 0.18                   | 21.17                   | –                               | –                           |
| 21 | AN523  | Lactobacillus reuteri | Small intestine of swine    | 1.23                   | 21.30                   | –                               | –                           |
| 22 | AN525  | Lactobacillus reuteri | Small intestine of swine    | 0.13                   | 21.28                   | –                               | –                           |
| 23 | AN527  | Lactobacillus reuteri | Small intestine of swine    | 0.12                   | 20.95                   | –                               | –                           |
| 24 | AN530  | Lactobacillus reuteri | Small intestine of swine    | 0.46                   | 21.21                   | –                               | –                           |
| 25 | AN540  | Lactobacillus reuteri | Small intestine of swine    | 1.00                   | 21.36                   | –                               | –                           |
| 26 | AN543  | Lactobacillus reuteri | Small intestine of swine    | 0.10                   | 21.42                   | –                               | –                           |
| 27 | AN546  | Lactobacillus reuteri | Small intestine of swine    | 3.58                   | 21.42                   | –                               | –                           |
| 28 | AN548  | Lactobacillus reuteri | Small intestine of swine    | 0.24                   | 21.32                   | –                               | –                           |
| 29 | AN703  | Lactobacillus reuteri | Small intestine of swine    | 8.13                   | 21.46                   | –                               | –                           |
| 30 | AN704  | Lactobacillus reuteri | Small intestine of swine    | 0.42                   | 21.37                   | –                               | –                           |
| 31 | AN705  | Lactobacillus reuteri | Small intestine of swine    | 1.38                   | 20.89                   | –                               | –                           |
| 32 | AN708  | Lactobacillus reuteri | Small intestine of swine    | 5.99                   | 21.38                   | –                               | –                           |
| 33 | AN709  | Lactobacillus reuteri | Small intestine of swine    | 3.36                   | 21.42                   | –                               | –                           |
| 34 | AN711  | Lactobacillus reuteri | Small intestine of swine    | 2.64                   | 21.41                   | –                               | –                           |
| 35 | AN722  | Lactobacillus reuteri | Small intestine of swine    | 4.81                   | 21.24                   | –                               | –                           |
| 36 | AN724  | Lactobacillus reuteri | Small intestine of swine    | 0.12                   | 21.34                   | –                               | –                           |
| 37 | AN727  | Lactobacillus reuteri | Small intestine of swine    | 2.48                   | 21.22                   | –                               | –                           |
| 38 | AN832  | Lactobacillus reuteri | Small intestine of swine    | 7.69                   | 21.39                   | –                               | –                           |
| 39 | AN903  | Lactobacillus reuteri | Small intestine of swine    | 1.03                   | 21.47                   | –                               | –                           |
| 40 | AN904  | Lactobacillus reuteri | Small intestine of swine    | 3.63                   | 21.49                   | –                               | –                           |
| 41 | AN906  | Lactobacillus reuteri | Small intestine of swine    | 9.73                   | 18.93                   | –                               | –                           |
| 42 | AN919  | Lactobacillus reuteri | Small intestine of swine    | 12.51                  | 18.90                   | –                               | –                           |
| 43 | AN924  | Lactobacillus reuteri | Small intestine of swine    | 7.59                   | 18.78                   | –                               | –                           |
| 44 | AN925  | Lactobacillus reuteri | Small intestine of swine    | 9.31                   | 19.01                   | –                               | –                           |
| 45 | AN926  | Lactobacillus reuteri | Small intestine of swine    | 9.90                   | 21.05                   | –                               | –                           |
| 46 | AN933  | Lactobacillus reuteri | Small intestine of swine    | 4.88                   | 19.54                   | –                               | –                           |
| 47 | AN941  | Lactobacillus reuteri | Small intestine of swine    | 10.13                  | 19.27                   | –                               | –                           |
| 48 | AN943  | Lactobacillus reuteri | Small intestine of swine    | 0.75                   | 20.79                   | –                               | +                           |
| 49 | AN947  | Lactobacillus reuteri | Small intestine of swine    | 9.92                   | 18.72                   | –                               | –                           |
| 50 | AN1002 | Lactobacillus reuteri | Small intestine of swine    | 12.09                  | 19.00                   | –                               | –                           |
| 51 | AN1009 | Lactobacillus reuteri | Small intestine of swine    | 9.69                   | 18.99                   | –                               | –                           |
| 52 | AN1016 | Lactobacillus reuteri | Small intestine of swine    | 4.47                   | 20.87                   | –                               | –                           |
| 53 | AN1019 | Lactobacillus reuteri | Small intestine of swine    | 8.83                   | 20.52                   | –                               | –                           |
| 54 | AN1026 | Lactobacillus reuteri | Small intestine of swine    | 7.55                   | 19.04                   | –                               | –                           |
| 55 | AN1033 | Lactobacillus reuteri | Small intestine of swine    | 2.38                   | 20.96                   | –                               | –                           |
| 56 | AN1034 | Lactobacillus reuteri | Small intestine of swine    | 5.58                   | 19.42                   | –                               | –                           |

Continued
| No | ID         | Strain Origin | Residual glucose (g/L) | Residual glycerol (g/L) | 1,3-PDO production (g/L) | Reuterin production a |
|----|------------|---------------|------------------------|------------------------|-------------------------|----------------------|
| 57 | AN1109     | Small intestine of swine | 8.60 | 21.03 | - | - |
| 58 | SBF301     | Lactobacillus reuteri Infant feces | 0.51 | 5.70 | ++ | + |
| 59 | SBF302     | Lactobacillus reuteri Infant feces | 1.30 | 7.37 | ++ | + |
| 60 | SBF303     | Lactobacillus reuteri Infant feces | 1.04 | 7.09 | ++ | ++ |
| 61 | SBF304     | Lactobacillus reuteri Infant feces | 2.31 | 8.33 | ++ | + |
| 62 | SBF305     | Lactobacillus reuteri Infant feces | 10.85 | 15.80 | + | + |
| 63 | SBF306     | Lactobacillus reuteri Infant feces | 0.32 | 5.98 | +++ | + |
| 64 | SBF307     | Lactobacillus reuteri Infant feces | 0.59 | 6.27 | +++ | ++ |
| 65 | SBF309     | Lactobacillus reuteri Infant feces | 3.99 | 9.26 | ++ | - |
| 66 | SBF310     | Lactobacillus reuteri Infant feces | 3.57 | 9.09 | ++ | + |
| 67 | SBF311     | Lactobacillus reuteri Infant feces | 0.08 | 5.20 | ++ | ++ |
| 68 | SBF312     | Lactobacillus reuteri Infant feces | 4.47 | 10.15 | ++ | + |
| 69 | SBF313     | Lactobacillus reuteri Infant feces | 7.43 | 12.87 | ++ | + |
| 70 | SBF314     | Lactobacillus reuteri Infant feces | 5.78 | 11.30 | ++ | + |
| 71 | SBF315     | Lactobacillus reuteri Infant feces | 4.40 | 9.93 | ++ | + |
| 72 | SBF316     | Lactobacillus reuteri Infant feces | 1.33 | 8.92 | ++ | - |
| 73 | SBF317     | Lactobacillus reuteri Infant feces | 12.47 | 16.74 | + | + |
| 74 | SBF318     | Lactobacillus reuteri Infant feces | 5.78 | 11.44 | ++ | + |
| 75 | SBF320     | Lactobacillus reuteri Infant feces | 11.91 | 16.46 | + | + |
| 76 | SBF321     | Lactobacillus reuteri Infant feces | 9.37 | 14.92 | + | + |
| 77 | SBF325     | Lactobacillus reuteri Infant feces | 0.32 | 5.27 | +++ | + |
| 78 | SBF326     | Lactobacillus reuteri Infant feces | 6.18 | 11.47 | ++ | + |
| 79 | SBF327     | Lactobacillus reuteri Infant feces | 5.54 | 10.86 | ++ | +++ |
| 80 | SBF328     | Lactobacillus reuteri Infant feces | 0.95 | 14.94 | + | +++ |
| 81 | SBF329     | Lactobacillus reuteri Infant feces | 1.13 | 7.73 | +++ | - |
| 82 | SBF330     | Lactobacillus reuteri Infant feces | 8.63 | 14.70 | + | + |
| 83 | SBF331     | Lactobacillus reuteri Infant feces | 0.39 | 6.30 | +++ | ++ |
| 84 | SBF332     | Lactobacillus reuteri Infant feces | 0.77 | 7.17 | +++ | ++ |
| 85 | SBF333     | Lactobacillus reuteri Infant feces | 1.59 | 8.07 | +++ | +++ |
| 86 | SBF334     | Lactobacillus reuteri Infant feces | 1.65 | 8.12 | ++ | ++ |
| 87 | SBF335     | Lactobacillus reuteri Infant feces | 0.32 | 6.50 | +++ | +++ |
| 88 | SBF336     | Lactobacillus reuteri Infant feces | 4.58 | 10.57 | ++ | + |
| 89 | SBF341     | Lactobacillus reuteri Infant feces | 0.29 | 6.41 | +++ | + |
| 90 | SBF401     | Lactobacillus reuteri Infant feces | 1.27 | 9.55 | ++ | + |
| 91 | SBF417     | Lactobacillus reuteri Infant feces | 0.53 | 7.07 | +++ | + |
| 92 | SBF418     | Lactobacillus reuteri Infant feces | 5.69 | 11.51 | ++ | + |
| 93 | SBF419     | Lactobacillus reuteri Infant feces | 0.32 | 5.36 | +++ | + |
| 94 | SBF420     | Lactobacillus reuteri Infant feces | 0.29 | 6.19 | +++ | + |
| 95 | SBF447     | Lactobacillus reuteri Infant feces | 0.66 | 6.86 | +++ | + |
| 96 | MBF301     | Lactobacillus reuteri Infant feces | 0.99 | 7.02 | +++ | + |
| 97 | MBF302     | Lactobacillus reuteri Infant feces | 0.25 | 5.39 | +++ | + |
| 98 | MBF303     | Lactobacillus reuteri Infant feces | 0.47 | 5.20 | +++ | + |
| 99 | MBF304     | Lactobacillus reuteri Infant feces | 0.41 | 4.18 | +++ | + |
| 100 | MBF307    | Lactobacillus reuteri Infant feces | 12.83 | 17.83 | + | + |
| 101 | MBF312    | Lactobacillus reuteri Infant feces | 1.38 | 7.84 | +++ | + |
| 102 | MBF326    | Lactobacillus reuteri Infant feces | 0.29 | 5.54 | +++ | + |
| 103 | MBF327    | Lactobacillus reuteri Infant feces | 7.63 | 13.25 | + | + |
| 104 | MBF329    | Lactobacillus reuteri Infant feces | 12.96 | 17.58 | + | + |
| 105 | MBF335    | Lactobacillus reuteri Infant feces | 0.86 | 4.86 | +++ | + |
| 106 | MBF342    | Lactobacillus reuteri Infant feces | 0.25 | 4.87 | +++ | + |
| 107 | MBF344    | Lactobacillus reuteri Infant feces | 0.27 | 5.55 | +++ | + |
| 108 | MBF351    | Lactobacillus reuteri Infant feces | 0.27 | 5.49 | +++ | + |
| 109 | MBF355    | Lactobacillus reuteri Infant feces | 0.25 | 4.77 | +++ | + |
| 110 | MBF356    | Lactobacillus reuteri Infant feces | 0.28 | 4.76 | +++ | + |
| 111 | MBF358    | Lactobacillus reuteri Infant feces | 0.49 | 5.88 | +++ | + |
| 112 | MBF359    | Lactobacillus reuteri Infant feces | 0.62 | 6.25 | +++ | + |

Continued
Antimicrobial activity of LRS against oral pathogenic bacteria, especially \( \textit{P. gingivalis} \). Treatment of the three oral pathogenic bacteria with LRS revealed a minimum inhibitory volume (MIV) of approximately 10% (v/v) for \( \textit{P. gingivalis} \). The MIV was 20% and 40% (v/v) for \( \textit{S. mutans} \) and \( \textit{F. nucleatum} \). However, the growth of \( \textit{P. gingivalis} \) was significantly inhibited in 10, 20 and 40% of LRS treatment (80% reduction in growth rate) (Fig. 2A). Prolonged treatment for up to 48 h with 40% (v/v) LRS resulted in a stable continuation of the inhibitory effect in all three strains (Fig. 2B).

**LRS effectively reduces the viability of oral pathogenic bacteria.** In fluorescent cell-staining assays, LRS increased the number of dead pathogenic bacteria and decreased the number of live pathogenic bacteria. To evaluate the effect of LRS on the viability of the three pathogenic bacterial strains, a mixture of SYTO9 green fluorescence nucleic acid stain and propidium iodide was used for cell staining. The cells were observed by fluorescence microscopy. Compared with the negative control (MRS or Brain Heart Infusion [BHI] medium), the intensity of green fluorescence emitted by live bacteria decreased in \( \textit{P. gingivalis} \), \( \textit{F. nucleatum} \), and \( \textit{S. mutans} \). After treatment with LRS, bacterial death was observed over time. As a result, it was observed that \( \textit{P. gingivalis} \) died rapidly starting 8 h after LRS treatment (Fig. 3A). These results were consistent with those of LIVE/DEAD BacLight analysis, with LRS treatment substantially reducing colony forming units compared with the MRS treatment.

**Antimicrobial activity of LRS against oral pathogenic bacteria, especially \( \textit{P. gingivalis} \).** To confirm the antibiofilm activity of LRS against the biofilm formation during the early stage of bacterial colonization, LRS was added to \( \textit{P. gingivalis} \) and \( \textit{S. mutans} \) cultures immediately after bacterial inoculations, so that the effects of LRS on biofilm formation during the initial attachment phase could be examined. LRS treatment substantially reduced the fluorescence intensity compared with the control treatment (Fig. 4A), which was consistent with the quantitative results. To determine the concentration of LRS that eradicates established \( \textit{P. gingivalis} \) biofilms, biofilms developed for 5 days were treated with LRS and stained with crystal violet. Compared with the control treatment,
Figure 1. Evaluation of the antimicrobial activity of culture supernatants of *L. reuteri* strains against *E. coli*, *P. aeruginosa*, *S. mutans*, and *P. gingivalis*. (A) The antimicrobial activity of culture supernatants from newly isolated and reference *L. reuteri* strains against *E. coli*, *P. aeruginosa* and *S. mutans* was analyzed through disk diffusion assays following 24 h incubation at 37 °C. (B) Growth curve of *P. gingivalis* at OD<sub>600</sub> measured using a disposable curvet 48 h after treatment with the supernatants of each strains. Significant differences from the control (*p* < 0.01) are indicated by **. (C) The effect of various concentrations of LRS and BE on *P. gingivalis* growth, measured by OD<sub>600</sub> (left panel) and intracellular ATP levels (right panel) following a 24 h incubation at 37 °C. MRS medium was used as the negative control. Significant differences from the control (*p* < 0.001) are indicated by ***. (D) The effect of 20% (v/v) and 40% (v/v) LRS on *P. gingivalis* growth (left panel) and intracellular ATP levels (right panel) over time. MRS medium was used as the negative control. Significant differences from the control (*p* < 0.001) are indicated by ***.
10%, 20%, and 30% (v/v) treatment of LRS achieved substantial removal of biofilms (Fig. 4B). Furthermore, we observed a significant reduction in the expression of \( \text{rgpA} \), \( \text{rgpB} \), \( \text{hagA} \), \( \text{hagB} \), and \( \text{kgp} \), all of which are genes involved in biofilm attachment and formation, in \( \text{P. gingivalis} \) following LRS treatment (Fig. 4C,D).

Whole genomic sequencing of \( \text{L. reuteri} \) AN417. To determine the antibacterial and antibiofilm molecules produced by \( \text{L. reuteri} \) AN417, whole genome sequencing was performed using PacBio RSII single-molecule real-time (SMRT) sequencing technology. As shown in Supplementary Fig. S2, the complete genome consisted of a single circular chromosome of 2,069,421 bp and four circular plasmids of 93,397 bp (pLreu417A of 57,676 bp, pLreu417B of 16,368 bp, pLreu417C of 10,268 bp, and pLreu417D of 9085 bp) with 38.95% G + C content. A total of 2151 genes were predicted in the genome of this strain. Of these, 2034 were identified as protein-coding genes. The total length of the coding regions was 1,850,478 bp. A total of 1571 protein-coding genes were assigned putative functions. The remainder were annotated as hypothetical proteins.

Genomic analysis and characterization of \( \text{L. reuteri} \) AN417. Genome-genome relatedness of \( \text{L. reuteri} \) AN417 was also analyzed by calculating the average nucleotide identity (ANI) and constructing a phylogenomic tree of 31 genome sequences with fewer than 30 scaffolds among the genomes of \( \text{L. reuteri} \) strains from the GenBank/EMBL/DDBJ database. \( \text{L. reuteri} \) genomes were an average size of 2.10 Mb and G + C ratio of 38.43–39.31 (Table 2). The whole genome of \( \text{L. reuteri} \) AN417 showed ANI values ranging from 94.8 to 99.6% with \( \text{L. reuteri} \) strains. The highest ANI values were obtained for the pig strains (Fig. 5A). Additionally, whole genome phylogenetic analysis was performed with 27 \( \text{L. reuteri} \) genomes, excluding those with many contigs. A phylogenetic tree of \( \text{L. reuteri} \) strains was constructed using the amino acid alignments of 766 core genes using the maximum likelihood approach. The tree showed clear separation of \( \text{L. reuteri} \) strains into five host-defined phylogenetic lineages (Fig. 5B). This analysis also revealed that \( \text{L. reuteri} \) AN417 isolated from pigs clustered in lineage IV, which is contained in strains originating from pigs.

The whole genome sequence of \( \text{L. reuteri} \) AN417 encodes the urease complex UreABCEFDG unlike general strains isolated from human and pigs, contributing to its viability under acidic conditions (Fig. 5C). The mucin binding protein, Muc2, contributes to host adaptation and adhesion to mucus. However, genes encoding reuteran, reutericyclin, and reuterin, which are important in the antimicrobial activity of \( \text{L. reuteri} \) strains, were absent in...
the genome (Fig. 5C). In addition, reuterin production was also measured in L. reuteri AN417 (Table 2), demonstrating that L. reuteri AN417 did not produce reuterin. This result was consistent with the genomic analysis results (Table 1).

**Antibacterial activity of LRS is mediated by the presence of fatty acids and sugars.** To categorize the type of metabolites responsible for the activity of LRS against periodontopathogens, the antibacterial effect of LRS against P. gingivalis in the presence of proteinase K, lipase, and α-amylase was evaluated. Treatment of LRS with lipase or α-amylase eliminated the inhibitory effect of LRS on P. gingivalis growth (Fig. 6A–C). These findings suggested that the antibacterial effect of LRS against oral pathogenic bacteria could be attributed to the presence of a fatty acid and/or sugar.
Discussion

The present study reports the antibacterial effects of cell-free culture supernatant from *L. reuteri* strain AN417 (LRS), a strain that was isolated from the porcine small intestine, against selected oral pathogenic bacteria. Based on our results, we anticipate that *L. reuteri* AN417 can positively affect oral health. Recently, there has been growing interest in the potential and utilization of microbial metabolites, termed postbiotics\(^2\). The cell-free culture supernatant of *L. reuteri* AN417 exhibited greater antimicrobial activity than those of the known *Lactobacillus* reference strains KCTC 3594 and KCTC 3678, which also inhibited periodontopathic bacteria (Fig. 1A,B). In our study, multiple lines of scientific evidence validated the antimicrobial activity of LRS.

Evaluation of the antimicrobial effects of naturally-derived agents has largely focused on their activity against *P. gingivalis*, *F. nucleatum*, and *S. mutans* because these bacteria have received the most attention in relation to oral diseases and are implicated in periodontal diseases, dental caries, and endocarditis\(^7\)–\(^10\). To date, the antimicrobial effects of naturally derived agents against these bacteria have been evaluated in numerous studies, with...
a recent study reporting the antimicrobial activities of L. reuteri supernatant against P. gingivalis\textsuperscript{21}. Hence, we also focused on these three oral pathogens in our study. The major antibacterial compounds produced by L. reuteri strains are reuterin, reutericyclin, and reuteran. Genomic analysis of L. reuteri AN417 revealed the absence of a pdu-cbi-cob-hem gene cluster for the biosynthesis of reuterin and cobalamin (vitamin B\textsubscript{12}), and genes for the synthesis of reutericycin and reuteran. However, L. reuteri AN417 encoded an inulin-type fructansucrase. The strain also encoded the UreABCEFGD urease complex, which was not found in the other strains isolated from humans and pigs, contributing to its viability under acidic conditions.

We compared the activity of concentrated L. reuteri AN417 cell-free culture medium (LRS) to the activity of L. reuteri AN417 cell extracts using organic solvents. The results showed that L. reuteri AN417 cell extracts had no antimicrobial activity, whereas LRS did (Fig. 1C). From these data, secondary metabolites produced during growth are thought to play a crucial role in antimicrobial activities against oral pathogenic bacteria\textsuperscript{24}. Bungenstock et al. reported that the antibacterial effect of probiotics against fermented foodborne pathogens is attributable to their production of lactic acid and the associated increase in acidity\textsuperscript{25}. However, the antimicrobial activity of LRS observed in this study may not have been due to lactic acid alone. Probiotic Lactobacillus spp. are

| Species          | Strains          | Genome assembly acc. No | Countries | Origin | No. of contigs | Genome size (bp) | GC ratio (%mol) | Notes               |
|------------------|------------------|-------------------------|-----------|--------|----------------|------------------|-----------------|-------------------|
| L. reuteri       | 121              | GCA_001889975.1         | Netherlands | Pig    | 14             | 2,302,234        | 39.01           |                   |
| L. reuteri       | 2010             | GCA_005703885.1         | USA       | Rat    | 38             | 2,220,255        | 38.53           |                   |
| L. reuteri       | 100–23           | GCA_000168255.1         | New Zealand | Rat    | 2              | 2,305,557        | 38.73           |                   |
| L. reuteri       | ATCC 53,608      | GCA_000236455.2         | Sweden    | Pig    | 3              | 2,091,243        | 38.75           |                   |
| L. reuteri       | ATG-F4           | GCA_004208615.1         | Korea     | Human  | 1              | 2,041,516        | 38.89           |                   |
| L. reuteri       | Byun-re-01       | GCA_003316895.1         | Korea     | Mouse  | 1              | 2,244,514        | 38.88           |                   |
| L. reuteri       | CNI-KCA2         | GCA_012275185.1         | Nigeria   | Chicken | 1             | 2,072,001        | 38.92           |                   |
| L. reuteri       | CRL 1098         | GCA_001657495.1         | Germany   | Sourdough | 45          | 1,963,029        | 38.74           |                   |
| L. reuteri       | DSM 20,016       | GCA_000016825.1         | Korea     | Human  | 1              | 1,999,618        | 38.87           | Type strain       |
| L. reuteri       | 149              | GCA_001688685.2         | Switzerland | Mouse | 1              | 2,044,771        | 38.76           |                   |
| L. reuteri       | IS007            | GCA_000410995.1         | China     | Pig    | 7              | 2,093,275        | 38.93           |                   |
| L. reuteri       | IRT              | GCA_0011046835.1        | Korea     | Human  | 1              | 1,993,967        | 38.90           |                   |
| L. reuteri       | L6798            | GCA_900093565.1         | Sweden    | Mouse  | 35             | 2,108,374        | 38.43           |                   |
| L. reuteri       | LL7              | GCA_007633215.1         | USA       | Mouse  | 2              | 2,384,717        | 38.81           |                   |
| L. reuteri       | LTH2584          | GCA_000712555.1         | Germany   | Sourdough | 55          | 2,066,054        | 38.53           |                   |
| L. reuteri       | LTH5448          | GCA_000758185.1         | Germany   | Sourdough | 36          | 1,980,298        | 38.44           |                   |
| L. reuteri       | LTR1318          | GCA_009184725.1         | China     | Human  | 2              | 2,047,619        | 38.98           |                   |
| L. reuteri       | Marseille-P4870  | GCA_901600665.1         | France    | Yogurt | 287            | 2,039,591        | 38.98           | For only ANI calculation |
| L. reuteri       | Marseille-P4904  | GCA_901600705.1         | France    | Yogurt | 282            | 2,039,591        | 38.98           | For only ANI calculation |
| L. reuteri       | Marseille-P5460  | GCA_901600695.1         | France    | Yogurt | 316            | 2,039,540        | 39.21           | For only ANI calculation |
| L. reuteri       | Marseille-P5461  | GCA_901600675.1         | France    | Yogurt | 311            | 2,039,572        | 39.21           | For only ANI calculation |
| L. reuteri       | MM4-1A           | GCA_000159475.2         | Finland   | Human  | 7              | 2,067,914        | 38.88           |                   |
| L. reuteri       | SD2112           | GCA_000159455.2         | Peru      | Human  | 5              | 2,316,838        | 39.04           |                   |
| L. reuteri       | SKKU-OGDONS-01   | GCA_003316935.1         | Korea     | Chicken | 1             | 2,259,968        | 38.86           |                   |
| L. reuteri       | TDI              | GCA_000439275.1         | USA       | Rat    | 1              | 2,145,445        | 38.78           |                   |
| L. reuteri       | TMW1.112         | GCA_000722353.2         | Germany   | Sourdough | 12          | 2,032,034        | 38.45           |                   |
| L. reuteri       | TMW1.656         | GCA_000712563.2         | Germany   | Sourdough | 17          | 1,949,539        | 38.49           |                   |
| L. reuteri       | UBLRU-87         | GCA_003719715.1         | India     | Fermented food | 91    | 1,821,307        | 38.70           | For only ANI calculation |
| L. reuteri       | WHH1689          | GCA_0003072625.1        | China     | Highland barley wine | 1 | 2,044,184        | 39.31           |                   |
| L. reuteri       | YSIL-12          | GCA_000874665.1         | China     | Pig    | 3              | 2,151,788        | 38.93           |                   |
| L. reuteri       | ZLR003           | GCA_001618905.1         | China     | Pig    | 1              | 2,234,097        | 38.66           |                   |
| L. reuteri       | AN417            | In this study           | Korea     | Pig    | 5              | 2,162,818        | 38.95           |                   |
| L. gastricus     | LG045            | GCA_009648555.1         | Korea     | Human  | 3              | 1,905,155        | 41.65           | Outgroup for tree |
| L. reuteri       | DSM 17,896       | GCA_001437055.1         | Germany   | Sourdough | 15          | 1,646,143        | 47.72           | Outgroup for tree |

Table 2. Lactobacillus reuteri genomes used for phylogeny reconstruction and comparative genomics. Whole genome phylogenetic analysis was performed with 27 L. reuteri genomes except for them with large numbers of contigs.
Figure 5. ANI values between genomes of *L. reuteri* strains and their phylogenetic position. (A) ANI values between genomes of *L. reuteri* strains. (B) Phylogenetic tree reconstructed using the amino acid alignments of 766 core genes using the maximum likelihood approach. Numbers above branches show maximum likelihood bootstrap supports from 500 non-parametric replicates. The tree was rooted using *L. gastricus* LG045 and *L. secaliphilus* DSM 17896 as outgroups. The scale bar represents the number of substitutions per site. (C) Genes encoding proteins related to environmental adaptation and antimicrobial compounds.
also known to produce various metabolites that defend against **S. mutans** colonization speakers. The level of intracellular ATP, the energy source for viable oral pathogenic bacteria, was reduced significantly by LRS, leading to the growth inhibition of oral pathogenic bacteria (Fig. 1D, right). The inhibitory effect was maintained for 96 h, indicating that the bioactive substances in the LRS were stable over time. Growth of **S. mutans** (a Gram-positive bacterium), **F. nucleatum** (Gram-negative), and **P. gingivalis** (Gram-negative) was inhibited by adding 30% (v/v), 20% (v/v), and 10% (v/v) of LRS, respectively (Fig. 2A), demonstrating that bacterial growth inhibition by LPS was the most potent and most specific for **P. gingivalis**. In addition, we speculate that higher concentrations of antimicrobial substances are required to inhibit the growth of Gram-positive bacteria, which have thick peptidoglycan cell walls.

Endotoxins, such as lipopolysaccharides (LPS), are produced from oral bacterial biofilms, including oral plaques. Endotoxins destroy alveolar bone and induce a series of inflammatory reactions that ultimately lead to tooth loss. To determine whether the effect originated from bacterial membrane damage, SYTO9 and propidium iodide staining was performed. LRS treatment resulted in a marked decrease in viable bacterial cells (Fig. 3). This result may imply that bacterial membrane integrity was weakened by treatment with LRS, which led to the inhibition of bacterial growth and a reduction in the continuous release of endotoxins.

Biofilms are the cause of dental bacterial infections. Biofilms are comprised of extracellular polymeric substances secreted by bacteria during metabolic processes. The biofilm structure confers antibiotic resistance. Biofilms in the oral cavity generally contribute to periodontitis. Within biofilms, resident microbes are resistant to external attacks that include antibacterial agents, and the bacteria dispersed from the biofilms can cause infection. Biofilm dispersal agents might be the most suitable targets for the prevention of periodontitis and dental caries. In previous studies, supernatants from cultures of **Lactobacillus** sp. inhibited biofilm formation and reduced the expression of genes related to the production of exopolysaccharides. In the current study, LRS significantly reduced biofilm formation (Fig. 4), which could contribute to the prevention of dental caries and periodontitis.

LRS has excellent inhibitory abilities against the growth and biofilm formation of the tested bacteria. In particular, **P. gingivalis** required the lowest amounts of LRS to inhibit its growth and biofilm formation among the bacteria tested in our study. Thus, we focused on **P. gingivalis** in our other analyses, as this was the pathogen most strongly affected by LRS.

The antimicrobial activity of **L. reuteri** has been attributed to its production of organic acids, hydrogen peroxide, and bacteriocin-like compounds, such as reuterin, reuteran, and reutericyclin. However, **L. reuteri** AN417 lacks the ability to produce them because of the absence of the required genes. Thus, to identify the antimicrobial substance in LRS, various enzymes such as α-amylase, lipase, and proteinase were added to LRS to catabolize substances secreted by bacteria during metabolic processes. The biofilm structure confers antibiotic resistance. Biofilms in the oral cavity generally contribute to periodontitis. Within biofilms, resident microbes are resistant to external attacks that include antibacterial agents, and the bacteria dispersed from the biofilms can cause infection. Biofilm dispersal agents might be the most suitable targets for the prevention of periodontitis and dental caries. In previous studies, supernatants from cultures of **Lactobacillus** sp. inhibited biofilm formation and reduced the expression of genes related to the production of exopolysaccharides. In the current study, LRS significantly reduced biofilm formation (Fig. 4), which could contribute to the prevention of dental caries and periodontitis.

According to a previous study, soluble or immobilized PLNC8 β bacteriocins from **L. plantarum** strains NC8 and 44048 prevent **P. gingivalis** colonization and pathogenicity. Therefore, it is necessary to conduct additional experiments under various conditions to support the findings of this study. Furthermore, the bioactive substances in LRS should be identified and purified for practical use.

In this study, we show that supernatants derived from **L. reuteri** AN417 cultures are able to suppress the growth and biofilm formation of oral pathogenic bacteria. Interestingly, **L. reuteri** AN417 does not produce reuteran, reutericyclin, and reuterin, which are important in the antimicrobial activity of reported **L. reuteri** strains. Although further studies are required, the antibacterial substance in LRS is suspected to be a fatty acid or a sugar, which has not yet been reported. Thus, LRS has potential as a novel bioactive substance for the prevention and treatment of pathogens associated with periodontitis.
Methods

Strain isolation and identification. Novel Lactobacillus reuteri strains were isolated from seven infants aged 3–7 years and from the small intestines of 13 6-month-old female pigs in the Republic of Korea. The pigs were fed a mixed diet. The intestinal contents from each child or pig were resuspended and serially diluted in sterile 0.85% NaCl. Aliquots were cultured anaerobically on MRS agar in an atmosphere of CO₂:H₂:N₂ (7:7:86) atmosphere. After 2–3 days, single colonies were subcultured on fresh MRS agar. All colonies were selected irrespective of their shape and size. Genomic DNA (gDNA) was extracted and purified from cells grown on MRS agar as described previously. The gDNA was used for 16S rRNA gene amplification and sequencing, and whole genome sequencing. The complete 16S rRNA gene sequence was amplified using universal primers: 27F (5’TAC GGY TAC CTT GTT ACG ACTT-3’) and 1492R (5’TAC GGY TAC CTT GTT ACG ACTT-3’). The amplified genes were sequenced and compared with sequences obtained from the EzBioCloud and GenBank/EMBL/DDBJ databases.

Identification of isolates using a MALDI-TOF Biotyper. One colony of each bacterial isolate was subcultured for 24 h and used for MALDI-TOF Biotyper analysis. The colony was acquired using a toothpick and spotted onto a polished steel MALDI target plate. One microliter of formic acid (70% in water) was added to the stock and dried. Subsequently, 1 μL of MALDI matrix (10 mg/mL solution of α-cyano-4-hydroxycinnamic acid (HCCA) in 50% acetonitrile/2.5% trifluoroacetic acid) was added to the spot and dried. The MALDI target plate was placed in the MALDI-TOF/Microflex LT instrument (Bruker Daltonics, Billerica, MA, USA) for automated measurement and data interpretation. The MALDI Biotyper output is a log (score) between 0 and 3.0, which is calculated from a comparison of the peak list from an unknown isolate with the reference MSP in the database. A log (score) ≥ 1.7 was indicative of a close relationship at the genus level. A log (score) ≥ 2.0 was set as the threshold for a match at the species level. Isolates with a log (score) ≥ 2.0 were accepted as the correct identification.

Bacteria, media, and culture. Lactobacillus reuteri strains KCTC 3594, KCTC 3678, KCTC 3679, KCTC 3680, KCTC 3682, and KCTC 3683 were used as reference strains. All samples were purchased from the Korean Collection for Type Cultures (KCTC, Daejeon, Korea). Reference strains and newly isolated L. reuteri strains (AN417, AN306, AN403, AN413, AN507, AN509, AN510, AN511, AN513, AN519, AN523, AN705, AN711, and RI-7) were grown on MRS agar plates. P. gingivalis strain BAA-308 and F. nucleatum KCTC 15573 were purchased from the American Type Culture Collection (Manassas, VA, USA) and KCTC, respectively. They were grown in trypticase soy broth (TSB; BD, Germany) comprised of (per L): 30 g trypticase soy broth, 5 g yeast extract, 1 mg vitamin K₁, and 15 g agar, or blood TSB agar (TSB medium plus 15 g/L agar and supplemented with 3% sheep blood). For all experiments, P. gingivalis and F. nucleatum were also cultured in TSB broth for at least 12 h prior to inoculation. Bacteria were grown and maintained at 37 °C in an anaerobic chamber in an atmosphere of CO₂:H₂:N₂ (5:10:85). S. mutans (KCTC 3065) strains were grown in brain heart infusion (BHI) medium at 37 °C under aerobic conditions.

Preparation of the test sample. Lactobacillus reuteri AN417 was grown in MRS broth for 48 h at 37 °C under anaerobic conditions to produce stationary phase cultures. The supernatant from L. reuteri AN417 culture (LRS) was collected via centrifugation at 4 °C for 30 min at 1500×g. The LRS was filtered through a 0.2 μm membrane filter to remove the remaining bacteria and debris. L. reuteri AN417 cell extract (BE) was prepared by the addition of 0.3 L ethyl acetate to the cell pellet, stirring the mixture for 24 h, and passing the mixture through a Phenex Telfon Polytetrafluoroethylene filter membrane. After centrifugation at 1500×g for 20 min, the cell pellet was removed. The cell-free supernatant and BEs were concentrated to 20× using a rotary evaporator and stored at 4 °C until required.

Determination of 1, 3-PDO production. Lactobacillus reuteri strains were grown in MRS medium (10 g/L proteose peptone No. 3, 10 g/L beef extract, 5 g/L yeast extract, 20 g/L dextrose, 1 g/L polysorbate 80, 2 g/L ammonium citrate, 5 g/L sodium acetate, 0.2 g/L MgSO₄·0.5 g/L MnSO₄, and 2 g/L dipotassium sulfate) supplemented with 20 g/L glycerol for 12 h under anaerobic conditions. The concentration of 1,3-PDO was determined using a 1200 series HPLC system (Agilent, Santa Clara, CA, USA) with a refractive index detector (RID) and an ion-exchange column (Aminex HPX-87H; Bio-Rad, Hercules, CA, USA) with a refractive index detector (RID) and an ion-exchange column (Aminex HPX-87H; Bio-Rad, Hercules, CA, USA). The mobile phase was 2.5 mM H₂SO₄, flow rate was 0.6 mL/min, column temperature was 65 °C, and RID was maintained at 45 °C.

Measurement of the concentration of reuterin (3-HPA). Cultivation to stationary phase was performed in a 250 mL round-bottom flask containing 100 mL of MRS medium for 24 h at 37 °C. Cells were harvested by centrifugation at 16,000×g for 5 min and washed twice in 50 mM sodium phosphate buffer (pH 7.5). The washed cells were resuspended in 200 mM glycerol solution and incubated at 30 °C for 3-HPA production. To measure the 3-HPA conversion yield, samples were collected at 1 h intervals. The samples were centrifuged at 16,000×g for 5 min and the supernatant was used to determine 3-HPA content. A previously described colorimetric method was used to determine the content of 3-HPA in the samples. The reaction consisting of 1 mL of the sample, 3 mL of HCl (37%), and 0.75 mL of 10 mM tryptophan-HCl. The mixture was mixed and incubated at 37 °C for 20 min, and the absorbance at 560 nm was measured using a SpectraMax 190 ELISA reader (Molecular Devices Corp., Sunnyvale, CA, USA). The amount of 3-HPA was calculated from the absorbance at 560 nm with acrolein as a standard.
Disk diffusion assay. Antimicrobial activity was determined using the disk diffusion assay. Cell-free supernatants of *L. reuteri* strains were prepared using 48-h cultures. Agar plates were inoculated with *E. coli, P. aeruginosa*, or *S. mutans*. Filter paper discs, approximately 6 mm in diameter, soaked with 50 μL of supernatant, were placed on the agar surface. After 24–48 h of incubation, the absence or presence of a clear zone around the disk was observed.

Growth rate measurement. To study the growth rates of the selected pathogenic bacteria in the presence or absence of LRS, bacteria were grown in 1 mL of the appropriate medium containing different concentrations of LRS at 37 °C in an anaerobic chamber or orbital shaker. Growth rates were determined by measuring the culture optical density at 600 nm (OD600) at various times.

ATP bioluminescence assay. ATP levels in cultures of the selected pathogenic bacteria treated with various concentrations of LRS were measured using a BacTiter-Glo ATP Assay Kit (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Bioluminescence measurements were obtained in triplicate for each sample. Pure culture media were used as negative controls. Luminescence was measured using a luminometer (Promega).

Minimal inhibitory volume (MIV) assay. The MIV of LRS was determined using a modified method of a previously described protocol. Pathogenic bacterial cultures generated in suitable liquid culture media as detailed above were diluted with nutrient broth to an OD600 of 0.005 or 0.05. The diluted bacterial suspensions were then treated with either MRS medium (control) or 1× LRS, dispensed into the first well of a 96-well plate, and serially diluted into consecutive wells. Plates were incubated at 37 °C for 24 h, and the absorbance at 600 nm was measured using a microplate reader.

LIVE/DEAD BacLight viability assay. The LIVE/DEAD BacLight Kit (Molecular Probes-Invitrogen, Carlsbad, CA, USA) was used to distinguish live and dead bacterial cells. The assay, which is based on membrane integrity and nucleic acid staining, was performed according to the manufacturer’s instructions. SYTO9 green fluorochrome (Thermo Fischer Scientific, Waltham, MA, USA) can penetrate the intact bacterial membrane, whereas the larger red fluorochrome, propidium iodide, only penetrates the membranes of damaged bacteria. Cells treated with LRS or MRS medium (control) for 24 h were stained in the dark for 15 min. The cells were mounted on slides and evaluated by fluorescence microscopy.

Biofilm inhibition assay. The bacterial suspension was prepared by diluting an overnight culture of bacteria in TSB broth. Dilutions of LRS in bacterial suspensions were prepared in polystyrene-coated flat-bottomed 24-well plates and incubated at 37 °C for 5 days without shaking to allow the development of multilayer biofilms. A pure culture medium served as a negative control. Biofilm biomass was assayed using the modified crystal violet staining assay and a LIVE/DEAD BacLight Kit (Molecular Probes-Invitrogen, Carlsbad, CA, USA) assay.

Time-kill assay. The time-to-kill *P. gingivalis* following LRS treatment was determined based on a previously described protocol. A bacterial suspension (OD600 = 1) was treated with LRS and incubated anaerobically at 37 °C. An aliquot (100 μL) of this bacterial culture was removed at 0, 4, 8, 24, 48, and 72 h following LRS treatment, and plated on TSB agar to quantify the number of colony forming units (CFUs) in the treated cultures. Pure MRS medium was used as a negative control.

Real-time quantitative polymerase chain reaction (RT-qPCR) analysis. The potential of LRS to prevent biofilm formation and/or destroy established biofilms was investigated. *P. gingivalis* (OD600 = 0.6) was dispensed into a polystyrene-coated 6-well plate and treated with MRS medium (control) or LRS for 48 h in an anaerobic incubator. In addition, *P. gingivalis* biofilms were established in a polystyrene-coated 6-well plate for 5 days, after which these biofilms were treated with either MRS medium (control) or LRS for 48 h in anaerobic conditions. Total RNA was extracted with TRIzol reagent, and cDNA was synthesized using a PrimeScript RT reagent Kit (TaKaRa Bio, Shiga, Japan). RT-qPCR analysis of cDNA was performed according to the manufacturer’s instructions (Bioneer, Daejeon, South Korea) to investigate the mRNA expression of the selected genes related to biofilm formation. The primers were used: hagA, Forward: 5’-ACAGCCTACGCGGATTTCC-3’, Reverse: 5’-CGAATTCATTGCCACCTTCT-3’; hagB, Forward: 5’-TGGTGACGGAAATATGCCTAAAC-3’; Reverse: 5’-CTGGCTTGTCCTGAGAAGCATA-3’; rgpA, Forward: 5’-GCCGAGATTGGTCTGAAACG-3’; Reverse: 5’-CGGAGCGAATTGGAATGGAAGGACTCTTGGAAC-3’; Forward: 5’-CGGAATACCATGCGGTG-3’; kpg, Forward: 5’-AGCTGACAAAGGGTGAGACCAAGG-3’; Reverse: 5’-TGGTGACATGTTTTTGCCGAACGGT-3’; and 16S rRNA, Forward: 5’-TGTAGTAGACTGTGTTCTCGGAG-3’; Reverse: 5’-ACGTGTACGACATCCCGATCT-3’. The reaction procedure involved incubation at 95 °C for 5 min followed by 30 cycles of 95 °C for 10 s, 55 °C for 30 s, and 72 °C for 30 s. Three independent reactions were conducted in triplicate for each gene.

Effect of enzymes on antibacterial activity. *Lactobacillus reuteri* AN417 cell-free supernatants were treated with enzymes to evaluate the effect of enzymes on antibacterial substances. The LRS were treated with proteinase K (1 mg/mL), lipase (700 units/mg), or α-amylase (150 units/mg). For lipase and α-amylase treatment, the pH of the LRS was adjusted to 6.5 with NaOH to facilitate enzymatic activity. Enzymes were activated by incubation of the enzyme-treated supernatant at 37 °C for 3 h, after which the enzymes were immediately activated.
inactivated at 95 °C for 3 min. LRS-enzyme suspensions were centrifuged, and the supernatant was stored at 4 °C until further use.

**Whole genome sequencing and analysis.** Whole genome sequencing of strain 417 was performed using PacBio RS II (Pacific Biosciences, Menlo Park, CA, USA) SMRT sequencing technology. A standard PacBio library with an average of 20 kb inserts was prepared and sequenced, yielding >371.74 x average genome coverage. De novo assembly of the 128,664 subreads with 7,806 nucleotides on average (1,004,367,686 bp in total) was conducted using the hierarchical genome-assembly process pipeline in SMRT Analysis v2.3.0 [41]. To correct the sequencing errors that can occur at both ends of a contig, the SMRT resequencing protocol was performed with assembly in which the first half of the contig was switched with the second half. Protein-coding genes were predicted using Prodigal v2.6.3. Ribosomal RNA, transfer RNA, and miscellaneous features were predicted using Rfam v12.0 [42]. CRISPR loci were predicted using the CRISPR recognition tool. ANI values were calculated using an online ANI calculator [43].

**Statistical analyses.** All statistical analyses were performed using Student’s t-test. The results are expressed as the mean ± standard deviation for each group. Multiple group data were analyzed using one-way analysis of variance, followed by Dunnett’s multiple range test. The threshold for significance was set at p < 0.05. Data shown are representative of three independent experiments, except for Fig. 1B, in which the data are from two independent experiments.

**Data availability** Whole genome sequences were deposited with Bioproject PRJNA637956 and Biosample SAMN15162791, respectively. GenBank accession numbers are CP054657 for single chromosome and CP054658–CP054661 for the four plasmids, respectively.

Received: 14 July 2020; Accepted: 28 December 2020
Published online: 15 January 2021

**References**

1. Hoare, A., Soto, C., Rojas-Celis, V. & Bravo, D. Chronic inflammation as a link between periodontitis and carcinogenesis. *Mediat. Inflamm.* 2019, 1029857. https://doi.org/10.1155/2019/1029857 (2019).
2. Takenaka, S., Ohsumi, T. & Noiri, Y. Evidence-based strategy for dental biofilms: Current evidence of mouthwash on dental biofilm and gingivitis. *Ipn. Dent. Sci. Rev.* 55, 33–40 (2019).
3. Kononen, E., Gursoy, M. & Gursoy, U. K. Periodontitis: A multifaceted disease of tooth-supporting tissues. *J. Clin. Med.* 8, 1135. https://doi.org/10.3390/jcm8081135 (2019).
4. Seguier, S., Gogly, B., Bodineau, A., Godeau, G. & Brousse, N. Is collagen breakdown during periodontitis linked to inflammatory cells and expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human gingival tissue? *J. Periodontol.* 72, 1398–1406 (2001).
5. Tanner, A. C. Anaerobic culture to detect periodontal and caries pathogens. *J. Oral. Biosci.* 57, 18–26 (2015).
6. Shaddox, L. M. et al. Local inflammatory markers and systemic endotoxin in aggressive periodontitis. *J. Dent. Res.* 90, 1140–1144 (2011).
7. Mysak, J. et al. Porphyromonas gingivalis: Major periodontopathic pathogen overview. *J. Immunol. Res.* 2014, 746068. https://doi.org/10.1155/2014/746068 (2014).
8. Signat, B., Roques, C., Poulet, P. & Duffaut, D. Fusobacterium nucleatum in periodontal health and disease. *Carr. Issues Mol. Biol.* 13, 25–36 (2011).
9. Jain, S. & Darveau, R. P. *Periodontitis: A multifaceted disease of tooth-supporting tissues*. New York, NY: VHC (2011).
10. Li, X., Kolltveit, K. M., Tronstad, L. & Olsen, I. *Systemic diseases caused by oral infection*. New York, NY: H.L. (2011).
11. Li, X., Kolltveit, K. M., Olsen, I. & Osbed, A. Systemic diseases caused by oral infection. *Clin. Microbiol. Rev.* 13, 547–558 (2000).
12. S. J. Lactic acid bacteria and human health. *Ann. Med.* 22, 37–41 (1990).
13. Cardoso, R. S. et al. Effects of *Bifidobacterium animalis* subsp. lactis HN019 on ligature-induced periodontitis in rats with experimental rheumatoid arthritis. *Benef. Microbes* 11, 33–46 (2020).
14. Khalil, D., Hultin, M., Rashid, M. U. & Lund, B. Oral microflora and selection of resistance after a single dose of amoxicillin. *Clin. Microbiol. Infect.* 22, 949E1–949E4. https://doi.org/10.1016/j.cmi.2016.08.008 (2016).
15. Lien, H. M. et al. Antimicrobial activity of Antrodia camphorata extracts against oral bacteria. *PLoS ONE* 9, e105286. https://doi.org/10.1371/journal.pone.0105286 (2014).
16. More, G., Thikkalange, T. E., Lall, N., Botha, F. & Meyer, J. J. Antimicrobial activity of medicinal plants against oral microorganisms. *J. Ethnopharmacol.* 119, 473–477 (2008).
17. Choi, H. A. et al. Antimicrobial and anti-biofilm activities of the methanol extracts of medicinal plants against dental pathogens *Streptococcus mutans* and *Candida albicans*. *J. Microbiol. Biotechnol.* 27, 1242–1248 (2017).
18. Uraz, A., Karaduman, B., Isler, S. C., Gonen, S. & Cetiner, D. Ozone application as adjunctive therapy in chronic periodontitis: Clinical, microbiological and biochemical aspects. *J. Dent. Sci.* 14, 27–37 (2019).
19. Haukioja, A. Probiotics and oral health. *Eur. J. Dent.* 4, 348–355 (2010).
20. Lin, H. S., Yeo, J. E., Hong, S. P. & Kang, M. S. Characterization of antibacterial cell-free supernatant from oral care probiotic *Weissella cibaria*. *CMU. Molecules* 23, 1984 (2018).
21. Geraldo, B. M. C. et al. Heat-killed *Lactobacillus reuteri* and cell-free culture supernatant have similar effects to viable probiotics during interaction with Porphyromonas gingivalis. *J. Periodontol. Res.* 55, 215–220 (2020).
22. Wegh, C. A. M., Geerlings, S. Y., Knol, J., Roeselers, G. & Belzer, C. Postbiotics and their potential applications in early life nutrition and beyond. *Int. J. Mol. Sci.* 20, 4673. https://doi.org/10.3390/ijms20154673 (2019).
23. Ren, D., Zhu, J., Geng, S., Liu, H. & Yu, H. Antimicrobial characteristics of lactic acid bacteria isolated from homemade fermented foods. *Biomed. Res. Int.* 2018, 516725. https://doi.org/10.1155/2018/516725 (2018).
24. Craney, A., Ahmed, S. & Nodwell, J. Towards a new science of secondary metabolism. *J. Antimicrob. (Tokyo)* 66, 387–400 (2013).
25. Bungenstock, L., Abdulmawjood, A. & Reich, I. Evaluation of antibacterial properties of lactic acid bacteria from traditionally and industrially produced fermented sausages from Germany. PLoS ONE 15, e0230345. https://doi.org/10.1371/journal.pone.0230345 (2020).
26. Wasfi, R., Abd El-Rahman, O. A., Zafer, M. M. & Ashour, H. M. Probiotic Lactobacillus sp. inhibit growth, biofilm formation and gene expression of caries-inducing Streptococcus mutans. J. Cell Mol. Med. 22, 1972–1983 (2018).
27. How, K. Y., Song, K. P. & Chan, K. G. Porphyromonas gingivalis: An overview of periodontopathic pathogen below the gum line. Front. Microbiol. 7, 53. https://doi.org/10.3389/fmicb.2016.00503 (2016).
28. Bjarnsholt, T. The role of bacterial biofilms in chronic infections. APMIS Suppl. 121, 1–51 (2013).
29. Saini, R., Saini, S. & Sharma, S. Biofilm: A dental microbial infection. J. Nat. Sci. Biol. Med. 2, 71–75 (2011).
30. Jayathilake, P. G. et al. Extracellular polymeric substance production and aggregated bacteria colonization influence the competition of microbes in biofilms. Front. Microbiol. 8, 1865. https://doi.org/10.3389/fmicb.2017.01865 (2017).
31. Guilhaum, C., Forestier, C. & Balestrino, D. Biofilm dispersal: Multiple elaborate strategies for dissemination of bacteria with unique properties. Mol. Microbiol. 105, 188–210 (2017).
32. Kang, M. S. et al. Inhibitory effect of Lactobacillus reuteri on periodontopathic bacteria. J. Microbiol. 49, 193–199 (2011).
33. Cleuxis, V., Lacroix, C., Vollenweider, S., Duboux, M. & Le Blay, G. Inhibitory activity spectrum of reuterin produced by Lactobacillus reuteri against intestinal bacteria. BMC Microbiol. 7, 101. https://doi.org/10.1186/1471-2180-7-101 (2007).
34. Mu, Q., Tavella, V. J. & Luo, X. M. Role of Lactobacillus reuteri in human health and diseases. Front. Microbiol. 9, 757. https://doi.org/10.3389/fmicb.2018.00757 (2018).
35. Khalaf, H. et al. Antibacterial effects of Lactobacillus and bacteriocin PLNC8 alphabeta on the periodontal pathogen Porphyromonas gingivalis. BMC Microbiol. 16, 188. https://doi.org/10.1186/s12866-016-0810-8 (2016).
36. Chun, I. & Goodfell, M. A phylogenetic analysis of the genus Nocardiia with 16S rRNA gene sequences. Int. J. Syst. Bacteriol. 45, 240–245 (1995).
37. Yoon, S. H. et al. Introducing EzBioCloud: A taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. Int. J. Syst. Evol. Microbiol. 67, 1613–1617 (2017).
38. Slininger, P. J., Bothast, R. J. & Smiley, K. L. Production of 3-hydroxypropionaldehyde from glycerol. Appl. Environ. Microbiol. 46, 62–67 (1983).
39. Spinder, J. K. et al. Human-derived probiotic Lactobacillus reuteri demonstrate antimicrobial activities targeting diverse enteric bacterial pathogens. Anaerobe 14, 166–171 (2008).
40. Appiah, T., Boakye, Y. D. & Agyare, C. Antimicrobial activities and time-kill kinetics of extracts of selected Ghanaian mushrooms. Evid. Based Complement Alternat. Med. 2017, 453430. https://doi.org/10.1155/2017/453430 (2017).
41. Chin, C. S. et al. Nonhybrid, finished microbial genome assemblies from long-read SMRT sequencing data. Nat. Methods 10, 563–569 (2013).
42. Griffiths-Jones, S., Bateman, A., Marshall, M., Khanna, A. & Eddy, S. R. Rfam: An RNA family database. Nucleic Acids Res. 31, 439–441 (2003).
43. Kook, J. K. et al. Genome-based reclassification of Fusobacterium nucleatum subspecies at the species level. Curr. Microbiol. 74, 1137–1147 (2017).

Acknowledgements

This work was supported by a National Research Foundation of Korea (NRF) Grant funded by the Korean Government (MSIP) (No. 2019R1F1A1051382 and No. 2016M3A9A5919255).

Author contributions

K.M.Y., J.-S.K., H.-W.J., D.-S.P. and K.-H.B conceived and designed the experiments. K.M.Y. and J.-S.K. analyzed the data. K.M.Y., D.-S.P. and K.-H.B. wrote the manuscript. H.-S.K., Y.-Y.K. and J.-K.O. provided help with critical discussion, editing, and final approval of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-020-80921-x.

Correspondence and requests for materials should be addressed to D.-S.P or K.-H.B.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2021