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Effects of *Lactiplantibacillus plantarum* LM1215 on *Candida albicans* and *Gardnerella vaginalis*

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Purpose: The aim of this study was to identify novel vaginal probiotics with the potential to prevent vulvovaginal candidiasis (VVC) and bacterial vaginosis (BV).

Materials and Methods: Eighteen strains of *Lactiplantibacillus plantarum* were isolated from healthy Korean women, and their antimicrobial effects against *Candida albicans* and *Gardnerella vaginalis* were assessed. Three strains (*L. plantarum* LM1203, LM1209, and LM1215) were selected for further investigation, focusing on their growth inhibition, biofilm regulation, and cellular mechanisms against these vaginal pathogens. Additionally, electron microscopy revealed damage to *G. vaginalis* induced by *L. plantarum* LM1215, and genomic analysis was conducted on this strain.

Results: *L. plantarum* LM1203, LM1209, and LM1215 showed approximately 1 and 2 Log CFU/mL growth reduction in *C. albicans* and *G. vaginalis*, respectively. These *L. plantarum* strains effectively inhibited biofilm formation and eliminated the mature biofilms formed by *C. albicans*. Furthermore, *L. plantarum* LM1215 decreased tricarboxylic acid cycle activity by 51.75 ($p<0.001$) and respiratory metabolic activity by 52.88% ($p<0.001$) in *G. vaginalis*. *L. plantarum* induced cellular membrane damage, inhibition of protein synthesis, and cell wall collapse in *G. vaginalis*. Genomic analysis confirmed *L. plantarum* LM1215 as a safe strain for vaginal probiotics.

Conclusion: The *L. plantarum* LM1215 is considered a safe probiotic agent suitable for the prevention of VVC and BV.

Key Words: *Lactiplantibacillus plantarum*, probiotics, microbial sensitivity test, vulvovaginal candidiasis, bacterial vaginosis

INTRODUCTION

The vaginal tract harbors various microbiomes, constituting approximately 9% of the total human microbiota.[1] In a healthy vaginal environment, the predominant lactic acid bacteria (LAB) play a pivotal role in maintaining the equilibrium of the vaginal microbiome niche.[1,2] The microflora in a healthy vaginal tract mainly consists of *Lactobacillus* spp., such as *Lactobacillus crispatus*, *Lactobacillus gasseri*, and *Lactobacillus jensenii*.[1,3] Conversely, vulvovaginal candidiasis (VVC) or bacterial vaginosis (BV) is associated with an abundance of *Candida albicans*,[4] *Gardnerella vaginalis*, *Prevotella bivia*, and *Fannyhessea vaginae*.[1,2]

Antibiotics are the conventional treatment for VVC and BV.[2,4,5] Typically, antibiotic treatments exhibit efficacy within a week;[5] however, more than 50% of women treated with antibiotics experience recurrences within 12 months.[5,6] Antibiotics, while effective against the causative agent, cannot directly restore the vaginal microflora, which contributes to the high recurrence rates. It has been reported that many women who have experienced BV are using probiotics as an alternative therapy to prevent the recurrence of vaginosis, although their safety and efficacy have not yet been proven.[5]

Lactiplantibacillus plantarum has more extensive genomic profile than other LAB species.[7] Its large genome (approximate-

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ly 3.3 Mbp) allows *L. plantarum* to inhabit various niches, including the gastrointestinal tract,[7,8] vaginal mucosa,[1,8] vegetables,[7,8] fruits, meat, and dairy products.[7] Genomic diversity also affects the antimicrobial activity of *L. plantarum*. Plantaricin, a member of the bacteriocin family, is the most common antimicrobial compound produced by *L. plantarum*. Additionally, *L. plantarum* secretes other compounds, such as organic acids, biosurfactants, and phenolic compounds. These antimicrobial agents can disrupt the cell membrane, induce leakage of intracellular molecules, and inhibit biofilm formation.[8]

In this study, we aimed to isolate novel probiotic strains from the vaginal tracts of healthy Korean women and investigate their antimicrobial efficacy against *C. albicans* and *G. vaginalis*. The isolated strains were evaluated for their ability to inhibit mature biofilm formation by *C. albicans* and the growth of *G. vaginalis* via cellular membrane damage. Furthermore, genome informatics was employed to predict potential antimicrobial agents. The results of this study may contribute to the prevention of vaginosis recurrence and regulation of vaginal microflora.

MATERIALS AND METHODS

Reagents and microorganisms

Crystal violet solution, iodonitrotetrazolium chloride (INT), and N-phenyl-1-naphthylamine (NPN) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Resazurin was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

C. albicans ATCC 11006 and *G. vaginalis* ATCC 14018 were obtained from the American Type Culture Collection (ATCC). *C. albicans* ATCC 11006 was cultured in yeast mold (YM) broth (MBCell, Seoul, Korea) at 24°C for 48 h. *G. vaginalis* ATCC 14018 was cultured in modified Brain Heart Infusion broth (BD Biosciences, Franklin Lakes, NJ, USA) containing 10% of horse serum (MBCell), 1% of yeast extract (BD Biosciences), 0.1% of maltose (Sigma-Aldrich), and 0.1% of glucose (Sigma-Aldrich) (BHYMG) at 37°C for 24 h under anaerobic condition.[2] Anaerobic conditions were maintained using a 20% CO_2 gas pack within an anaerobic jar (Mitsubishi Gas Chemical Company, Tokyo, Japan). These strains were preserved in media containing 10% glycerol at -80°C until use.

Collection of vaginal fluid

This study included healthy reproductive Korean women aged 18–35 years, who visited Kyung Hee University Hospital at Gangdong for general checkups between September 2022 and October 2022. The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of Kyung Hee University Hospital at Gangdong (IRB No. KHNMC 2022-06-044). Vaginal samples from all volunteers were tested for Nugent scoring, and only women with a score of <4 were interpreted as normal and included in this study. The exclusion criteria were current vaginitis or cervicitis, current use of oral contraceptives, antibiotic use within 14 days, probiotic use within 2 months, history of pelvic organ surgery, premenstrual syndrome, severe dysmenorrhea, history of alcohol or drug addiction, heavy smoking, and use of any medication for medical or psychological diseases. Ten healthy women were enrolled in this study, and vaginal fluid was collected from the lateral vaginal walls and posterior fornix using standard swabs (eSwab®; Copan Diagnostics, Murietta, CA, USA). The swabs were immediately transferred to the laboratory at 4°C.

Isolation of LAB from vaginal fluid

Human vaginal fluid was spread onto de Man–Rogosa–Sharpe (MRS) agar (BD Biosciences) and incubated at 37°C for 72 h. After incubation, each colony was isolated and further cultured on MRS agar supplemented with 0.0035% (w/v) bromocresol purple to investigate acid production. The acid-producing colonies were subsequently purified from the newly prepared MRS. Single and pure colonies were identified as gram-positive, catalase-negative, rod-type strains. The isolated strains were designated LM1202–LM1219 and identified as *L. plantarum* through 16S rRNA sequencing. A total of 18 strains were stored in MRS containing 10% glycerol at -80°C until use.[4]

Screening of antimicrobial effects of isolated strains

The antimicrobial effects of the isolates were assessed by evaluating the inhibition zone,[9] minimum inhibitory concentration (MIC),[4,9,10] and biofilm inhibition rate.[9] Isolated strains were cultured in MRS broth and sub-cultured three times at 12-h intervals. *C. albicans* and *G. vaginalis* were cultured in YM and BHYMG broths, respectively.

To measure the inhibition zones of *L. plantarum* strains, 5 μL of each *L. plantarum* culture spotted on MRS agar and incubated at 37°C for 24 h. After incubation, 7 mL of YM or BHYMG agar were overlaid on the isolated *L. plantarum* colonies and solidified for 30 min. Approximately 7 Log CFU/mL of *C. albicans* were spread on YM-overlaid MRS agar, and 8 Log CFU/mL of *G. vaginalis* were spread on BHYMG-overlaid MRS agar. Inhibition zones were measured around *L. plantarum* colonies after 24 h of incubation.

The MIC values were determined using the cell-free supernatant (CFS) of *L. plantarum*. The CFSs were prepared by centrifugation at 10000×g at 4°C for 10 min. After centrifugation, each supernatant was filtered using a 0.45-μm cellulose acetate membrane filter. The filtrates were diluted two-fold in YM or BHYMG broth in a 96-well microplate. After dilution, approxi-mately 6 Log CFU/mL of *C. albicans* or *G. vaginalis* were added to each well and incubated for 24 h. MIC was determined as the lowest concentration that visually inhibited fungal or bacterial growth.

To investigate biofilm inhibition, approximately 6 Log

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CFU/mL of *C. albicans* were in-cubated with 12.5% CFS in a 96-well microplate at 24°C for 24 h. After incubation, biofilms were washed with phosphate-buffered saline (PBS) and dried at 37°C for 30 min. The rate of biofilm formation was determined through crystal violet staining for 30 min. The crystal violet in mature biofilms was extracted using 100% ethanol and quantified using a microplate reader (SpectraMax iD3; Molecular Devices, San Jose, CA, USA).

Microbial growth curve

The growth rates of *C. albicans* and *G. vaginalis* with CFS or *L. plantarum* strains were estimated as previously described,[10] with minor modifications. Briefly, approximately 5 Log CFU/mL of *C. albicans* or 6 Log CFU/mL of *G. vaginalis* were incubated with CFS or planktonic cells of *L. plantarum*. Viable *C. albicans* cells were enumerated on YM agar (for CFS-treated) and YM agar containing 10% of citric acid (for planktonic cell-treated). The viability of *G. vaginalis* treated with CFS was assessed on BHYMG agar plates. The number of viable *L. plantarum* strains was determined on MRS agar plates.

Tricarboxylic acid (TCA) cycle activity

Inhibition of the TCA cycle in *C. albicans* and *G. vaginalis* was measured as formazan, which is related to a reduction in INT.[4,9,10] Each microorganism was incubated with 25% CFS to inhibit the TCA cycle. After incubation, the cells were harvested by centrifugation at 10000×g at 4°C for 10 min, washed twice with PBS, and diluted to an OD_{600} of 0.2. Subsequently, 1 mM of INT solution was added, and INT reduction was performed at 37°C for 30 min. After the reaction, the absorbance of formazan was measured at 630 nm using a microplate reader.

Biofilm regulation

Inhibition of biofilm formation and eradication of mature biofilm[9] were assessed to elucidate the biofilm regulation of *L. plantarum*.

Inhibition of biofilm formation was performed as described in the previous section (screening of antimicrobial effects of isolated strains). The CFS treatment ranges were determined as 6.25, 12.5, and 25%.

Approximately 6 Log CFU/mL of *C. albicans* were added to a 96-well microplate and incubated for 24 h. After incubation, the mature biofilm was washed with PBS, and planktonic cells were removed. The washed biofilm was treated with each CFS and further incubated for 24 h. Eradication of the mature biofilm was evaluated using crystal violet, as described above.

Cellular damage measurement

Cellular damage to *G. vaginalis* was assessed by bacterial metabolic activity using the resazurin indicator,[11] cellular membrane damage using the NPN fluorescence indicator,[10] and bacterial protein analysis using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).[12] *G. vaginalis* was incubated with the MIC of CFS at 37°C for 24 h under anaerobic conditions. After incubation, *G. vaginalis* was harvested by centrifugation at 10000×g at 4°C for 10 min, washed twice with PBS, and subjected to cellular damage analysis.

The CFS-treated *G. vaginalis* was diluted to an OD_{600} of 0.2 and reacted with 30 μg/mL of resazurin at 37°C for 30 min. The reduction of resazurin to resorufin was measured using a microplate reader with excitation and emission wavelengths of 540 and 590 nm, respectively.

To measure cellular membrane damage, CFS-treated *G. vaginalis* was incubated with 10 μM of NPN and incubated at 24°C for 30 min. The absorption of NPN was measured using a microplate reader at an excitation wavelength of 340 nm and an emission wavelength of 420 nm.

The bacterial proteins were extracted using B-PER™ Bacterial Protein Extraction Reagent (Thermo Fisher Scientific). The lysed bacterial cells were centrifuged at 13000×g at 4°C for 30 min, and the supernatants were collected for protein analysis. Protein concentration was measured using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). SDS-PAGE was performed on a 15% resolving gel with a 5% stacking gel. Briefly, 20 μg of proteins were separated in acrylamide gel and stained Coomassie Brilliant Blue R-250 solution (Bio-Rad, Hercules, CA, USA) four times at 30-min intervals. After staining, Coomassie Brilliant Blue R-250 Destaining Solution (Bio-Rad Laboratories) was used to visualize the proteins. SDS-PAGE was visualized using the FastGene® GelPic LED Box (Nippon Genetics Europe, Düren, Germany).

Microscopic observation

CFS-induced cellular damage to *G. vaginalis* was observed through field-emission scanning electron microscopy (FE-SEM) and transmission electron microscopy (TEM). For FE-SEM observations, CFS-treated *G. vaginalis* were harvested by centrifugation (10000×g at 4°C for 10 min) and fixed with Karnovsky's fixative solution at 4°C. The fixed cells were washed with 50-mM sodium cacodylate buffer and further fixed with 1% osmium tetroxide (OsO_4) dissolved in 100-mM sodium cacodylate buffer at 4°C for 1 h. After fixation, excess OsO_4 was removed, and dehydration was performed using 30%, 50%, 70%, 80%, 90%, and 100% ethanol for 20 min. The cells were visualized using an FE-SEM (JSM-6700F, JEOL, Tokyo, Japan).[9]

For TEM observation, the fixed cells were washed with distilled water and stained using 0.5% uranyl acetate (in water) at 4°C for overnight. After staining, the samples were washed with distilled water and dehydrated in 30%, 50%, 70%, 80%, 90%, and 100% ethanol for 20 min. The dehydrated samples were immersed in propylene oxide twice for 15 min, followed by a mixture of propylene oxide and Spurr's resin (1:1) for 2 h, propylene oxide and Spurr's resin (1:2) for 2 h, Spurr's resin at 4°C for 24 h, and Spurr's resin at 25°C for 3 h, consecutively. Each sample was polymerized at 70°C for 24 h and sectioned using an ultra-microtome (EM UC7; Leica, Wetzlar, Germany). The

thinly sliced sections were subjected to TEM (JEM1010, JEOL).[13]

Probiotics properties

The essential probiotic properties, including resistance to gastric conditions and bile salts, adhesion to intestinal[4] and vaginal[14] epithelial cells, and auto-aggregation and hydrophobicity to hexadecane,[5] were measured as previously described.

Cultured *L. plantarum* LM1203, LM1209, and LM1215 were incubated in MRS containing 0.3% pepsin or 0.1% oxgall at 37°C for 2 h (for measuring pepsin resistance) and 24 h (for measuring bile salt resistance), respectively. After incubation, the viable cells were spread onto MRS agar. *L. plantarum* ATCC 14917 (type strain) was used as a control.

For investigating adhesion to epithelial cells, HT-29 intestinal epithelial cells and VK2/E6E7 were used (ATCC). HT-29 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution at 37°C in a humidified atmosphere containing 5% CO_2. VK2/E6E7 cells were maintained in keratinocyte serum-free medium supplemented with keratinocyte supplements composed of bovine pituitary extract and human recombinant epidermal growth factor (EGF) (Gibco). During incubation, the medium was refreshed every 2–3 days, and each cell line was grown to 80% confluence. Once the cell lines reached 80% confluence, adherent cells were trypsinized with trypsin-ethylenediaminetetraacetic acid solution (0.25%) and harvested by centrifugation. The harvested cells were seeded in 24-well plates (1×10^5 cells/well) and incubated in different media to form a monolayer. Cultured *L. plantarum* LM1203, LM1209, and LM1215 were diluted to approximately 8 Log CFU/mL, and the HT-29 or VK2/E6E7 monolayers were treated with these diluted *L. plantarum* strains for 2 h without antibiotics. After 2 h, non-adherent bacterial cells were removed by washing with PBS, and adherent bacterial cells were collected using a 1% (v/v) Triton-X solution. Adherent bacterial cells were spread on MRS agar, and viable cells were estimated. *L. plantarum* ATCC 14917 was used as a control.

Cultured *L. plantarum* was harvested by centrifugation (10000×g at 4°C for 10 min) and washed twice with PBS. The washed cells were resuspended in PBS and adjusted to an OD_{600} of 0.5. To evaluate auto-aggregation, the adjusted suspension was allowed to stand and incubated at 37°C. The upper suspension was collected, and the absorbance was measured at 600 nm. Auto-aggregation was calculated and compared to that at 0 h. To evaluate hydrophobicity, 2 mL of adjusted suspension was mixed with 1 mL of hexadecane. The mixture was incubated at 25°C for 30 min. After incubation, the aqueous phase was separated, and its absorbance was measured at 600 nm. Hydrophobicity was calculated and compared to that at 0 min. *L. plantarum* ATCC 14917 was used as a control.

Antibiotics resistance and hemolysis

Antibiotic resistance was evaluated according to the European Food Safety Authority (EFSA) guidelines. Susceptibilities to ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, and tetracycline were measured using ETEST® strips (bioMérieux, Marcy-l'Étoile, France) and interpreted based on cut-off values.

Hemolysis was assessed on Columbia blood agar (Oxoid, Basingstoke, United Kingdom) containing 5% sheep blood.[4]

Genome analysis

Extraction of genomic DNA (gDNA) and genome analysis

Cultured *L. plantarum* LM1215 was harvested by centrifugation (10000×g at 4°C for 10 min) and washed with PBS. The gDNA was extracted using the TaKaRa MiniBEST Bacteria Genomic DNA Extraction Kit (Takara Bio, Kusatsu, Japan) according to the manufacturer's guidelines.

A DNA library was constructed and sequenced using single-molecule real-time (SMRT) sequencing technology (Pacific Biosciences, Menlo Park, CA, USA). Briefly, 5 μg of gDNA were used for SMRTbell library construction using the SMRTbell Template Prep Kit 1.0 and DNA/Polymerase Binding kit P6. The SMRT library was sequenced with one SMRT cell using C4 chemistry (DNA sequencing reagent 4.0), and 240 min movies were captured for each SMRT cell using the PacBio RS II system by Insilicogen (Yongin, Korea). The genome coverage (depth of coverage) was 195×, and HiFi long-read sequencing was performed for analysis.

Genome assembly was performed using a hierarchical genome assembly process (HGAP) and visualized using Artemis[15] and DNAPlotter.[16] Coding sequences were predicted using GLIMMER 3.0. Gene ontology (GO) analysis was performed using Blast2GO, and clusters of orthologous groups of protein (COG) were predicted using the Basic Local Alignment Search Tool.

Phylogenetic relationship and average nucleotide identity (ANI) analysis

The phylogenetic relationships of *L. plantarum* LM1215 were analyzed based on the ortholog gene sequences. Whole genome sequences of *L. plantarum*, *Lactiplantibacillus pentosus*, *Levilactobacillus brevis*, *Limosilactobacillus reuteri*, *Limosilactobacillus fermentum*, *Lentilactobacillus buchneri*, and *Leuconostoc citreum* were obtained from the National Center for Biotechnology Information (NCBI) database. Ortholog analysis was performed using OrthoFinder v2.5.5,[17] and species trees were inferred using the STAG algorithm and rooted using the STRIDE algorithm in OrthoFinder. The tree species was illustrated using Dendroscope 3.[18]

ANI values were calculated using the OrthoANI algorithm[19] through EzBioCloud (https://www.ezbiocloud.net/tools/ani). The results of the ANI distance, combined with the Newick tree,

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were illustrated using the heatmap plot function of TBtools.[20]

Multiple genome comparison
Eight *L. plantarum* genomes were compared using MAUVE[21] by constructing multiple genome alignments.

Prediction of gene cluster
Antimicrobial secondary metabolite gene clusters were predicted using antiSMASH bacterial version 7.0.[22]

Detection of antibiotic resistance genes (ARGs) and virulence factors
ARGs and virulence factors were analyzed using ResFinder version 4.3.3 (http://genepi.food.dtu.dk/resfinder) and virulence factor database (VFDB).[23]

Determination of pathogenicity
The pathogenicity of *L. plantarum* LM1215 was determined using the PathogenFinder.[24]

Genomic island (GI), insertion sequence (IS), prophage, plasmid related sequence, and CRISPR-Cas
Mobile genetic elements (MGEs) were analyzed using VRprofile2.[25] Plasmid-related sequences were analyzed using PlasmidFinder.[26] The CRISPR-Cas system was analyzed using CRISPRCasFinder.[27]

Statistical analysis
Statistical analyses were performed using SPSS Statistics version 18 software (IBM, Armonk, NY, USA). Mean values were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's range test or the Games-Howell post-hoc test. Statistical significance was set at $p<0.05$.

RESULTS

Antimicrobial effects of *L. plantarum* isolated from the human vaginal tract
Eighteen strains of *L. plantarum* were isolated from vaginal fluid. The antimicrobial effects of these strains are listed in Table 1. *L. plantarum* strains did not inhibit the growth of *C. albicans* ATCC 11006; however, nine strains inhibited the growth of *G. vaginalis* ATCC 14018. The three strains with the largest inhibition zones were *L. plantarum* LM1203 (21 mm), *L. plantarum* LM1209 (20 mm), and *L. plantarum* LM1215 (21 mm). The MIC of the CFS against *G. vaginalis* was 25% for most isolates except for *L. plantarum* LM1202, which was not effective. All isolates inhibited biofilm formation by *C. albicans*, irrespective of their growth inhibition capabilities. *L. plantarum* LM1202 was the most effective at inhibiting biofilm formation (91.44%), while *L. plantarum* LM1219 was the least effective (64.65%). Based on these results, three strains (*L. plantarum* LM1203, *L. plantarum* LM1209, and *L. plantarum* LM1215) were selected for further investigation.

Fungistatic effects of *L. plantarum*
Fig. 1 shows the impact of *L. plantarum* and its CFS on the

Table 1. Antimicrobial Properties of *Lactiplantibacillus plantarum* Against *Candida albicans*, and *Gardnerella vaginalis*

Microorganism	Inhibition zone (mm)		Minimum inhibitory concentration (%)		Biofilm inhibition rate (%)
	C. albicans	*G. vaginalis*	*C. albicans*	*G. vaginalis*	*C. albicans*
L. plantarum LM1202	Not effective	Not effective	Not effective	Not effective	91.44±1.60
L. plantarum LM1203	Not effective	21.00±1.63	Not effective	25	89.26±0.76
L. plantarum LM1204	Not effective	18.00±0.82	Not effective	25	86.11±2.46
L. plantarum LM1205	Not effective	16.00±0.82	Not effective	25	84.67±2.30
L. plantarum LM1206	Not effective	16.00±1.41	Not effective	25	70.18±2.68
L. plantarum LM1207	Not effective	Not effective	Not effective	25	74.85±7.29
L. plantarum LM1208	Not effective	Not effective	Not effective	25	66.03±2.55
L. plantarum LM1209	Not effective	20.00±0.82	Not effective	25	72.82±4.58
L. plantarum LM1210	Not effective	14.00±2.16	Not effective	25	88.01±1.36
L. plantarum LM1211	Not effective	16.33±2.05	Not effective	25	86.16±2.77
L. plantarum LM1212	Not effective	Not effective	Not effective	25	81.03±1.15
L. plantarum LM1213	Not effective	18.67±0.47	Not effective	25	83.83±3.39
L. plantarum LM1214	Not effective	Not effective	Not effective	25	87.68±0.98
L. plantarum LM1215	Not effective	21.00±0.82	Not effective	25	85.82±0.53
L. plantarum LM1216	Not effective	Not effective	Not effective	25	77.36±4.57
L. plantarum LM1217	Not effective	Not effective	Not effective	25	86.48±1.39
L. plantarum LM1218	Not effective	Not effective	Not effective	25	73.99±1.63
L. plantarum LM1219	Not effective	Not effective	Not effective	25	64.65±2.81

Data are shown as means±standard deviations of three independent experiments.

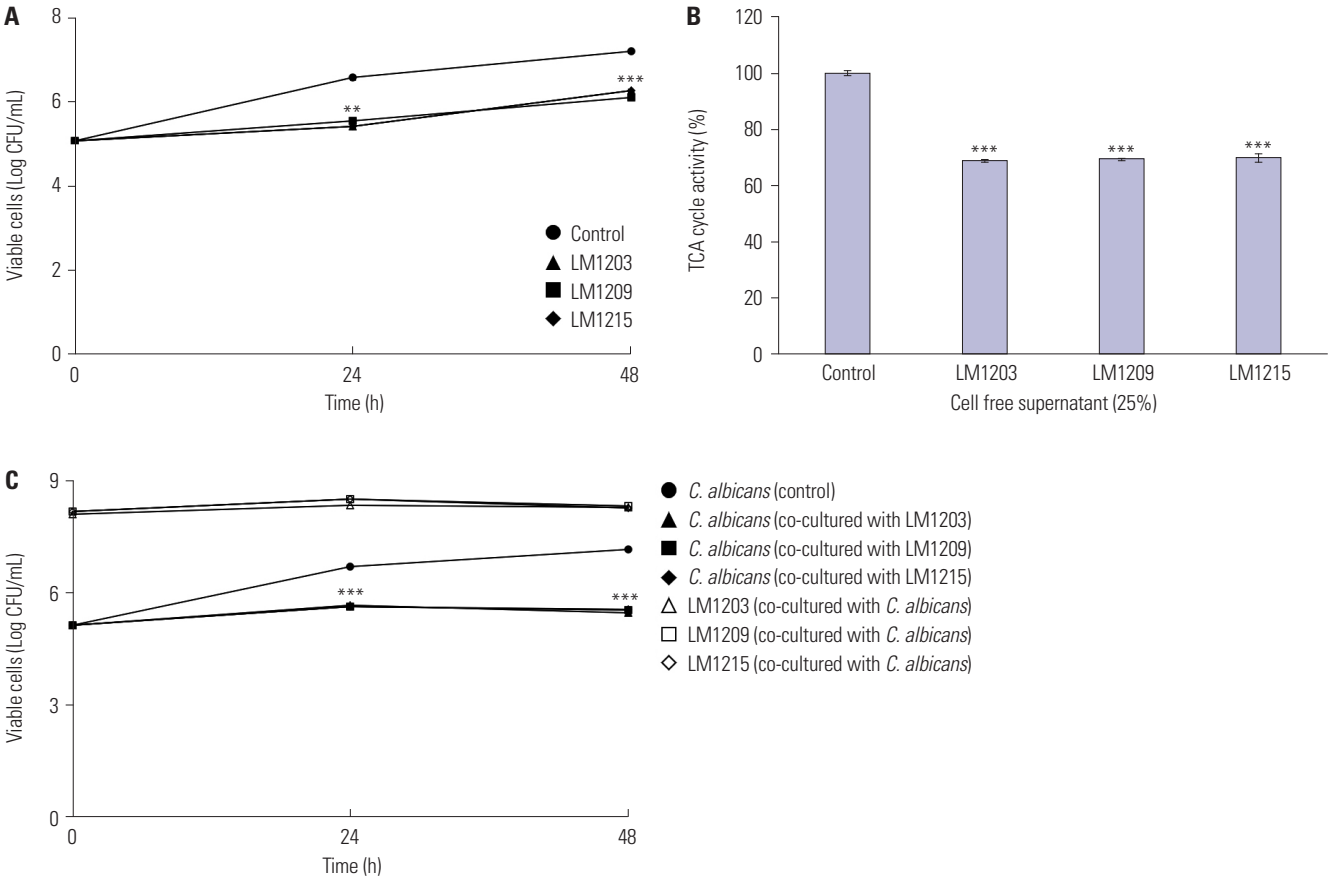

Fig. 1. Fungistatic effects and tricarboxylic acid (TCA) cycle inhibition of *Lactiplantibacillus plantarum*. (A) Fungistatic effects of cell-free supernatants (CFS). (B) TCA cycle inhibition in *Candida albicans* through CFS. (C) Cell viability of *C. albicans* and *L. plantarum* during co-incubation. Significant differences compared to the control are indicated by asterisks (**p<0.01 and ***p<0.001).

growth rate of *C. albicans*. The CFS significantly inhibited the growth rate of *C. albicans*. Specifically, the CFS of *L. plantarum* LM1203 inhibited *C. albicans* growth to 5.41 (p<0.01) and 6.26 (p<0.001) Log CFU/mL at 24 h and 48 h, respectively. Similarly, *L. plantarum* LM1209 and LM1215 inhibited the growth of *C. albicans* to 6.11 (p<0.001) and 6.26 Log CFU/mL (p<0.001) at 48 h, respectively (Fig. 1A). Conversely, the control (non-treated) cells reached 6.57 and 7.19 Log CFU/mL at 24 h and 48 h, respectively.

These fungistatic effects were also observed during TCA cycle inhibition (Fig. 1B). TCA cycle activity decreased to 68.81% (CFS of *L. plantarum* LM1203), 69.37% (CFS of *L. plantarum* LM1209), and 69.92% (CFS of *L. plantarum* LM1215) (p< 0.001). During co-culture with planktonic cells, cell viabilities of *C. albicans* were measured at 5.46 (co-cultured with *L. plantarum* LM1203), 5.54 (co-cultured with *L. plantarum* LM1209), and 5.55 Log CFU/mL (co-cultured with *L. plantarum* LM1215) (p<0.001). The planktonic cells of *L. plantarum* were maintained at 8.28 (*L. plantarum* LM1203), 8.31 (*L. plantarum* LM1209), and 8.25 Log CFU/mL (*L. plantarum* LM1215) (Fig. 1C).

Biofilm regulation by *L. plantarum*

The regulation of biofilm formation by *C. albicans* using *L. plantarum* is shown in Fig. 2. *L. plantarum* LM1203 CFS at concentrations of 6.25%, 12.50%, and 25% inhibited biofilm formation by 89.77%, 92.44%, and 94.07%, respectively (p<0.001). *L. plantarum* LM1209 inhibited biofilm formation by 87.30%, 89.97%, and 92.71%, respectively (p<0.001), and *L. plantarum* LM1215 showed 88.59% (p<0.001), 89.12% (p<0.05), and 91.29% (p<0.001) biofilm inhibition, respectively, at the same concentrations of CFS (Fig. 2A).

As shown in Fig. 2B, *L. plantarum* effectively removed mature biofilms. *L. plantarum* LM1203 and *L. plantarum* LM1215 removed 29.39% (p<0.01) and 33.89% (p<0.001) of mature biofilms, respectively, at CFS concentration of 25%. *L. plantarum* LM1209 removed 1.31%, 36.43% (p<0.05), and 40.39% (p<0.01) of mature biofilms at CFS concentrations of 6.25%, 12.5%, and 25%, respectively.

Growth inhibition and bacterial damage in *G. vaginalis*

The bacterial growth inhibition and membrane damage effects of *L. plantarum* on *G. vaginalis* are shown in Fig. 3. The cell viability of the untreated *G. vaginalis* increased from 6.06 to 8.95

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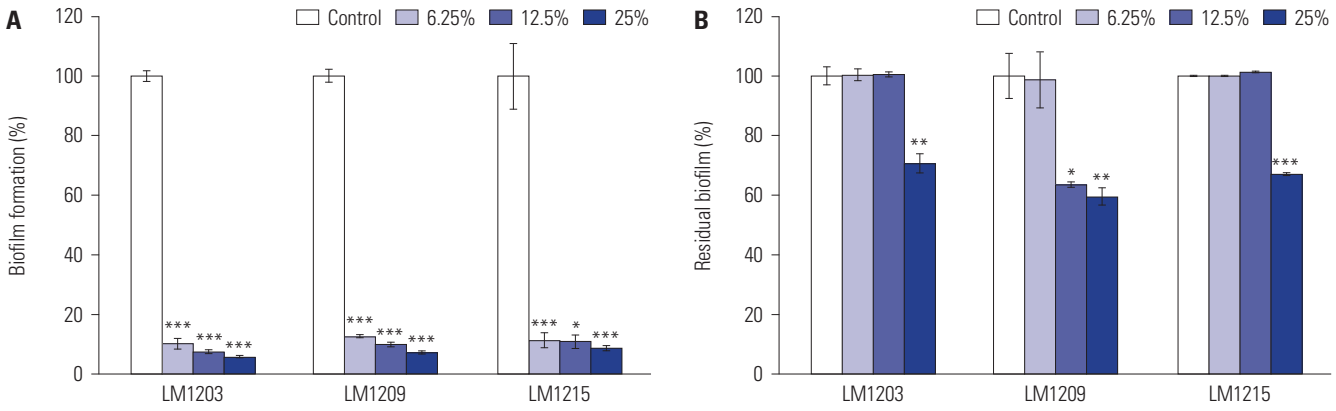

Fig. 2. *Candida albicans* biofilm regulation by *Lactiplantibacillus plantarum*. (A) Inhibition of biofilm formation. (B) Eradication of mature biofilm. Significant differences compared to the control are indicated by asterisks (*p<0.05, **p<0.01, and ***p<0.001).

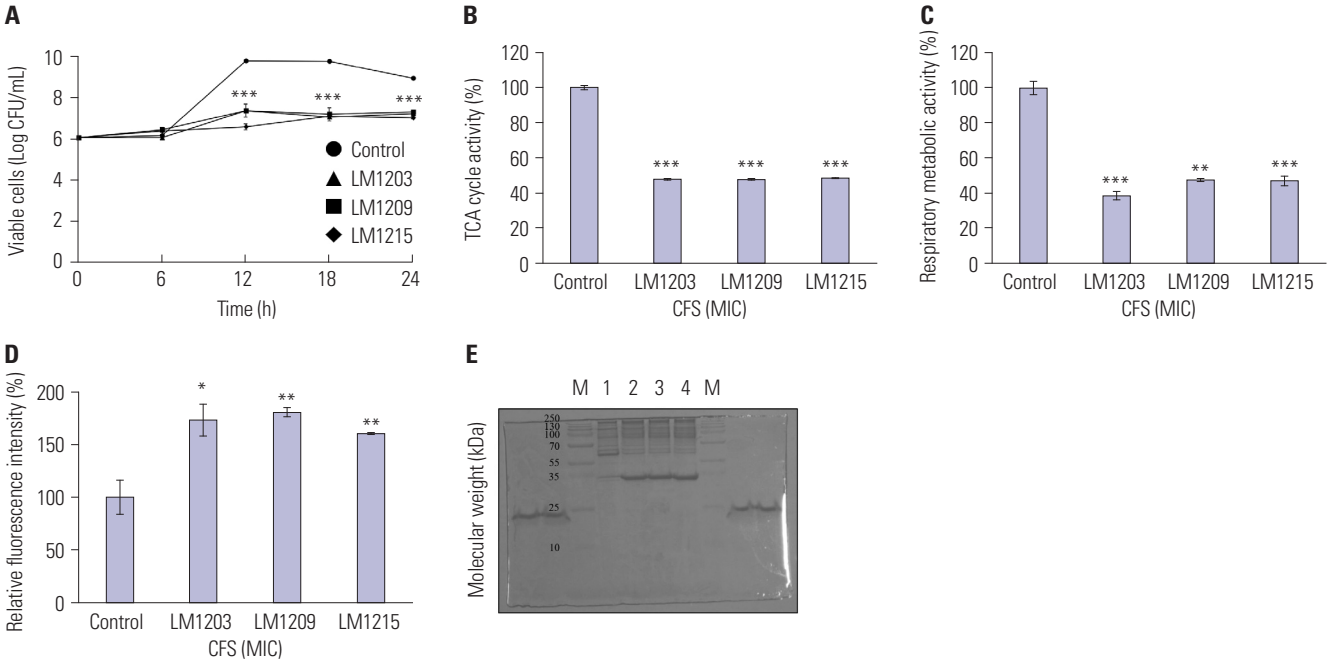

Fig. 3. Antibacterial effects of *Lactiplantibacillus plantarum* against *Gardnerella vaginalis*. (A) Bacteriostatic effects of *L. plantarum*. (B) Tricarboxylic acid cycle inhibition by *L. plantarum*. (C) Inhibition of respiratory metabolism by *L. plantarum*. (D) N-phenyl-1-naphthylamine uptake in damaged *G. vaginalis*. (E) Change of cellular proteins in damaged *G. vaginalis*. (M, protein size marker; 1, Control (non-treated *G. vaginalis*); 2, CFS of *L. plantarum* LM1203-treated *G. vaginalis*; 3, CFS of *L. plantarum* LM1209-treated *G. vaginalis*; 4, CFS of *L. plantarum* LM1215-treated *G. vaginalis*). Significant differences compared to the control are indicated by asterisks (*p<0.05, **p <0.01, ***p<0.001). CFS, cell-free supernatants; MIC, minimum inhibitory concentration.

Log CFU/mL after 24 h. Compared to the control, *L. plantarum* LM1203, LM1209, and LM1215 reduced *G. vaginalis* cell growth to 7.24, 7.31, and 7.03 Log CFU/mL, respectively, after 24 h (p< 0.001). The TCA cycle activity of *G. vaginalis* was reduced by 47.83%, 47.69%, and 48.25% to *L. plantarum* LM1203, LM1209, and LM1215, respectively (p<0.001). Respiratory metabolic activity was reduced by 61.27% (p<0.001), 52.46% (p<0.01), and 52.88% (p<0.001) by *L. plantarum* LM1203, LM1209, and LM1215, respectively. Furthermore, *L. plantarum* promoted NPN absorption into the cell membrane of *G. vaginalis*, resulting in increased fluorescence intensities of 177.33% (*L. plantarum* LM1203) (p<0.05), 180.96% (*L. plantarum* LM1209) (p< 0.01), and 160.48% (*L. plantarum* LM1215) (p<0.01). Notably, intracellular proteins (approximately 55 kDa) of damaged *G. vaginalis* were degraded into smaller proteins (<35 kDa).

Electron microscope observation

Fig. 4 shows the FE-SEM and TEM images of the damage inflicted by *L. plantarum* CFS on *G. vaginalis*. CFS-treated *G. vaginalis* showed cell surface blebs and wrinkles compared to the control (Fig. 4A). Additionally, CFS-treated *G. vaginalis* had disrupted cell walls and adopted a hexagonal shape. These structural distortions resulted in the leakage of cellular components (Fig. 4B).

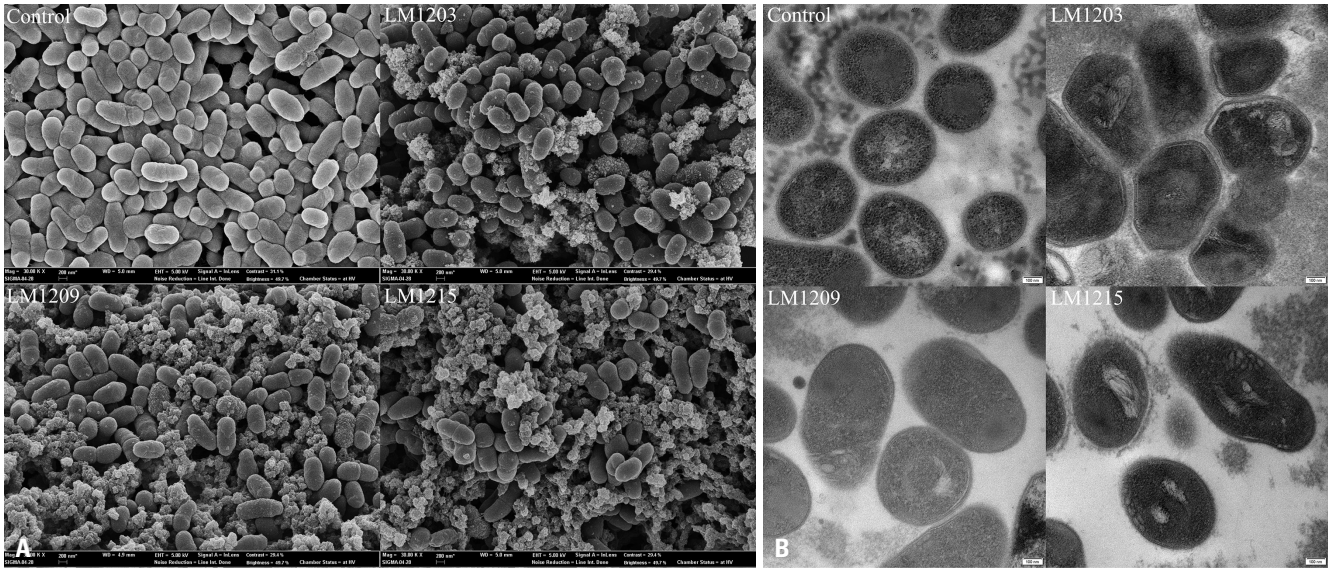

Fig. 4. Field-emission scanning electron microscopy (FE-SEM) and transmission electron microscopy (TEM) observation of damaged *Gardnerella vaginalis*. (A) SEM image of damaged *G. vaginalis* compared to untreated control cells (30000× magnification). (B) TEM image of damaged *G. vaginalis* compared to the control cells (20000× magnification).

Table 2. Probiotic Properties of *Lactiplantibacillus plantarum* Isolated from Human Vaginal Tract

Probiotic properties (%)	Microorganisms			
	L. plantarum ATCC 14917	*L. plantarum* LM1203	*L. plantarum* LM1209	*L. plantarum* LM1215
Resistance				
Pepsin (0.3)	61.75±3.69	79.63±2.62	108.33±4.71	121.21±5.67
Bile salt (0.1)	26.91±1.20	192.17±6.44	125.74±2.06	67.20±3.43
Adhesion				
HT-29	2.84±0.18	3.40±0.13	3.32±0.28	6.09±0.12
VK2/E6E7	2.98±0.20	5.00±0.21	5.04±0.48	4.06±0.25
Auto-aggregation	47.05±0.91	45.10±0.82	52.34±0.59	40.91±2.10
Hydrophobicity	5.87±0.32	6.00±1.11	9.38±1.13	7.91±1.04

Data are shown as means±standard deviations of three independent experiments.

Probiotic properties

Table 2 summarizes the probiotic properties of *L. plantarum* strains isolated from the human vaginal tract. After incubation with 0.3% pepsin, *L. plantarum* LM1203, LM1209, and LM1215 exhibited acid tolerance of 79.63%, 108.33%, and 121.21%, respectively, while *L. plantarum* ATCC 14917 showed 61.75% acid tolerance. Regarding bile salt resistance, *L. plantarum* LM1203 exhibited the highest tolerance of 192.17%, followed by *L. plantarum* LM1209 with 125.74%. *L. plantarum* LM1215 showed 67.20% of bile salt tolerance and *L. plantarum* ATCC 14917 showed the lowest value of bile salt tolerance (26.91%). Regarding the adhesion to HT-29 cell, *L. plantarum* LM1215 showed the highest adherence ratio (6.09%), whereas other strains exhibited 3.40% (LM1203), 3.32% (LM1209), and 2.84% (ATCC 14917). For adherence to VK2/E6E7 cells, *L. plantarum* LM1203, LM1209, and LM1215 showed adherence rates of 5.00%, 5.04%, and 4.06%, respectively, while *L. plantarum* ATCC exhibited a rate of 2.98%. *L. plantarum* LM1209 showed higher auto-aggregation ability (52.34%) than that of *L. plantarum* ATCC 14917 (47.05%), while *L. plantarum* LM1203 and LM1215 showed 45.40% and 40.91%, respectively. *L. plantarum* LM1203 (6.00%), LM1209 (9.38%), and LM1215 (7.91%) showed higher hydrophobicity than that of *L. plantarum* ATCC 14917 (5.87%).

Antibiotics susceptibility and hemolysis

Table 3 presents the antibiotic resistance and hemolysis of *L. plantarum*. The *L. plantarum* LM1215 did not exhibit antibiotics resistance, with MICs of 0.094 μg/mL for ampicillin, 1.5 μg/mL for chloramphenicol, 0.016 μg/mL for clindamycin, 0.19 μg/mL for erythromycin, 6 μg/mL for gentamicin, 64 μg/mL for kanamycin, and 4 μg/mL for tetracycline. However, both *L. plantarum* LM1203 and LM1209 showed kanamycin resistance (MICs >256 μg/mL). Hemolysis was not detected in any of the *L. plantarum* strains.

Overall, *L. plantarum* LM1215 showed antimicrobial effects and probiotic properties. Therefore, further investigations were conducted to analyze its genome.

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Complete genome analysis

General genome information

As shown in Fig. 5A, the size of the entire genome sequence of *L. plantarum* LM1215 was 3221233 bp, with a GC content of 44.62%. Moreover, it contained 3208 protein-coding sequences (CDSs), as well as 16 rRNA and 68 tRNA genes. This Whole Genome project has been deposited in DDBJ/ENA/GenBank

Table 3. Antibiotics Susceptibility and Hemolysis of *Lactiplantibacillus plantarum* Isolated from the Human Vaginal Tract

Antibiotic	Cut-off values (μg/mL)	Antibiotic susceptibility		
		Minimum inhibitory concentration (μg/mL)		
		L. plantarum LM1203	*L. plantarum* LM1209	*L. plantarum* LM1215
Ampicillin	2	0.19	0.19	0.094
Chloramphenicol	8	2	2	1.5
Clindamycin	2	0.125	0.38	0.016
Erythromycin	1	0.25	0.38	0.19
Gentamicin	16	8	6	6
Kanamycin	64	>256	>256	64
Tetracycline	32	3	4	4
Hemolysis		γ-hemolysis	γ-hemolysis	γ-hemolysis

Experiments were conducted in triplicate.

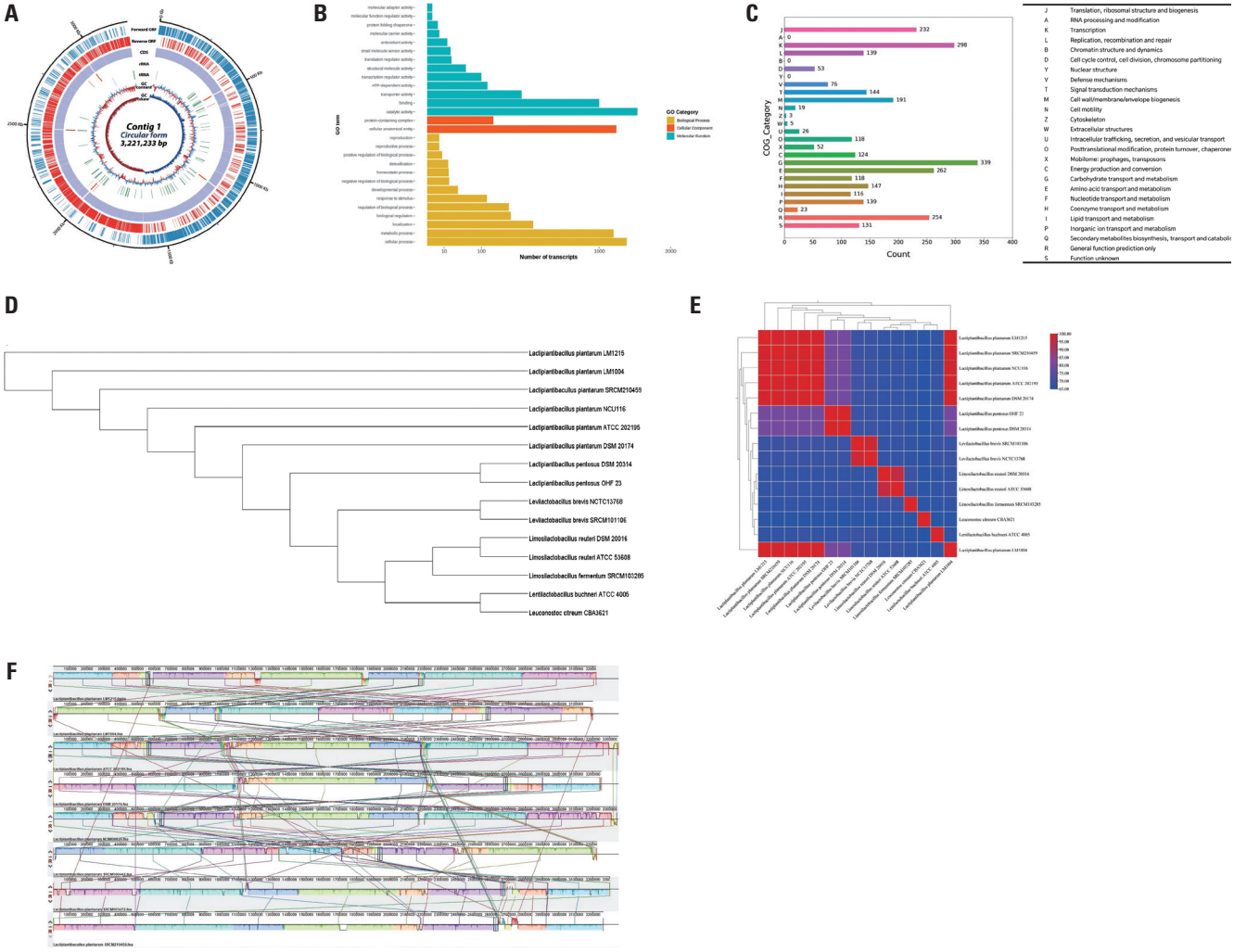

Fig. 5. General genomic information of *Lactiplantibacillus plantarum* LM1215. (A) Circular gene map of *L. plantarum* LM1215. Each circle, from outside to inside, indicates protein-coding sequences (CDS) on the forward strand, CDS on the reverse strand, tRNA, rRNA, GC content, and GC skew. (B) Gene Ontology analysis. (C) Clusters of Orthologous Groups of proteins analysis. (D) Phylogenetic tree based on ortholog gene. (E) Comparison of average nucleotide identity value within the Lactobacillaceae family. (F) Multiple genome alignment and genome comparison of *L. plantarum*. tRNA, transfer RNA; rRNA, ribosomal RNA; GC content, guanine-cytosine content.

under the accession number CP128990.

GO and COG analyses

As shown in Fig. 5B, 8687 transcripts were annotated and categorized into 16 biological processes, two cellular components, and 13 molecular function categories. These genes were mainly enriched for catalytic activity (1491 transcripts), cellular processes (1346 transcripts), cellular anatomical entities (1209 transcripts), metabolic processes (1172 transcripts), and binding (1000 transcripts).

According to the COG analysis (Fig. 5C), 3009 CDSs (93.80%) were specifically assigned to clusters of COG families comprising 23 functional categories. Most functional proteins were involved in carbohydrate transport and metabolism (339 CDSs). Other abundant CDSs were classified as transcription (298 CDSs); amino acid transport and metabolism (262 CDSs), general function prediction only (254 CDSs); and translation, ribosomal structure, and biogenesis (232 CDSs).

Phylogenetic orthology and ANI

Fig. 5D and 5E show the phylogenetic relationships and ANI distances of *L. plantarum* LM1215 compared to those of other LAB. *L. plantarum* LM1215 was closely associated with other Lactiplantibacillus species (*L. plantarum* and *L. pentosus*) and distinct from *Levilactobacillus*, *Limosilactobacillus*, *Lentilactobacillus*, and *Leuconostoc*. In the ANI distance analysis, *L. plantarum* LM1215 showed a high genomic similarity to other *L. plantarum* strains (98.98%–99.96%). Compared with other *Lactiplantibacillus*, *L. pentosus* DSM 20314 and *L. pentosus* OHF 23 showed 80.18% and 80.14% genomic similarity, respectively. Other LAB showed 66.17%–69.08% genomic similarity wih *L. plantarum* LM1215.

Comparative genome alignments

A comparative genomic analysis of *L. plantarum* strains is shown in Fig. 5F. Genome rearrangements were observed within *L. plantarum*, and genome deletions occurred in several strains, including *L. plantarum* ATCC 202195, DSM 20174, NCIMB8826, SRCM100442, 103472, and SRCM210459. *L. plantarum* LM1215 was most similar to *L. plantarum* LM1004, which underwent genome rearrangement without deletions.

Prediction of gene clusters

Antimicrobial secondary metabolite gene clusters are shown in Fig. 6. *L. plantarum* LM1215 is predicted to have four types of antimicrobial secondary metabolites: plantaricin E/F, type III polyketide synthases, terpenes, and cyclic lactone autoinducers.

Antibiotic resistance genes (ARGs) and virulence factors

As shown in Table 4, intrinsic ARGs and acquired ARGs were not detected in *L. plantarum* LM1215. Compared to the VFDB, 10 alignments exhibited matching virulence factors with bit scores exceeding 50. However, several alignments, such as *sdrE* (E-value:4E-49), *sdrC* (E-value:2E-48), *clpC* (E-value:3E-10), *tufA* (E-value:3E-10), *hasC* (E-value:2E-08), *lap* (E-value:2E-08), and *bsh* (E-value:6E-05), exhibited E-values below E-50. Conversely, *clfA*, *clfB*, and *sdrD* genes had E-values of 2E-79, 2E-69, and 7E-51, respectively (Table 5).

Pathogenicity

As shown in Table 6, *L. plantarum* LM1215 was not identified as a pathogen. Fourteen non-pathogenic proteins were matched to those of other LAB, including *Pediococcus pentosaceus* ATCC

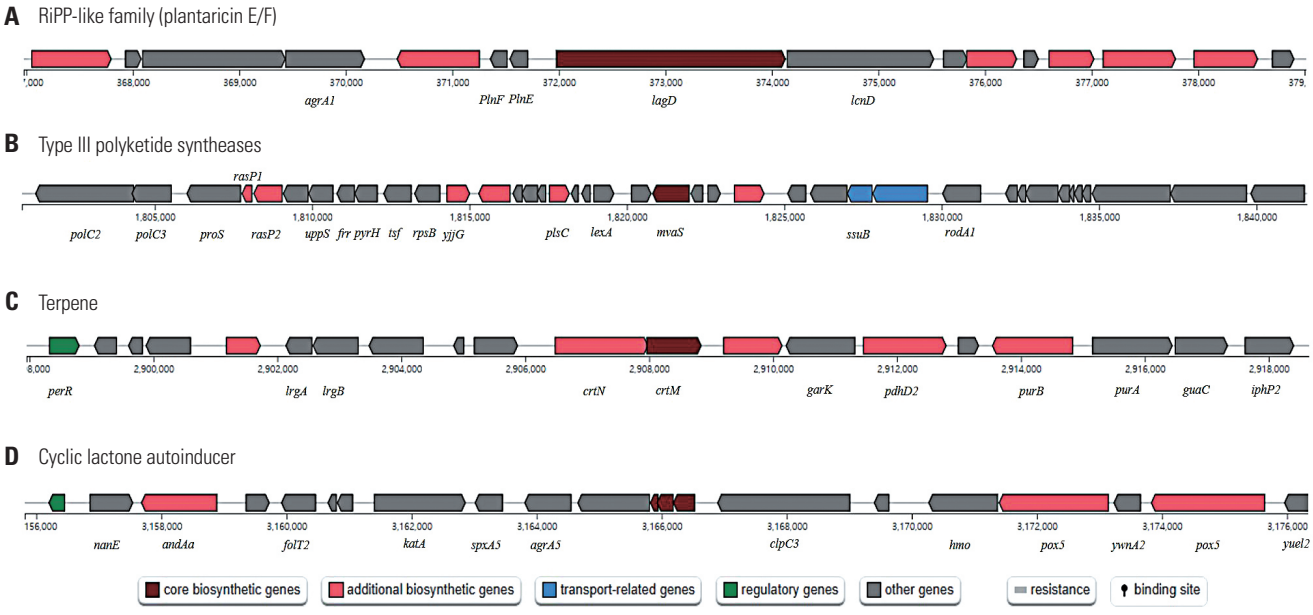

Fig. 6. General gene cluster of antimicrobial secondary metabolites in *Lactiplantibacillus plantarum*. (A) Prediction of plantaricin E/F gene cluster. (B) Prediction of type III polyketide synthases gene cluster. (C) Prediction of terpene gene cluster. (D) Prediction of cyclic lactone autoinducer gene cluster.

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25745, *Leuconostoc mesenteroides* spp. *mesenteroides* ATCC 8293, *Leuconostoc kimchii* IMSNU 11154, *Oenococcus oeni* PSU-1, and *L. reuteri* Lc 705.

Horizontal gene transfer (HGT)

Table 7 summarizes the presence of GIs, ISs, prophages, plasmid-related sequences, and the CRISPR-Cas system in *L. plantarum* LM1215. *L. plantarum* LM1215 contained seven GIs, five ISs, and five prophages; however, these MGEs did not carry ARGs or virulence factors. No plasmid-related sequences were detected in *L. plantarum* LM1215, which possessed a CRISPR sequence with evidence level 1.

Table 4. Intrinsic and Acquired Antibiotic Resistance Genes in *Lactiplantibacillus plantarum* LM1215

Intrinsic antibiotic resistance genes	
Class of antibiotics	**Related resistance genes**
Aminocyclitol	Not detected
Aminoglycoside	Not detected
Amphenicol	Not detected
Diaminopyrimidines	Not detected
Glycopeptide	Not detected
Ionophores	Not detected
β-Lactam	Not detected
Lincosamide	Not detected
Macrolide	Not detected
Nitroimidazole	Not detected
Oxazolidinone	Not detected
Phosphonic acid derivatives	Not detected
Pleuromutilin derivatives	Not detected
Polymyxin	Not detected
Pseudomonic acid	Not detected
Quinolone	Not detected
Rifamycin	Not detected
Steroid antibacterial agent	Not detected
Streptogramin A	Not detected
Streptogramin B	Not detected
Sulfonamide	Not detected
Tetracycline	Not detected
Acquired antibiotic resistance genes	Not detected

DISCUSSION

To date, clinical studies have been conducted on BV and VVC to establish the beneficial effects of probiotics using different types and routes. *L.crispatus* containing vaginal probiotics has been found to be clinically effective and safe to prevent the recurrence of BV in a phase 2b trial in which vaginal probiotics were administered to women who had completed a course of vaginal antibiotics gel during 11 weeks.[28] The recurrence rate of BV was significantly decreased by 34% at week 12% and 27% at week 24 in the group using *L.crispatus* compared to the placebo group.[28] A randomized clinical trial of a short-duration of administration of *L. acidophilus* containing vaginal probiotics showed no additional beneficial effects on long-term BV recurrence after the completion of antibiotics.[29] A recent meta-analysis, including 17 randomized controlled trials on the treatment of BV, revealed that probiotics adjunctive to antibiotics were more effective than antibiotics alone, and probiotics were significantly more effective than placebo.[30] A few clinical trials were conducted on VVC compared to BV. *L. gasseri* containing vaginal probiotics[31] or *L. acidiophilus*[32] have not been found to have any beneficial effects on recurrent VVC, and meta-analyses did not show definitive findings due to a limited number of clinical studies of probiotics on recurrent VVC.[33,34]

C. albicans is a biofilm-forming yeast,[3] commonly found in patients with VVC, whereas other non-albicans *Candida* infections are considered minor infections of the vaginal tract.[3,35] These biofilms facilitate surface adhesion and enhance resistance to environmental stressors, host immune responses, and therapeutic agents.[36] Moreover, biofilm formation can lead to histological damage in mucosal epithelial cells and local inflammation in the vaginal epithelium. Another significant concern is that biofilms hinder the efficacy of antifungal agents in VVC treatment.[35] Sun, et al.[37] reported that azole compounds, such as clotrimazole, up-regulate efflux pumps within biofilms

Table 5. Detection of Virulence Factor-Related Genes in *Lactiplantibacillus plantarum* LM1215

Genes	Products	Identities	Gaps	Score (bits)	E-value
clfA	Clumping factor A, fibrinogen-binding protein	438/533 (82)	6/533 (1)	305	2E-79
clfB	Clumping factor B, adhesin	435/531 (81)	6/531 (1)	293	6E-76
sdrD	Ser-Asp rich fibrinogen-binding bone sialoprotein-binding protein	354/437 (81)	6/437 (1)	210	7E-51
sdrE	Ser-Asp rich fibrinogen-binding bone sialoprotein-binding protein	349/431 (80)	-	204	4E-49
sdrC	Ser-Asp rich fibrinogen-binding bone sialoprotein-binding protein	333/410 (81)	-	202	2E-48
clpC	Endopeptidase Clp ATP-binding chain C	119/146 (81)	-	76	3E-10
tufA	Elongation factor Tu	97/114 (85)	2/114 (1)	76	3E-10
hasC	UTP--glucose-1-phosphate uridylyltransferase HasC	71/83 (85)	-	70	2E-08
lap	*Listeria* adhesion protein Lap	62/71 (87)	-	70	2E-08
bsh	Bile salt hydrolase	65/77 (84)	-	58	6E-05

Table 6. The Prediction Probability of *Lactiplantibacillus plantarum* LM1215 as a Human Pathogen

Human pathogen prediction		Non-pathogenic bacteria		
Probability of a human pathogen		0.189		
Matched pathogenic families		0		
Matched non-pathogenic families		14		
Matched sequence detail				
Start	**End**	**Matched microorganisms**	**Matched protein**	**Identity (%)**
174186	176345	*Pediococcus pentosaceus* ATCC 25745	α-Galactosidase	98.19
333957	334403	*Leuconostoc mesenteroides* spp. *mesenteroides* ATCC 8293	Ribonuclease HI	98.65
337217	338548	*Leuconostoc kimchii* IMSNU 11154	Glutathione reductase	100
1849138	1849470	*Pediococcus pentosaceus* ATCC 25745	Head-tail connector protein	91.82
1851679	1852704	*Pediococcus pentosaceus* ATCC 25745	Hypothetical protein	97.07
1852891	1854789	*Pediococcus pentosaceus* ATCC 25745	Phage terminase-like protein, large subunit	93.83
1854792	1855250	*Pediococcus pentosaceus* ATCC 25745	Phage terminase, small subunit	90.79
3039746	3040066	*Oenococcus oeni* PSU-1	Thiol-disulfide isomerase and thioredoxin	100
3036552	3038456	*Oenococcus oeni* PSU-1	Cation transport ATPase	98.11
3040090	3040359	*Oenococcus oeni* PSU-1	Metal-sensitive transcriptional regulator	97.75
3040760	3041314	*Oenococcus oeni* PSU-1	DNA-binding ferritin-like protein (oxidative damage protectant)	95.65
3041749	3042414	*Oenococcus oeni* PSU-1	Crp-like transcriptional regulator	98.64
3081284	3083164	*Limosilactobacillus reuteri* Lc 705 (plasmid)	β-galactosidase (GH42)	99.52
3083148	3083492	*Limosilactobacillus reuteri* Lc 705 (plasmid)	β-galactosidase small subunit	100

Table 7. Mobile Genetic Elements, Plasmid Related Sequence, and CRISPR-Cas in *Lactiplantibacillus plantarum* LM1215

Mobile genetic elements	Details	Start	End	GC contents (%)	ARGs	Virulence genes
Genomic island	-	351044	378900	40.07	Not detected	Not detected
Genomic island	-	570297	590667	50.78	Not detected	Not detected
Genomic island	-	1028102	1044143	36.83	Not detected	Not detected
Genomic island	-	2792187	2805572	38.89	Not detected	Not detected
Insertion sequence/Transposon	ISP1	537345	538607	39.94	Not detected	Not detected
Insertion sequence/Transposon	ISLpl2	822165	822713	42.52	Not detected	Not detected
Insertion sequence cluster/Transposon	ISP1/ISP1	1024558	1025618	40.28	Not detected	Not detected
Insertion sequence/Transposon	ISP1	1756417	1757679	40.02	Not detected	Not detected
Insertion sequence/Transposon	ISP2	2779405	2780898	41.66	Not detected	Not detected
Prophage	-	549799	560673	43.98	Not detected	Not detected
Prophage	-	1192545	1230073	41.41	Not detected	Not detected
Prophage	-	1826999	1882244	41.85	Not detected	Not detected
Prophage	-	2145402	2218664	41.69	Not detected	Not detected
Prophage	-	2406578	2413360	49.09	Not detected	Not detected
Plasmid related sequence						
Inc18	**RepA_N**	**Rep3**	**Pep_trans**	**Rep2**	**NT_Rep**	**Rep1**	**RepL**
Not detected	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
CRISPR						
Start	**End**	**Direction**	**Consensus repeat**	**Number of repeats**	**Number of spacers**	**Evidence level**
2742366	2742451	Unknown	TAAGAAACTTAAAGTGTCTTATT	2	1	1

and extracellular matrices, whereas the presence of β-glucan within biofilms inhibits the effectiveness of amphotericin B, a polyene antifungal agent. Thus, controlling *C. albicans* biofilm formation is crucial for VVC treatment and the development of natural antifungal agents. In this study, *L. plantarum* LM1203, LM1209, and LM1215 exhibited fungistatic (Fig. 1) and biofilm removal (Fig. 2) effects.

LAB produce a wide range of antimicrobial metabolites that depolarize membranes,[38,39] inhibit cell wall enzyme synthesis,[38] cause cell wall lysis,[39] damage proteins and nucleic acids through protonated acids,[38,40] generate oxidative stress through the accumulation of reactive oxygen species,[38] and reduce in-

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tracellular ATP levels.[39] Similarly, LAB produce antifungal metabolites that induce cell surface distortions, disrupt the hyphae of fungal cells, and induce the leakage of cytoplasmic content.[40] As shown in Fig. 6, *L. plantarum* LM1215 was predicted to have four types of antimicrobial secondary metabolite production gene clusters. One of these clusters encodes for plantaricin E/F, a two-peptide bacteriocin consisting of plnE and plnF.[41,42] Plantaricin E/F acts as an antagonist against gram-positive bacteria and fungi,[41] and is more effective than plantaricin A.[42] Gram-positive bacteria subjected to plantaricin E/F exhibit cell membrane damage and dissipation of cellular proton motive forces, ultimately resulting in cell death.[43] Therefore, the fungistatic (Fig. 1) and bacteriostatic effects, as well as cellular membrane damage (Figs. 3 and 4) observed in this study may be attributed to the action of plantaricin E/F produced by *L. plantarum* LM1215. Additionally, aromatic polyketides, terpenes, and cyclic lactone autoinducers have been predicted in *L. plantarum* LM1215. These genetic properties contributed to the regulation of *C. albicans* and *G. vaginalis* by *L. plantarum* LM1215.

Probiotics are generally regarded as safe for dietary consumption; however, recent studies have raised concerns about the emergence of antibiotic-resistant non-pathogenic bacteria.[44] Antibiotic resistance is classified as intrinsic or acquired, with the latter often resulting from HGT between different bacterial genera via conjugation, transformation, and transduction.[45,46] LAB are intrinsically resistant to certain antibiotic classes, including aminoglycosides, quinolones, and diaminopyrimidines. Moreover, *Lactiplantibacillus* spp. are generally resistant to vancomycin. Furthermore, conjugation can occur between LAB and other pathogenic bacteria via conjugation pili.[46] Considering these concerns, the EFSA recommends comprehensive screening for ARGs using whole-genome sequencing when probiotics are used in humans and animals.[45,46] In the present study, *L. plantarum* LM1215 did not possess any intrinsic or acquired ARGs, including MGEs. Additionally, *L. plantarum* LM1215 possesses a CRISPR-Cas system, which acts as a defense mechanism against HGT in bacteria.[47] Thus, *L. plantarum* LM1215 is considered a safe probiotic agent suitable for the prevention of VVC and BV.

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REFERENCES

1. Saraf VS, Sheikh SA, Ahmad A, Gillevet PM, Bokhari H, Javed S. Vaginal microbiome: normalcy vs dysbiosis. Arch Microbiol 2021; 203:3793-802.
2. Kim H, Kim Y, Kang CH. In vivo confirmation of the antimicrobial effect of probiotic candidates against Gardnerella vaginalis. Microorganisms 2021;9:1690.
3. McKloud E, Delaney C, Sherry L, Kean R, Williams S, Metcalfe R, et al. Recurrent vulvovaginal candidiasis: a dynamic interkingdom biofilm disease of Candida and Lactobacillus. mSystems 2021; 6:e0062221.
4. Bae WY, Lee YJ, Jung WH, Shin SL, Kim TR, Sohn M. Draft genome sequence and probiotic functional property analysis of Lactobacillus gasseri LM1065 for food industry applications. Sci Rep 2023; 13:12212.
5. Chow K, Wooten D, Annepally S, Burke L, Edi R, Morris SR. Impact of (recurrent) bacterial vaginosis on quality of life and the need for accessible alternative treatments. BMC Womens Health 2023; 23:112.
6. Mendling W, Holzgreve W. Astodrimer sodium and bacterial vaginosis: a mini review. Arch Gynecol Obstet 2022;306:101-8.
7. Yilmaz B, Bangar SP, Echegaray N, Suri S, Tomasevic I, Manuel Lorenzo J, et al. The impacts of Lactiplantibacillus plantarum on the functional properties of fermented foods: a review of current knowledge. Microorganisms 2022;10:826.
8. Rocchetti MT, Russo P, Capozzi V, Drider D, Spano G, Fiocco D. Bioprospecting antimicrobials from Lactiplantibacillus plantarum: key factors underlying its probiotic action. Int J Mol Sci 2021;22: 12076.
9. Bae WY, Jung WH, Lee YJ, Shin SL, An YK, Kim TR, et al. Heat-treated Pediococcus acidilactici LM1013-mediated inhibition of biofilm formation by Cutibacterium acnes and its application in acne vulgaris: a single-arm clinical trial. J Cosmet Dermatol 2023;22: 3125-34.
10. Bae WY, Kim HY, Yu HS, Chang KH, Hong YH, Lee NK, et al. Antimicrobial effects of three herbs (Brassica juncea, Forsythia suspensa, and Inula britannica) on membrane permeability and apoptosis in Salmonella. J Appl Microbiol 2021;130:394-404.
11. Ouyang X, Hoeksma J, van der Velden G, Beenker WAG, van Triest MH, Burgering BMT, et al. Berkchaetoazaphilone B has antimicrobial activity and affects energy metabolism. Sci Rep 2021;

11:18774.
12. Huang Z, Jia S, Zhang L, Liu X, Luo Y. Inhibitory effects and membrane damage caused to fish spoilage bacteria by cinnamon bark (Cinnamomum tamala) oil. LWT 2019;112:108195.
13. Yoo JH, Baek KH, Heo YS, Yong HI, Jo C. Synergistic bactericidal effect of clove oil and encapsulated atmospheric pressure plasma against Escherichia coli O157:H7 and Staphylococcus aureus and its mechanism of action. Food Microbiol 2021;93:103611.
14. Pino A, Rapisarda AMC, Vitale SG, Cianci S, Caggia C, Randazzo CL, et al. A clinical pilot study on the effect of the probiotic Lacticaseibacillus rhamnosus TOM 22.8 strain in women with vaginal dysbiosis. Sci Rep 2021;11:2592.
15. Carver T, Harris SR, Berriman M, Parkhill J, McQuillan JA. Artemis: an integrated platform for visualization and analysis of high-throughput sequence-based experimental data. Bioinformatics 2012;28:464-9.
16. Carver T, Thomson N, Bleasby A, Berriman M, Parkhill J. DNAPlotter: circular and linear interactive genome visualization. Bioinformatics 2009;25:119-20.
17. Emms DM, Kelly S. OrthoFinder: phylogenetic orthology inference for comparative genomics. Genome Biol 2019;20:238.
18. Huson DH, Scornavacca C. Dendroscope 3: an interactive tool for rooted phylogenetic trees and networks. Syst Biol 2012;61:1061-7.
19. Yoon SH, Ha SM, Lim J, Kwon S, Chun J. A large-scale evaluation of algorithms to calculate average nucleotide identity. Antonie Van Leeuwenhoek 2017;110:1281-6.
20. Chen C, Chen H, Zhang Y, Thomas HR, Frank MH, He Y, et al. TBtools: an integrative toolkit developed for interactive analyses of big biological data. Mol Plant 2020;13:1194-202.
21. Darling AC, Mau B, Blattner FR, Perna NT. Mauve: multiple alignment of conserved genomic sequence with rearrangements. Genome Res 2004;14:1394-403.
22. Blin K, Shaw S, Augustijn HE, Reitz ZL, Biermann F, Alanjary M, et al. AntiSMASH 7.0: new and improved predictions for detection, regulation, chemical structures and visualisation. Nucleic Acids Res 2023;51:W46-50.
23. Liu B, Zheng D, Zhou S, Chen L, Yang J. VFDB 2022: a general classification scheme for bacterial virulence factors. Nucleic Acids Res 2022;50:D912-7.
24. Cosentino S, Voldby Larsen M, Møller Aarestrup F, Lund O. PathogenFinder--distinguishing friend from foe using bacterial whole genome sequence data. PLoS One 2013;8:e77302.
25. Wang M, Goh YX, Tai C, Wang H, Deng Z, Ou HY. VRprofile2: detection of antibiotic resistance-associated mobilome in bacterial pathogens. Nucleic Acids Res 2022;50:W768-73.
26. Carattoli A, Zankari E, García-Fernández A, Voldby Larsen M, Lund O, Villa L, et al. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrob Agents Chemother 2014;58:3895-903.
27. Couvin D, Bernheim A, Toffano-Nioche C, Touchon M, Michalik J, Néron B, et al. CRISPRCasFinder, an update of CRISRFinder, includes a portable version, enhanced performance and integrates search for Cas proteins. Nucleic Acids Res 2018;46:W246-51.
28. Cohen CR, Wierzbicki MR, French AL, Morris S, Newmann S, Reno H, et al. Randomized trial of lactin-V to prevent recurrence of bacterial vaginosis. N Engl J Med 2020;382:1906-15.
29. Bradshaw CS, Pirotta M, De Guingand D, Hocking JS, Morton AN, Garland SM, et al. Efficacy of oral metronidazole with vaginal clindamycin or vaginal probiotic for bacterial vaginosis: randomised placebo-controlled double-blind trial. PLoS One 2012;7:e34540.
30. Chen R, Li R, Qing W, Zhang Y, Zhou Z, Hou Y, et al. Probiotics are a good choice for the treatment of bacterial vaginosis: a meta-analysis of randomized controlled trial. Reprod Health 2022;19:137.
31. Witt A, Kaufmann U, Bitschnau M, Tempfer C, Ozbal A, Haytouglu E, et al. Monthly itraconazole versus classic homeopathy for the treatment of recurrent vulvovaginal candidiasis: a randomised trial. BJOG 2009;116:1499-505.
32. Mollazadeh-Narestan Z, Yavarikia P, Homayouni-Rad A, Samadi Kafil H, Mohammad-Alizadeh-Charandabi S, Gholizadeh P, et al. Comparing the effect of probiotic and fluconazole on treatment and recurrence of vulvovaginal candidiasis: a triple-blinded randomized controlled trial. Probiotics Antimicrob Proteins 2023;15:1436-46.
33. van de Wijgert J, Verwijs MC. Lactobacilli-containing vaginal probiotics to cure or prevent bacterial or fungal vaginal dysbiosis: a systematic review and recommendations for future trial designs. BJOG 2020;127:287-99.
34. Cooke G, Watson C, Deckx L, Pirotta M, Smith J, van Driel ML. Treatment for recurrent vulvovaginal candidiasis (thrush). Cochrane Database Syst Rev 2022;1:CD009151.
35. Sabbatini S, Visconti S, Gentili M, Lusenti E, Nunzi E, Ronchetti S, et al. Lactobacillus iners cell-free supernatant enhances biofilm formation and hyphal/pseudohyphal growth by Candida albicans vaginal isolates. Microorganisms 2021;9:2577.
36. Bapat P, Singh G, Nobile CJ. Visible lights combined with photosensitizing compounds are effective against Candida albicans biofilms. Microorganisms 2021;9:500.
37. Sun C, Zhao X, Jiao Z, Peng J, Zhou L, Yang L, et al. The antimicrobial peptide AMP-17 derived from Musca domestica inhibits biofilm formation and eradicates mature biofilm in Candida albicans. Antibiotics (Basel) 2022;11:1474.
38. Zapaśnik A, Sokołowska B, Bryła M. Role of lactic acid bacteria in food preservation and safety. Foods 2022;11:1283.
39. Kaveh S, Hashemi SMB, Abedi E, Amiri MJ, Conte FL. Bio-preservation of meat and fermented meat products by lactic acid bacteria strains and their antibacterial metabolites. Sustainability 2023;15:10154.
40. Mani-López E, Arrioja-Bretón D, López-Malo A. The impacts of antimicrobial and antifungal activity of cell-free supernatants from lactic acid bacteria in vitro and foods. Compr Rev Food Sci Food Saf 2022;21:604-41.
41. Li Z, Cheng Q, Guo H, Zhang R, Si D. Expression of hybrid peptide EF-1 in Pichia pastoris, its purification, and antimicrobial characterization. Molecules 2020;25:5538.
42. Abdulhussain Kareem R, Razavi SH. Plantaricin bacteriocins: as safe alternative antimicrobial peptides in food preservation—a review. J Food Saf 2019;40:e12735.
43. Wang Y, Wei Y, Shang N, Li P. Synergistic inhibition of plantaricin E/F and lactic acid against Aeromonas hydrophila LPL-1 reveals the novel potential of class IIb bacteriocin. Front Microbiol 2022;13:774184.
44. Wang K, Zhang H, Feng J, Ma L, de la Fuente-Núñez C, Wang S, et al. Antibiotic resistance of lactic acid bacteria isolated from dairy products in Tianjin, China. J Agric Food Res 2019;1:100006.
45. Nunziata L, Brasca M, Morandi S, Silvetti T. Antibiotic resistance in wild and commercial non-enterococcal lactic acid bacteria and bifidobacteria strains of dairy origin: an update. Food Microbiol 2022;104:103999.
46. Ojha AK, Shah NP, Mishra V. Conjugal transfer of antibiotic resistances in Lactobacillus spp. Curr Microbiol 2021;78:2839-49.
47. Tao S, Chen H, Li N, Liang W. The application of the CRISPR-Cas system in antibiotic resistance. Infect Drug Resist 2022;15:4155-68.

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