Park *et al. Annals of Microbiology* (2025) 75:2
https://doi.org/10.1186/s13213-024-01790-7

Annals of Microbiology

UNIVERSITÀ DEGLI STUDI DI MILANO

ORIGINAL ARTICLE

Open Access

Biosafety characteristics and antibacterial activity of probiotic strains against *Streptococcus mutans*, *Aggregatibacter actinomycetemcomitans*, and *Porphyromonas gingivalis*

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Abstract

Background Oral diseases with high prevalence worldwide are recognized as severe health problems. Probiotics are used to prevent oral diseases, including dental caries, oral malodor, periodontitis, and subgingival plaque. In this study, we aimed to confirm the antibacterial effect of probiotics on oral pathogens and to assess their characterization and safety as probiotics.

Methods The antibacterial effects of *Lacticaseibacillus rhamnosus* MG4706, *Lacticaseibacillus paracasei* MG4715, and *Limosilactobacillus reuteri* MG4722 on the growth biofilm formation of *Streptococcus mutans*, *Aggregatibacter actinomycetemcomitans*, and *Porphyromonas gingivalis* were evaluated. We also investigated the production of antibacterial substances (H_2O_2 and reuterin) by these strains and their ability to adhere to oral epithelial cells. The safety of *L. reuteri* MG4722 was verified through whole-genome sequencing analysis and antibiotic susceptibility, lactate dehydrogenase activity, hemolytic activity, and bile acid hydrolase activity. The reuterin biosynthesis genes of *L. reuteri* MG4722 were identified using genomic analysis.

Results *L. reuteri* MG4722 significantly inhibited the growth of *S. mutans*, *A. actinomycetemcomitans*, and *P. gingivalis* and suppressed the biofilm formation by *A. actinomycetemcomitans*. In addition, it showed considerable adhesion ability to oral epithelial cells. *L. reuteri* MG4722 produced H_2O_2 and reuterin as antibacterial substances, as confirmed by the presence of genes encoding the antibacterial compounds reuterin, reuteran, and reutericyclin. *L. reuteri* MG4722 showed no hemolysis, bile salt hydrolase activity, antibiotic resistance or toxicity to HT-29 cells, and no antibiotic-resistance genes were identified.

Conclusion *L. reuteri* MG4722 demonstrated antibacterial effects on oral pathogens by producing antibacterial substances and adhering to oral epithelial cells. These results suggest that *L. reuteri* MG4722 could be an effective probiotic for oral health.

Keywords *L. reuteri* MG4722, Oral pathogens, Hydrogen peroxide, Reuterin, Whole genome sequencing, Biosafety

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Background

Oral diseases are highly prevalent worldwide and are recognized as major health issues (Peres et al. 2019). According to the Centers for Disease Control and Prevention (CDC), more than 40% of adults experience oral discomfort and spend billions of dollars annually on treatment (Mann et al. 2021). More than 600 species of microorganisms are present in the oral cavity, and bacterial infections are responsible for most oral diseases. Various bacteria present in the oral cavity directly infiltrate vascular endothelial cells or damaged blood vessels and attach to specific organs, ultimately causing systemic diseases (Buchbauer et al. 1991).

Oral diseases include periodontal disease, dental caries, and halitosis (Haraguchi et al. 2014; Izidoro et al. 2022). Periodontal disease is a bacterial-induced inflammatory disease that destroys the tissue around teeth and is the leading cause of tooth loss during adulthood (Sang-Ngoen et al. 2021). The main bacteria associated with periodontal diseases are *Actinobacillus actinomycetemcomitans* (*A. actinomycetemcomitans*), *Porphyromonas gingivalis* (*P. gingivalis*), and *Treponema denticola*. *A. actinomycetemcomitans* is a gram-negative bacterium closely related to periodontitis, tooth loss, and neoplastic lesions (Damgaard et al. 2017). *P. gingivalis* is a gram-negative anaerobic bacterium, which initiates and progresses periodontal disease by decomposing proteins. Dental caries is a highly preventable disease worldwide that damages the calcified structure of tooth enamel (Chen et al. 2020). *Streptococcus mutans* (*S. mutans*) is a gram-positive facultative anaerobic bacterium that has long been considered pathogenic in dental caries and destroys tissues by excreting toxins or secondary products into periodontal tissues (Palombo 2011; Kulik et al. 2019). Halitosis is a disagreeable smell discharged from oral or nasal passages (Murata et al. 2002). Approximately 90% of cases of halitosis are attributed to conditions within the oral region, nasal cavity, upper respiratory tract, and upper digestive tract (Renvert et al. 2020). The main cause of halitosis is methyl mercaptan (CH_3SH), and the proportion of CH_3SH is relatively high in individuals with periodontal disease (Loesche and Kazor 2002). These compounds are synthesized by *P. gingivalis* (Kang et al. 2006).

Mouthwashes are commonly used to suppress pathogens in the oral cavity (Lee et al. 2021). However, these antibacterial substances can lead to an imbalance in the oral microbiome, eliminating beneficial bacteria and potentially leading to resistance (Kim et al. 2020). Probiotics can reduce and prevent oral diseases while resolving these side effects (How and Yeo 2021). Probiotic bacteria, such as *Lacticaseibacillus rhamnosus*, *Limosilactobacillus reuteri*, and *Lacticaseibacillus paracasei*, can rapidly colonize the oral cavity (Jiang et al. 2020). *L. rhamnosus* is safe for teeth and has been extensively studied as an oral probiotics (Elgamily et al. 2018). Studies on probiotics, such as *L. paracasei* and *L. reuteri*, have been conducted to prevent oral diseases or alleviate symptoms such as dental caries, oral malodor, periodontitis, and subgingival plaque, and *L. reuteri* has been reported to reduce the proportion of anaerobic bacteria in patients with chronic periodontitis (How and Yeo 2021).

In this study, we investigated the antibacterial effects and biological safety of *L. rhamnosus*, *L. paracasei*, and *L. reuteri* strains of oral origin against oral pathogens.

Materials and methods

Preparation of cell-free supernatant (CFS) of probiotic strains

L. rhamnosus MG4706, *L. paracasei* MG4715, and *L. reuteri* MG4722 were isolated from the oral cavity of a healthy human. Probiotic strains were confirmed by 16S rRNA gene sequencing (SolGent Co., Ltd., Daejeon, Republic of Korea) and registered on the NCBI database using BLAST (Table 1). Probiotics were cultured in de Man, Rogosa, and Sharp (MRS) broth (BD Bioscience, Franklin Lakes, NJ, USA) at 37°C for 24 h. Subsequently, the microbial load of probiotics was adjusted to an OD_{600} of 1.0 (10^8 CFU/mL) and subcultured at 37°C for 24 h. Cell-free supernatant (CFS) was obtained via centrifugation at 4,000 × g for 15 min at 4°C, adjusted to pH 7.4, and filtered using a 0.22-μm polytetrafluoroethylene membrane filter (ADVANTEC, Tokyo, Japan).

Bacterial cultures and antibacterial activity against oral pathogens

S. mutans KCTC3065, *A. actinomycetemcomitans* KCTC2581, and *P. gingivalis* KCTC5352 were purchased from the Korean Collection for Type Cultures (KCTC, Republic of Korea). *S. mutans* and *A. actinomycetemcomitans* were spread on a brain heart infusion (BHI) agar (Difco) plate and cultured at 37°C for 48 h. Single colony formed on the plate of *S. mutans* was cultured in BHI broth (Difco) for 24 h, adjusted to OD_{600} of 1.0 (1×10^8 CFU/mL), inoculated onto a 96-well plate (2×10^5 CFU/180 μL/well), treated with 10% CFS (20 μL), and incubated at 37°C for 24 h. A single colony formed on the plate of *A. actinomycetemcomitans* was cultured in BHI broth for 24 h, adjusted to an OD_{600} of 1.0 (1×10^8 CFU/

Table 1 Accession numbers and origins of probiotic strains used in this study

Strain	NCBI accession number	Origin
Lacticaseibacillus rhamnosus MG4706	OP077109	Human (oral)
Lacticaseibacillus paracasei MG4715	OP035523	
Limosilactobacillus reuteri MG4722	OP035530	

mL), inoculated onto a 96-well plate (2×10^6 CFU/180 µL/well), treated with 10% CFS (20 µL), and incubated at 37°C for 48 h. *P. gingivalis* was spread on Tryptic soy agar (TSA) containing 5 µg/mL hemin, 1 µg/mL vitamin K_1, and 5% sheep blood plate and cultured at 37°C for 7 days. Colonies formed on the plate were transferred on half-BHI medium containing yeast extract (5 mg/mL), hemin (5 µg/mL), and vitamin K_1 (1 µg/mL), adjusted to an OD_{600} of 1.0 (10^8 CFU/mL), inoculated onto 96-well plates (2×10^6 CFU/180 µL/well), treated with 10% CFS (20 µL), and incubated at 37°C for 4 days. Culture conditions for each strain were established through previous studies, and all strains were cultured under anaerobic conditions. The inhibitory effect of oral pathogens was assessed by measuring the absorbance at 600 nm using a microplate reader (BioTek, Winooski, VT, USA).

Biofilm formation

Biofilm formation by pathogens was assessed using crystal violet staining, as previously described, with some modifications (Zanetta et al. 2023). *S. mutans* was cultured in BHI broth for 24 h, adjusted to an OD_{600} of 1.0 (1×10^8 CFU/mL), inoculated onto a 96-well plate (1×10^4 CFU/180 µL/well), and cultured under anaerobic conditions for 12 h. Subsequently, 10% CFS (20 µL) was treated for an additional 24 h. *A. actinomycetemcomitans* was cultured in BHI broth for 24 h, adjusted to OD_{600} of 1.0 (1×10^8 CFU/mL), inoculated onto a 96-well plate (1×10^7 CFU/180 µL/well), and cultured under anaerobic conditions for 24 h. Subsequently, 10% CFS (20 µL) was added for an additional 24 h. *P. gingivalis* was suspended in half-BHI broth, adjusted to an OD_{600} of 1.0 (1×10^8 CFU/mL), and inoculated onto a 96-well plate (2×10^6 CFU/180 µL/well). After 5 days, the cells were treated with 10% CFS (20 µL) for 24 h. After cultivation, the pathogens were washed at least twice with distilled water and allowed to air dry. The pathogens were stained with 0.1% crystal violet for 2 min, washed thrice with distilled water, air-dried, and dissolved in 95% ethanol. The absorbance at 575 nm was measured using a microplate reader. Biofilm formation was calculated using the following equation:

$$Biofilm\ formation\ (\%) = 100 - \left[\frac{(ODcontrol - ODblank) - (ODsample - ODblank)}{ODcontrol - ODblank} \times 100\right]$$

OD_{blank}, Microbial culture medium; $OD_{control}$, Microbial suspension; OD_{sample}, Sample treated microbiological suspension.

Hydrogen peroxide (H2O2) production

The H_2O_2 production by the strains was evaluated using 3,3',5,5'-tetramethyl-benzidine (TMB)-MRS agar plates. The plates were prepared by autoclaving and drying MRS agar supplemented with 1.0 mM TMB and 10 µg/mL peroxidase (Sigma-Aldrich, St. Louis, MO, USA). The probiotics were suspended in MRS broth, spread on TMB-MRS agar plates, and then incubated under anaerobic conditions at 37°C for 48 h. Then, the plates were exposed to ambient air for 2 h to verify H_2O_2 production by the probiotic strains. The presence of H_2O_2 was indicated by the consumption of peroxidase, which catalyzes the oxidation of TMB, resulting in a blue coloration (Park et al. 2023).

Reuterin production

Reuterin was quantified using a colorimetric method, as previously reported, with some modifications (Cadieux et al. 2008). *L. reuteri* MG4722 was cultured at 37°C for 24 h and transferred to 300 mM glycerol under anaerobic conditions for 3 h. CFS was obtained by centrifugation at 4000 × g for 15 min at 4°C and filtered using a 0.22-µm polytetrafluoroethylene membrane filter. The supernatant (300 µL) was mixed with 10 mM tryptophan (225 µL), and 12 N HCl (900 µL) was added. After 30 min of incubation at 37°C, the absorbance at 450 nm was measured using a microplate reader. A standard curve was prepared using acrolein (AccuStandard, Inc., New Haven, CT, USA).

Adhesion assay on oral epithelial cells

The ability of the probiotic strains to adhere to mouth epidermal carcinoma (KB) cells was assessed as previously described, with some modifications (Park et al. 2023). Briefly, KB cells (Korea Cell Line Bank) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (P/S; Gibco). Briefly, KB cells were seeded in 24-well plates (2.0×10^5 cells/well) and incubated for 48 h to form a monolayer. Subsequently, cells were treated with probiotic strains (1×10^8 CFU/mL) for 2 h, washed thrice, and lysed with 10 mM phosphate-buffered saline (PBS, pH 7.4). The adhesion rate (%) was determined through colony counts on the MRS agar plates and calculated using the following equation:

$$Adhesion\ rate\ (\%) = \frac{\log(adherent\ counts)CFU/mL}{\log(initial\ counts)CFU/mL} \times 100$$

Initial count, initial bacterial count before attachment to cells; adherent counts, bacterial count after washing.

Antibiotic susceptibility

The antibiotic resistance of *L. reuteri* MG4722 was determined using the minimum inhibitory concentrations (MICs) of antibiotics (ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, streptomycin, and tetracycline). *L. reuteri* MG4722 was cultured in MRS broth at 37°C for 18 h, harvested by centrifugation at 4000 × g for 10 min at 4°C, and washed twice with

10 mM PBS (pH 7.4). Cells were resuspended in PBS at a McFarland standard turbidity of 0.5 and inoculated onto Mueller-Hinton agar and LAB susceptibility test medium (LSM; 90% Iso-Sensitest broth, 10% MRS broth, and 1.7% agar). MIC test strips (Liofilchem, Inc., Roseto degli Abruzzi, Italy) were placed on the plate according to the manufacturer's instructions and incubated at 37°C for 24 h. Antibiotic susceptibility was determined according to the European Food Safety Authority (EFSA) guidelines (FEEDAP et al. 2018).

Cytotoxicity

HT-29 cells (Korea Cell Line Bank) were cultured in 96-well plates (2.5×10^4 cells/well) in DMEM with 10% FBS and 1% P/S at 37°C under 5% CO_2 for 24 h. *L. reuteri* MG4722 (10^6–10^8 CFU/mL) was treated for 24 h. Cytotoxicity of *L. reuteri* MG4722 was determined using a Quanti-LDH PLUS cytotoxicity assay kit (Biomax, Seoul, Republic of Korea) following the manufacturer's instruction. Cytotoxicity was calculated using the following equation:

$$Cytotoxicity\ (\%) = 100 - \left[\frac{(Sample - Low\ control)}{(High\ control - Background\ control)} \times 100\right]$$

Low control, cell culture supernatant of cells only; High control, cell culture supernatant of cells after lysis; Background control, medium only.

Hemolytic activity

Hemolytic activity was determined using TSA containing 5% sheep blood (MBcell, Seoul, Republic of Korea). *L. reuteri* MG4722 was grown in MRS broth, streaked onto a TSA plate, and incubated for 48 h at 37°C. After 24 h, hemolytic activity was determined by evaluating the presence or absence of hemolysis around the colonies (Yasmin et al. 2020).

Bile salt hydrolase (BSH) activity

BSH activity was determined as previously described (Lee et al. 2023). *L. reuteri* MG4722 was grown in MRS broth, streaked onto a taurodeoxycholic acid hydrate (Sigma-Aldrich) agar plate, and incubated for 48 h at 37°C. BSH activity was assessed by examining the appearance of colonies surrounded by precipitated zones.

Morphology

L. reuteri MG4722 was cultured in MRS broth for 24 h, washed twice with PBS, and lyophilized for use. The morphology of *L. reuteri* MG4722 was assessed using a field emission-scanning electron microscope (SU5000 FE-SEM; Hitachi, Tokyo, Japan) as previously reported, with some modifications (Green Buzhor et al. 2024). The samples were prepared by vacuum-coating with a platinum bilayer. The surface and cross-sectional images of the strains were obtained at an acceleration voltage of 3.0 kV.

Whole genome sequencing (WGS)

The genomic DNA of *L. reuteri* MG4722 was extracted using a PureLink™ Microbiome DNA purification kit (Invitrogen, MA, USA) according to the manufacturer's instructions. A DNA library was prepared using a TruSeq Nano DNA library prep kit (Illumina, Inc., San Diego, CA, USA). WGS was performed using an Illumina Novaseq6000 instrument (Illumina, Inc.) for 2×150-bp sequencing on an Illumina platform by a certified service provider (DNA Link, Inc., Republic of Korea). The gene prediction of the coding sequences (CDS), ribosomal RNA (rRNA), and transfer RNA (tRNA) in the assembled gene was performed using Prokka v1.13. Gene annotation was performed using Blast2GO (BioBam Bioinformatics, Valencia, Spain).

Gene annotation was also reanalyzed according to the prokaryotic genome annotation pipeline. To identify the species based on genomic sequences, average nucleotide identity (ANI) values between *L. reuteri* MG4722 and several reference-type strains were compared using JSpecies v.1.2.1. Additionally, virulence factors were identified through homology searches using the Virulence Factor Database (VFDB) as a reference. The secondary metabolite biosynthetic gene cluster of *L. reuteri* MG4722 was identified using the antiSMASH ver. 7.0 database.

Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM) of three independent measurements. Normal distribution was verified using the Shapiro-Wilk test before further statistical analysis. In case the groups were normally distributed, a one-way analysis of variance (ANOVA) followed with the Dunnett's multiple comparisons test was performed. In case the groups were not normally distributed, the results were analyzed using the Kruskal–Wallis test followed with the Dunn's multiple comparisons to compare more than two group calculations. Statistical analysis was performed using Prism (ver. 10.4.0; GraphPad Software, San Diego, CA, USA). Statistical significance was set at $p < 0.05$.

Results

Growth and biofilm inhibitory effect of probiotic strains against oral pathogens

We investigated whether CFS of probiotic strains affected the growth of *S. mutans*, *A. actinomycetemcomitans*, and *P. gingivalis*. The growth of *S. mutans* was significantly inhibited by 31.4–42.2% by all strains, whereas the growth of *A. actinomycetemcomitans* and *P. gingivalis* was inhibited by 19.6% and 40%, respectively, by *L. reuteri* MG4722 (Fig. 1A).

We confirmed the antibiofilm activity of probiotic strains, showing that *L. rhamnosus* MG4706 and *L. paracasei* MG4715 significantly inhibited the biofilm formation by *S. mutans* ($p < 0.05$). All of the tested strains considerably inhibited biofilm formation by more than 50%, with *L. reuteri* MG4722 showing the highest inhibition of 80% ($p < 0.001$) against *A. actinomycetemcomitans. L. reuteri* MG4722 reduced the biofilm formation by *P. gingivalis;* however, the difference was not statistically significant (Fig. 1B).

Antibacterial substance (H_2O_2 and Reuterin) production of probiotic strains

We investigated whether the probiotic strains produce antibacterial substances. *L. reuteri* MG4722 showed H_2O_2 bioactivity. However, *L. rhamnosus* MG4706 and *L. paracasei* MG4715 did not produce H_2O_2 (Fig. 2). In addition, *L. reuteri* MG4722 showed a colorimetric change to blue, indicating the presence of reuterin. Table 2 shows the contents of H_2O_2 and reuterin produced by *L. reuteri* MG4722.

Adhesion ability of probiotic strains to oral epithelial cells

We confirmed the LAB strain's ability to adhere to oral epithelial KB cells. All strains showed high adhesion

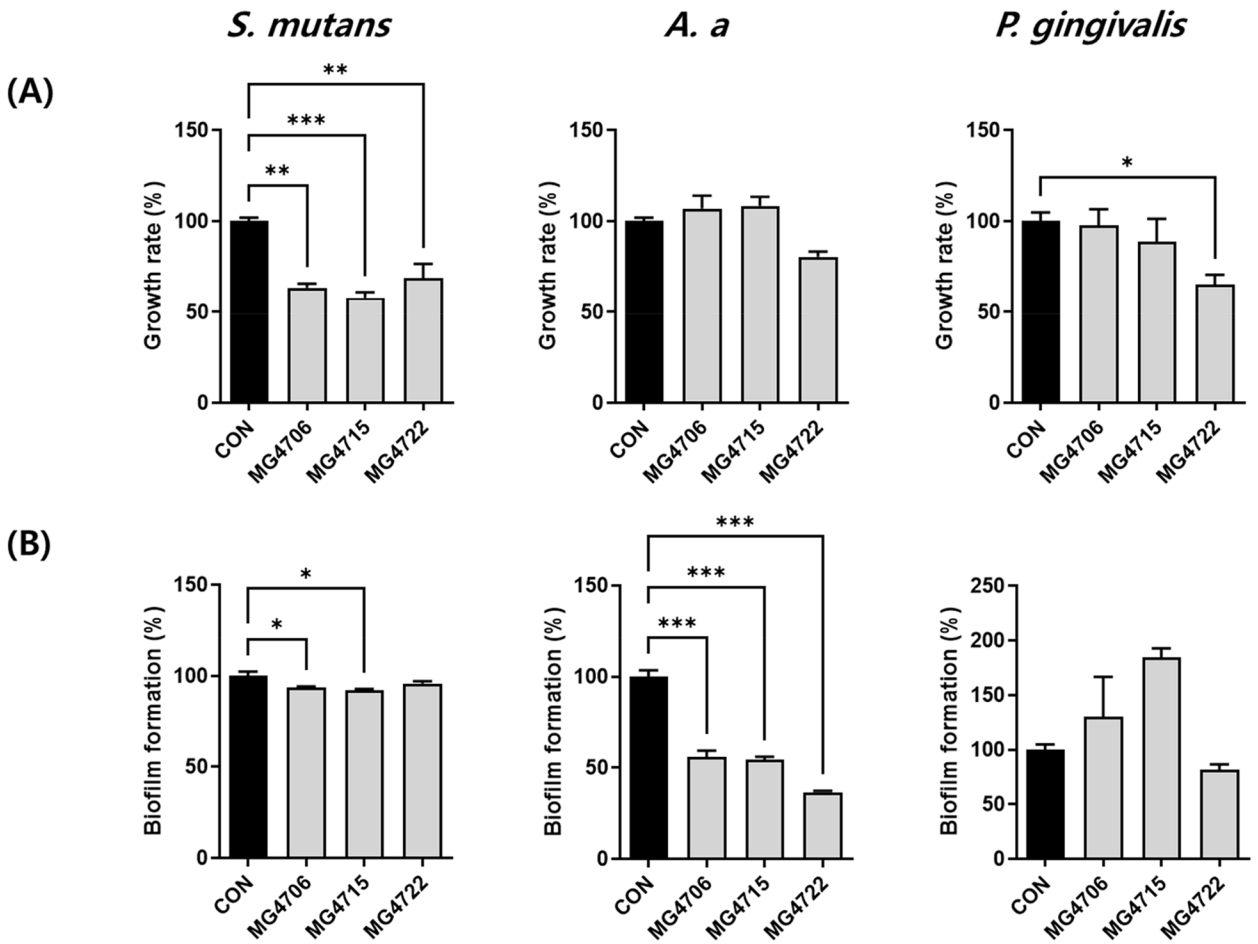

Fig. 1 Antimicrobial activity of LAB strains against *S. mutans*, *A. actinomycetemcomitans,* and *P. gingivalis*. Growth rate (**A**) and Biofilm formation (**B**). All values are represented as mean ± SEM ($n = 3$). Significant differences indicate the means at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ with Dunnett's multiple comparisons test

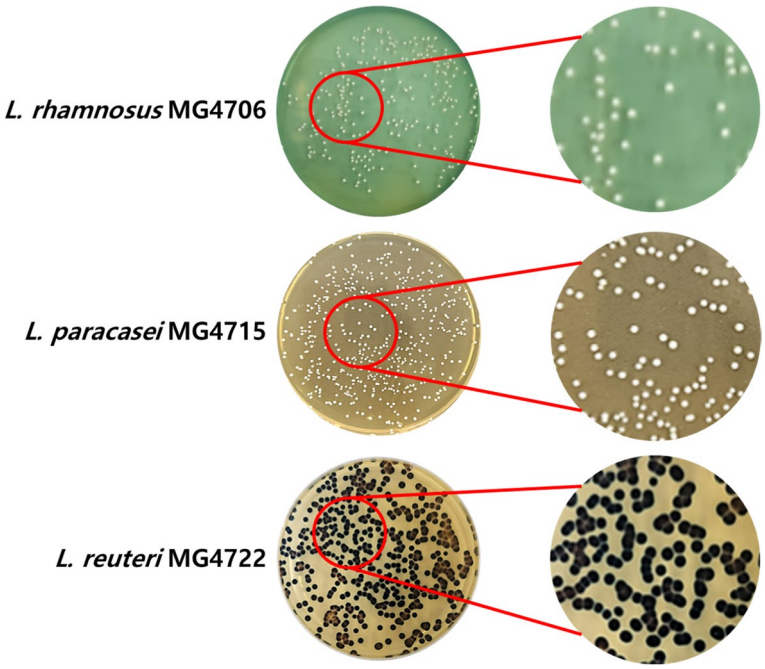

Fig. 2 H_2O_2 production of probiotic strains. The blue colonies on the TMB agar would be categorized as H_2O_2-positive

Table 2 Production of antimicrobial substances of *L. reuteri* MG4722

Strain	Antimicrobial substances	
	H_2O_2 (μM)	Reuterin (mM)
L. reuteri MG4722	30.38 ± 0.13	13.16 ± 0.15

All data are determined by a colorimetric method performed in triplicate and presented as the mean ± SEM ($n = 3$)

Table 3 Adhesion of LAB strains on oral epithelial KB cells

Strains	Number of probiotic strains adhering/1 KB cells	Adhesion rate (%)
L. rhamnosus MG4706	54.58 ± 1.58	86.05 ± 0.15
L. paracasei MG4715	54.50 ± 4.49	86.73 ± 4.49
L. reuteri MG4722	77.74 ± 5.34	93.49 ± 0.35

The adhesion rate is expressed as a percentage of the number of bacteria adhered to cells (Log CFU/mL) divided by the number of bacteria initially inoculated (Log CFU/mL). All data are presented as the mean ± SEM ($n = 3$)

to oral epithelial cells, with a range of 86.05–93.49% (Table 3).

Antibiotic susceptibility of L. Reuteri MG4722

We confirmed the antibiotic susceptibility of *L. reuteri* MG4722. As shown in Table 4, the MICs of all eight antibiotics against *L. reuteri* MG4722 were lower than the cut-off values in the EFSA guidelines. These results indicate that *L. reuteri* MG4722 is safe as a probiotic.

Table 4 Antibiotic susceptibility of *L. reuteri* MG4722

Antibiotics	MIC (μL/mL)	Cut-Off Value (μL/mL)
Ampicillin	0.19	2
Chloramphenicol	0.32	4
Clindamycin	3	4
Erythromycin	1	1
Gentamicin	0.25	8
Kanamycin	6	64
Streptomycin	6	64
Tetracycline	0.125	32

Microbiological cut-off values for antibiotics for *L. reuteri* as provided by EFSA guidelines (2018)

Morphology and safety of L. Reuteri MG4722

In terms of morphology, *L. reuteri* MG4722 had a short rod-shaped form (Fig. 3A). We determined the safety of *L. reuteri* MG4722 by assessing the cytotoxicity on HT-29 cells, and its hemolytic and BSH activities. *L. reuteri* MG4722 showed no cytotoxicity on HT-29 cells (Fig. 3B), no hemolytic activity (γ-hemolysis) on the host, and no BSH activity (Fig. 3C and D).

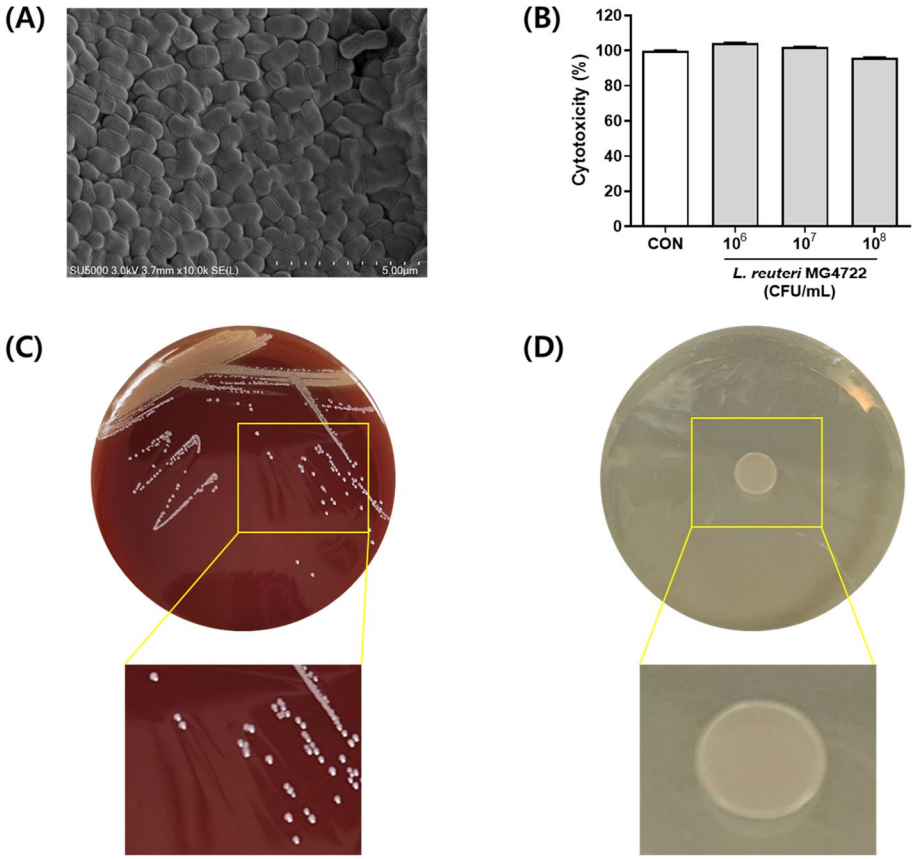

Fig. 3 Morphology and safety of*L. reuteri* MG4722. SEM micrographs (**A**), cytotoxicity (**B**), hemolysis (**C**), and BSH activity (**D**) of *L. reuteri* MG4722. SEM image showing the surface morphology of *L. reuteri* MG4722. Imaging was conducted at 10,000 x magnification with an accelerating voltage of 3 kV. The scale bar represents 5 μm. HT-29 cells were treated with *L. reuteri* MG4722 (10^6-10^8 cells/mL). Data are presented as the mean ± SEM ($n = 3$)

Genome analysis of L. Reuteri MG4722

Genomic analysis of *L. reuteri* MG4722 indicated a single circular chromosome of 1,943,662 bp and a GC content of 38.93% (Fig. 4). The chromosome of *L. reuteri* MG4722 (contig 1) contained 1,925 CDS, 15 rRNA genes (five each of the 5 S, 16 S, and 23 S rRNA operons), and 69 tRNA genes. A DNA plot was used to illustrate the structural and functional features of contig 1 and the chromosome of *L. reuteri* MG4722. The ANI analysis confirmed that the strain was an *L. reuteri* species, with 99.81% similarity to *L. reuteri* JCM1112 as the type strain using Jspecies ver 1.2.1 (Table 5).

In addition, no antibiotic-resistance genes were found in *L. reuteri* MG4722, as confirmed using ResFinder DB.

The search for secondary metabolite biosynthesis gene clusters using the AntiSmash database did not reveal any gene clusters containing bacteriocin, type 1 polyketide synthases (PKS), non-ribosomal peptide synthetases (NRPS), or post-translationally modified peptides (RiPPs).

Presence of the gene encoding reuterin

We noticed that the gene encoding reuterin was present in the genome of *L. reuteri* MG4722. The gene for reuterin biosynthesis genes in *L. reuteri* MG4722 was identified as containing the *pdu-cbi-cob-hem* cluster (Fig. 5; Table 6). Genomic analysis revealed that *L. reuteri* MG4722 produces reuterin, which is consistent with the results shown in Table 2.

Discussion

Numerous microorganisms are distributed throughout the human body, and several studies have reported a relationship between these microorganisms and human health (Nie et al. 2023). The oral cavity, which contains the second-largest number of microorganisms in the human body, is estimated to accommodate a diverse group of microorganisms, including bacteria, fungi, and viruses (Shoemark and Allen 2015). Oral health is generally affected by health status, nutritional status, lifestyle, and composition of the oral microbiome (Mahasneh and Mahasneh 2017). Biofilm formation is a type of periodontal disease initiated by oral

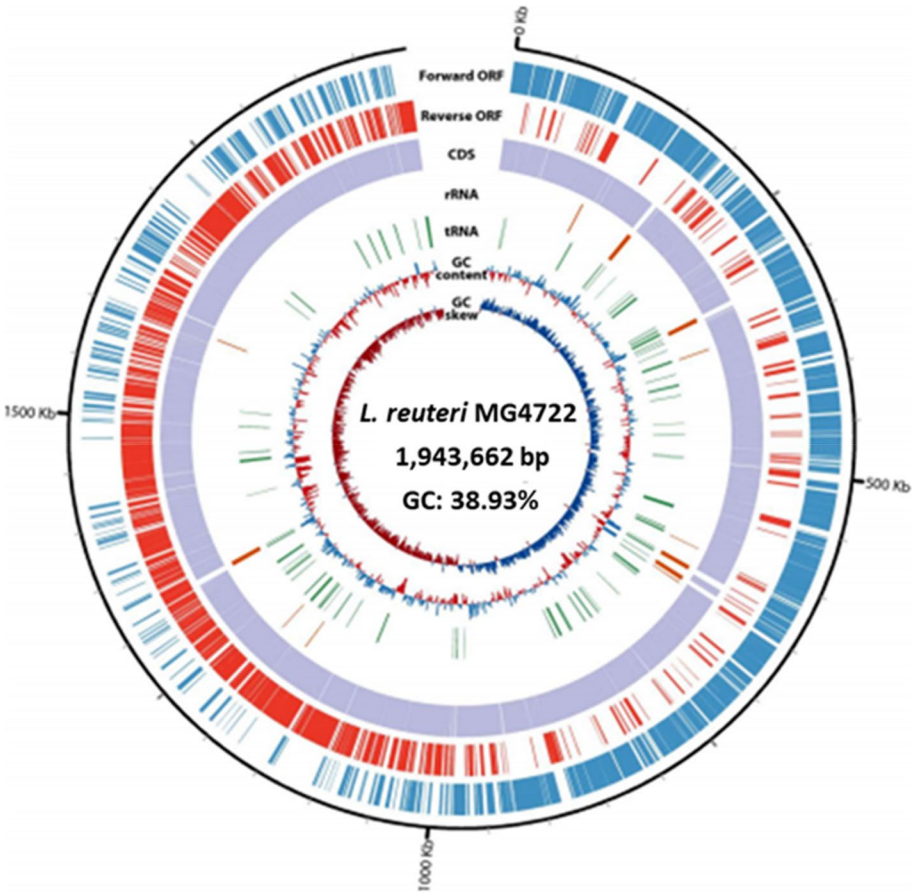

Fig. 4 Genomic map of *L. reuteri* MG4722. Marked genome characteristics are shown from outside to the center: CDS on the forward strand, CDS on the reverse strand, tRNA, rRNA, GC content, and GC skew

Table 5 ANI values between the genome of type strains belong to the genus *Limosilactobacillus*

Species	Strain	ANI between genome of *L. reuteri* MG4722
Limosilactobacillus reuteri subsp. reuteri	JCM1112	99.81
Limosilactobacillus reuteri subsp. murium	lpuph1	95.84
Limosilactobacillus reuteri subsp. porcinus	3c6	95.61
Limosilactobacillus reuteri subsp. rodentium	100-23	95.31
Limosilactobacillus reuteri subsp. suis	ATCC 53608	95.21
Limosilactobacillus balticus	BG-AF3-A	92.86
Limosilactobacillus agrestis	WF-MT5-A	89.97
Limosilactobacillus albertensis	Lr3000	81.15
Limosilactobacillus caviae	CCM 8609	80.99
Limosilactobacillus rudii	STM3_1	79.21
Limosilactobacillus oris	DSM 4864	71.03
Limosilactobacillus fermentum	DSM 20052	68.58

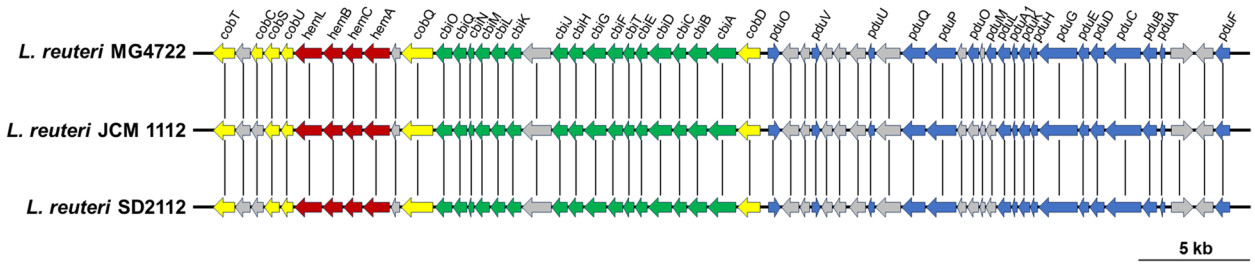

Fig. 5 Reuterin biosynthesis gene cluster comparison containing *pdu-cbi-cob-heme* gene cluster in *L. reuteri* MG4722, *L. reuteri* JCM1112, and *L. reuteri* SD2112. The arrows indicate the transcription direction in the *pdu-cbi-cob-heme* gene cluster, each with the same color. The blue arrows represent genes that are involved in the glycerol and propanediol utilization (*pdu*); The yellow and green arrows represent genes that are cobalamin biosynthesis (*cbi-cob*), respectively; The red arrows represent *hem* genes; The grey arrows are not related to reuterin production

pathogens that form complex structures on the tooth surface and destroy tooth-supporting tissues (How et al. 2016). Gingipain, a proteolytic enzyme of *Porphyromonas gingivalis*, is a major virulence factor responsible for causing periodontal disease and can help the survival by interacting with other species, including *Treponema denticola* and *Tannerella forsythia* (Bao et al. 2014). In the present study, we investigated the antibacterial activities of *Lactobacillus* strains against oral pathogens. *L. reuteri* MG4722 significantly inhibited the growth of *S. mutans*, *A. actinomycetemcomitans*, and *P. gingivalis* and biofilm formation by *A. actinomycetemcomitans*.

Probiotics can help to improve oral health by maintaining homeostasis of the oral microbiota and competing for binding sites with harmful microorganisms, thereby showing preventive and therapeutic effects against pathogenic bacteria (Nie et al. 2023). Probiotics also regulate immune responses and secrete substances with antibacterial properties (Gungor et al. 2015). Probiotics exert their antibacterial effects by producing organic acids that can inhibit pathogens or secreting compounds with antibacterial properties (Lee et al. 2013). Probiotics can produce H_2O_2 through electron transport, causing peroxidation of lipids and increasing membrane permeability, thereby destructing nucleic acids and cellular proteins of bacteria (Naidu et al. 1999). In this study, we confirmed the H_2O_2 production ability of *L. reuteri* MG4722, suggesting that this strain may have antibacterial properties.

Probiotics prevent the attachment of harmful bacteria and subsequent infections through their ability to adhere (Mann et al. 2021). The ability to adhere to oral epithelial cell monolayers has been used to increase the number of beneficial bacteria [2]. The *Lactobacillus* genus generally prevents pathogen attachment through the adhesion factors, including cholic acid and surface layer proteins, on the cell surface and may play an important role in subsequent immune regulation (Kaźmierczyk-Winciorek et al. 2021). In this study, *L. reuteri* MG4722 showed a high ability to adhere to KB epithelial cells, suggesting that its antibacterial activity by inhibiting the adhesion of harmful oral bacteria.

The safety of a strain to be used as a probiotic must be thoroughly evaluated, including by investigating its antibiotic resistance and virulence factors (Ruiz-Ramírez et al. 2023). According to the EFSA guidelines, ingestible probiotic must be assessed for resistance to antibiotics such as gentamicin, kanamycin, streptomycin, tetracycline, erythromycin, clindamycin, chloramphenicol, ampicillin, and vancomycin. In addition, probiotic strains must establish a comprehensive genetic evaluation to confirm the absence of acquired or transferable antibiotic resistance determinants and assess their genomic stability (Campedelli et al. 2019). In our study, *L. reuteri* MG4722 satisfied the safety requirements of the EFSA cut-off values. In addition, antibiotic-resistance genes were not detected in *L. reuteri* MG4722.

According to the safety evaluation guidelines of FAO/WHO, probiotic strains must be confirmed for safety, including BSH activity, hemolytic activity, and toxicity (Lee et al. 2023). Hemolytic activity is an important indicator, and the hemolytic properties of bacteria can cause cell lysis and dissolution of hemoglobin (Bitschar et al. 2017; Liu et al. 2021). BSH activity lowers cholesterol; however, excess cholesterol lowering can cause lipid dyspepsia and impair colonic mucosal function, potentially leading to gallstone formation (Lee et al. 2023). In this study, *L. reuteri* MG4722 did not exhibit hemolytic activity, BSH activity, or cytotoxicity in HT-29 cells.

WGS can be used to study the functional aspects of microorganisms by sequencing their entire genomes and comparing them with previously identified genetic information (Klaenhammer 1988). Genomes with ANI values exceeding 95% are classified as representing the same species (Kim et al. 2014; Greppi et al. 2020). *L. reuteri* MG4722 was confirmed by comparing with *L. reuteri* JCM 1112 (ANI 99.81%). Probiotics with high levels of antibiotic resistance

Table 6 Predicted functions of the genes from *L. reuteri* MG4722 containing*pdu-cbi-cob-heme* gene cluster

Locus tag	Start	End	Functions	Gene symbol
AB3U52_RS08795	1702676	1701624	nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase	cobT
AB3U52_RS08800	1703388	1702678	hypothetical protein	-
AB3U52_RS08805	1704015	1703425	histidine phosphatase family protein	cobC
AB3U52_RS08810	1704773	1704012	adenosylcobinamide-GDP ribazoletransferase	cobS
AB3U52_RS08815	1705372	1704782	bifunctional adenosylcobinamide kinase/adenosylcobinamide-phosphate guanylyltransferase	cobU
AB3U52_RS08820	1706766	1705435	glutamate-1-semialdehyde-2,1-aminomutase	hemL
AB3U52_RS08825	1707721	1706750	porphobilinogen synthase	hemB
AB3U52_RS08830	1708644	1707727	hydroxymethylbilane synthase	hemC
AB3U52_RS08835	1709899	1708634	glutamyl-tRNA reductase	hemA
AB3U52_RS08840	1710359	1709901	bifunctional precorrin-2 dehydrogenase/sirohydrochlorin ferrochelatase	-
AB3U52_RS08845	1711879	1710374	cobyric acid synthase	cobQ
AB3U52_RS08850	1712757	1711954	ATP-binding cassette domain-containing protein	cbiO
AB3U52_RS08855	1713446	1712769	cobalt ECF transporter T component CbiQ	cbiQ
AB3U52_RS08860	1713778	1713467	ABC-type cobalt transport system	cbiN
AB3U52_RS08865	1714521	1713775	energy-coupling factor ABC transporter permease	cbiM
AB3U52_RS08870	1715218	1714505	precorrin-2 C(20)-methyltransferase	cbiL
AB3U52_RS08875	1715999	1715220	sirohydrochlorin cobaltochelatase	cbiK
AB3U52_RS08880	1717386	1715992	uroporphyrinogen-III C-methyltransferase	-
AB3U52_RS08885	1718134	1717376	precorrin-6A reductase	cbiJ
AB3U52_RS08890	1718856	1718131	precorrin-3B C(17)-methyltransferase	cbiH
AB3U52_RS08895	1719924	1718869	cobalt-precorrin 5A hydrolase	cbiG
AB3U52_RS08900	1720688	1719927	cobalt-precorrin-4 methyltransferase	cbiF
AB3U52_RS08905	1721259	1720705	decarboxylating cobalt-precorrin-6B (C(15))-methyltransferase	cbiT
AB3U52_RS08910	1721854	1721252	cobalt-precorrin-7 (C(5))-methyltransferase	cbiE
AB3U52_RS08915	1723002	1721851	cobalt-precorrin-5B (C(1))-methyltransferase	cbiD
AB3U52_RS08920	1723666	1722983	cobalt-precorrin-8X methylmutase	cbiC
AB3U52_RS08925	1724631	1723672	cobalamin biosynthesis protein CobD	cobB
AB3U52_RS08930	1725992	1724628	cobyrinate a,c-diamide synthase	cbiA
AB3U52_RS08935	1727080	1725992	aminotransferase class I/II-fold pyridoxal phosphate-dependent enzyme	cobD
AB3U52_RS08940	1727443	1728009	Chain A, Atp Bound At The Active Site Of A Pduo Type Atp:co(i)rrinoid Adenosyltransferase From Lactobacillus Reuteri	pduO
AB3U52_RS08945	1728899	1728057	GNAT family N-acetyltransferase	-
AB3U52_RS08950	1729370	1728921	flavodoxin	-
AB3U52_RS08955	1729477	1729905	EutP/PduV family microcompartment system protein	pduV
AB3U52_RS08960	1730455	1729898	permease	-
AB3U52_RS08965	1731096	1730452	histidine phosphatase family protein	-
AB3U52_RS08970	1731987	1731193	tyrosine-protein phosphatase	-
AB3U52_RS08975	1732407	1732060	BMC domain-containing protein	pduU
AB3U52_RS08980	1733607	1732423	acetate kinase	ackA_2
AB3U52_RS08985	1734752	1733631	iron-containing alcohol dehydrogenase	pduQ
AB3U52_RS08990	1736203	1734770	CoA-acylating propionaldehyde dehydrogenase PduP	pduP
AB3U52_RS08995	1736679	1736206	heme-binding protein	-
AB3U52_RS09000	1737269	1736682	ATP:cob(I)alamin adenosyltransferase	pduO
AB3U52_RS09005	1737562	1737290	propanediol utilization protein PduN	-
AB3U52_RS09010	1738053	1737550	PduM family microcompartment protein	pduM
AB3U52_RS09015	1738729	1738085	phosphate propanoyltransferase	pduL

Table 6 (continued)

Locus tag	Start	End	Functions	Gene symbol
AB3U52_RS09020	1739048	1738758	BMC domain-containing protein	pduA_1
AB3U52_RS09025	1739630	1739061	BMC domain-containing protein	pduK
AB3U52_RS09030	1739997	1739638	propanediol dehydratase reactivation protein PduH	pduH
AB3U52_RS09035	1741834	1739984	diol dehydratase reactivase subunit alpha	pduG
AB3U52_RS09040	1742380	1741865	diol dehydratase small subunit	pduE
AB3U52_RS09045	1743103	1742393	propanediol/glycerol family dehydratase medium subunit	pduD
AB3U52_RS09050	1744797	1743121	propanediol/glycerol family dehydratase large subunit	pduC
AB3U52_RS09055	1745538	1744822	Chain A, Crystal Structure Of A Trimeric Bacterial Microcompartment Shell Protein Pdub With Glycerol Metabolites	pduB
AB3U52_RS09060	1745917	1745636	BMC domain-containing protein	pduA
AB3U52_RS09065	1746171	1747250	helix-turn-helix domain-containing protein	-
AB3U52_RS09070	1748124	1747291	ethanolamine utilization protein EutJ	-
AB3U52_RS09075	1748916	1748209	MULTISPECIES: aquaporin family protein	pduF

may pose safety concerns because antibiotic-resistant genes may be transmitted (Zhang et al. 2018). *L. reuteri* MG4722 confirmed that no antibiotic-resistance genes were identified and that pathogen transfer is impossible.

Lactobacillus spp. produce various metabolites that protect against colonization by oral periodontal pathogens (Wasfi et al. 2018). *L. reuteri* has high potential for application as a natural antibacterial agent to prevent pathogenic infections and increase beneficial bacteria through its high persistence and antibacterial activity (Greppi et al. 2020). *L. reuteri* AN417 inhibits the growth and biofilm formation of oral pathogens (Yang et al. 2021). Additionally, oral tablets containing *L. reuteri* reduce periodontal pathogens and improve halitosis in clinical trials (Kaźmierczyk-Winciorek et al. 2021). The primary antibacterial compounds produced by *L. reuteri* are organic acids, hydrogen peroxide, reuterin, reuteran, and reutericyclin (Yang et al. 2021). Reuterin, also known as 3-hydroxy propionaldehyde, is an important compound produced by *L. reuteri* for inhibiting the growth of pathogens, and its expression is regulated by the *pdu-cbi-cob-hem* cluster consisting of 58 genes (Lee et al. 2017). In this study, *L. reuteri* MG4722 was found to have the complete *pdu-cbi-cob-hem* operon, a reuterin biosynthesis gene. *L. reuteri* MG4722 did not possess secondary metabolite biosynthetic gene clusters containing bacteriocins, PKS, NRPS, and RiPP. We also confirmed that reuterin was produced in *L. reuteri* MG4722. In *L. reuteri* MG4722, only the *pdu-cbi-cob-heme* gene cluster was found, suggesting that its antibacterial activity may be related to reuterin production.

Conclusion

L. reuteri MG4722 exhibits antibacterial efficacy by attaching to the oral epithelium and secreting antibiotics, such as H_2O_2 and reuterin, into the oral cavity. *L. reuteri* MG4722 exhibited significant adhesion ability to oral epithelial cells and produced antibacterial substances such as H_2O_2 and reuterin, supported by identifying corresponding biosynthetic genes. Safety assessments confirmed the absence of hemolytic activity, bile salt hydrolase activity, and antibiotic resistance, with no detectable toxicity. Therefore, *L. reuteri* MG4722 is a potential probiotic candidate for oral hygiene and could be proposed as a functional food or therapeutic agent for oral health. In future studies, the efficacy of *L. reuteri* MG4722 should be validated in animal models and clinical trials.

Abbreviations

ANI	Average nucleotide identity
BHI	Brain heart infusion broth
BSH	Bile salt hydrolase
CDC	Centers for Disease Control and Prevention
CDS	Coding sequences
CFS	Cell-free supernatant
CFUs	Colony-forming units
CH3SH	Methyl mercaptan
DMEM	Dulbecco's Modified Eagle Medium
EFSA	European Food Safety Authority
FBS	Fetal bovine serum
FAO	Food and Agriculture Organization of the United Nations
KCTC	Korean Collection for Type Cultures
LSM	LAB susceptibility test medium
MICs	Minimum inhibitory concentrations
MRS	de Man, Rogosa, and Sharp broth
OD	Optical density
PBS	Phosphate-buffered saline
P/S	Penicillin–streptomycin
rRNA	Ribosomal RNA
SEM	Standard error of the mean
SPSS	Statistical Package for the Social Sciences
TSA	Tryptic soy agar
TMB	Tetramethyl-benzidine
WGS	Whole genome sequencing
WHO	World Health Organization

Acknowledgements
Not applicable.

Authors' contributions
J.-Y.P. was a major contributor to writing the manuscript. J.-Y.P. and J.Y.L. performed the experiments. All authors participated in data analysis and data curation. J.-Y.P. and B.K.K. were involved in visualization and methodology. B.-K.K. and S.-I.C. were involved in conceptualization and funding acquisition. S.-I.C. and B.K.K. participated in the discussion and revision of the manuscript and approved the final version. All authors read and approved the final manuscript.

Funding
Not applicable.

Data availability
The datasets generated and/or analyzed during the current study are available in the NCBI repository, *L. reuteri* MG4722 (CP162612 (NZ_CP162612)).

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 13 August 2024 Accepted: 18 December 2024
Published online: 15 January 2025

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