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Comparative anti-microbial and anti-biofilm activities of postbiotics derived from kefir and normal raw milk lactic acid bacteria against bovine mastitis pathogens

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ABSTRACT

Bovine mastitis, caused by pathogens including *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Escherichia coli*, poses a significant challenge to the dairy industry. This study aimed to investigate the underlying mechanisms of the anti-microbial and anti-biofilm activities of postbiotics derived from lactic acid bacteria against bovine mastitis pathogens. Kefir-derived *Lentilactobacillus kefiri* LK1 produced significantly more lactic acid than normal raw milk-derived *Enterococcus faecium* EFM2, which was attributed to differences in biochemical activities. At an optimum concentration of 25%, both *L. kefiri* LK1 (POS_LK1) and *E. faecium* EFM2 postbiotics (POS_EFM2) exhibited anti-microbial and anti-biofilm activities by modulating hydrophobicity, auto-aggregation, and exopolysaccharide (EPS) production phenotypes and genotypes of bovine mastitis pathogens. POS_LK1 treatment reduced auto-aggregation and EPS production by *E. faecalis* and downregulated *wspA* and *pelA* expression in *P. aeruginosa* compared to those in POS_EFM2. Therefore, our results suggest that POS_LK1 could be used in the dairy industry as an anti-biofilm agent to effectively prevent pathogenic biofilm-induced bovine mastitis.

1. Introduction

Bovine mastitis is an inflammatory response of the mammary gland caused by microbial infection, exerting negative effects on milk production, milk quality, and animal welfare (Cheng & Han, 2020). The symptoms of bovine mastitis include red and swollen udders, fever, and an elevated somatic cell count (SCC) due to immune responses (Cheng & Han, 2020). Bovine mastitis is identified through an elevated SCC in raw milk (≥200,000 per mL) (Yun, Yoon, & Lee, 2020). Pathogens that cause bovine mastitis are generally classified as contagious or environmental, depending on their primary reservoir and method of transmission (Bogni et al., 2011). *Staphylococcus aureus* is the most representative contagious pathogen transmitted among bovines through milking equipment from infected milk and teat skin (Bogni et al., 2011). *Enterococcus faecalis*, *Pseudomonas aeruginosa,* and *Escherichia coli* are the main environmental and opportunistic pathogens present in bedding and housing on dairy farms (Bogni et al., 2011; Kim, Youn, et al., 2022). High stocking densities, contaminated floors and workers, wet bedding, poor ventilation, and inadequate milking preparation can promote the growth and biofilm formation of these pathogens, thereby increasing the incidence of bovine mastitis (Cheng & Han, 2020).

Biofilms, a strategy employed by pathogens for survival and protection against adverse environments, are produced by bacterial attachment, maturation, and dispersal (Liu et al., 2017). Biofilm formation begins with bacteria adhering to a surface, a process influenced by the cell surface hydrophobicity of pathogens (Dwivedi & Sehgal, 2022). Subsequently, these pathogens form multicellular structures, connected through auto-aggregation and exopolysaccharide (EPS) production (Liu et al., 2020). The resulting biofilm protects pathogens from antibiotics, desiccation, extreme temperatures, competing microorganisms, and the host immune systems, ultimately making the biofilm difficult to remove (Vu, Chen, Crawford, & Ivanova, 2009). Furthermore, unremoved biofilms cause recurrent and chronic bovine mastitis, fostering the emergence and spread of antibiotic-resistant bacteria, thereby posing a substantial threat to human health (Pedersen et al., 2021).

Kefir and normal raw milk are rich in various beneficial lactic acid

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Abbreviations

(ANOVA) analysis of variance
(CFU) colony forming units
(Caco-2) colorectal adenocarcinoma
(MRS) De Man, Rogosa, and Sharpe
(EMEM) Eagle's minimum essential medium
(EPS) exopolysaccharide
(HI_FBS) heat-inactivated fetal bovine serum
(HPLC) high-performance liquid chromatography
(LAB) lactic acid bacteria
(MTT) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
(P/S) penicillin/streptomycin
(PBS) phosphate-buffered saline
(POS_LK1) *Lentilactobacillus kefiri* LK1 postbiotics
(POS_EFM2) *Enterococcus faecium* EFM2 postbiotics
(RT–qPCR) reverse transcription–quantitative polymerase chain reaction
(SCC) somatic cell count

bacteria (LAB) owing to their high content of nutrients, such as carbohydrates, fats, casein, proteins, vitamins, and minerals (Jha et al., 2022; Kim et al., 2017). Many studies have explored the health benefits associated with these LAB, including inhibiting pathogenic growth and biofilm formation (Ağagündüz et al., 2021; Sevin et al., 2021). Therefore, LAB represent a potential form of probiotics, which are live microorganisms that provide health benefits (FAO/WHO, 2002). Although numerous benefits of probiotics have been reported, recent attention has been drawn to safety concerns, such as sepsis, endocarditis, and bacteremia in vulnerable groups, as well as the acquisition and transmission of antibiotic-resistance genes (Piqué, Berlanga, & Miñana-Galbis, 2019). Postbiotics are metabolites of probiotics that maintain the beneficial functions of probiotics without their associated safety issues (Kerry et al., 2018). Therefore, the application of postbiotics in preventing bovine mastitis has been considered and studied in livestock (Bogni et al., 2011; Kerry et al., 2018).

This is the first study to compare the anti-microbial and anti-biofilm activities and their mechanisms against bovine mastitis pathogenic bacteria (*S. aureus*, *E. faecalis*, *P. aeruginosa*, and *E. coli*) using postbiotics derived from kefir and normal raw milk LAB. The mechanisms of anti-biofilm activity were demonstrated by analyzing biochemical analysis, organic acid quantification of postbiotics, and modulation in biofilm-related phenotypes and genotypes, such as hydrophobicity, auto-aggregation, and EPS production of pathogens.

2. Materials and methods

Fig. 1 shows a flow diagram of the experimental procedures to investigate LAB strains with anti-microbial and anti-biofilm activities derived from kefir and normal raw milk.

2.1. Isolation and identification of LAB

LAB isolation followed previously established procedures, with some modifications (Kim et al., 2017; Kim, Youn, et al., 2022). Kefir was prepared by adding 50 g of kefir grains into 1 L of sterilized milk (Seoul Milk, Seoul, South Korea) and fermenting the mixture at 25 °C for 48 h. For isolation LAB, kefir and normal raw milk (with an SCC of <200,000 per mL) were streaked onto De Man, Rogosa, and Sharpe agar (MRS; Difco, Detroit, MI, USA) and incubated anaerobically at 37 °C for 48 h. DNA from each LAB strain was extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. LAB identification was accomplished through 16S rRNA sequencing using 27F and 1492R primers (Bionics, Seoul, South Korea).

2.2. Biochemical analysis using the VITEK 2 system

Biochemical analysis of LAB strains was conducted using the VITEK 2

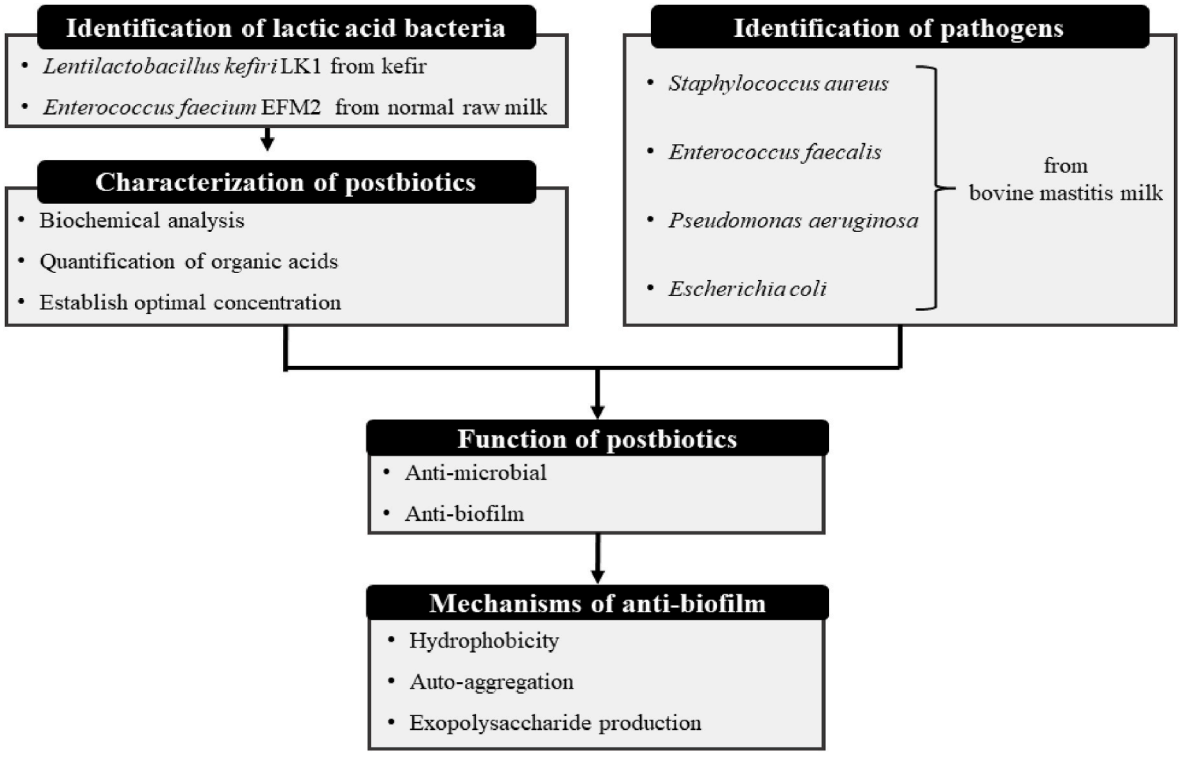

Fig. 1. Flow diagram of the experimental procedures.

system (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. Each LAB strain was suspended in 0.45% saline to a 3.0 McFarland standard and analyzed using a VITEK 2 ANC card (bioMérieux).

2.3. High-performance liquid chromatography (HPLC) analysis of postbiotics

Organic acids in postbiotics were measured using an Agilent 1260 HPLC ChemStation™ (Agilent Technologies, Palo Alto, CA, USA). Each LAB strain was incubated anaerobically at 37 °C for 48 h in MRS broth (Difco) to obtain postbiotics. Thereafter, each culture was centrifuged (7280×*g* at 4 °C for 10 min), and the supernatant was filtered using a 0.2-μm syringe filter (Millipore, Bedford, MA, USA). This filtered supernatant was considered as postbiotics (Żółkiewicz, Marzec, Ruszczyński, & Feleszko, 2020). The HPLC system was equipped with an Aminex HPX-87H column (300 × 7.8 mm; Bio-Rad Laboratories, Hercules, CA, USA) and an ultraviolet detector set to read at 210 nm. The eluent consisted of 5 mM sulfuric acid (Daejung Chemicals & Metals, Gyeonggi-do, South Korea) with an isocratic flow rate of 0.6 mL/min. During the 20 min run time, 10 μL of postbiotics was injected, and the column temperature was set to 50 °C. Peaks were identified by comparison with the retention times of the standards and quantification was performed using a standard curve with a correlation coefficient $R^2 > 0.999$.

2.4. Optimizing the concentration of the postbiotics

LAB postbiotics were assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) assay for optimizing postbiotics concentrations, with some modifications (Lee, Park, & Kim, 2022). Colorectal adenocarcinoma (Caco-2) cells were seeded in 96-well plates (SPL Life Sciences, Gyeonggi-do, South Korea) at a concentration of 1×10^6 cells/well with 200 μL of Eagle's minimum essential medium (EMEM; Welgene Inc., Gyeongsan-si, South Korea) supplemented with 20% heat-inactivated fetal bovine serum (HI_FBS; GW Vitek Inc., Seoul, South Korea) and penicillin/streptomycin (P/S; Welgene Inc.). Subsequently, the plates were incubated at 37 °C for 24 h in a 5% CO_2 atmosphere until 95−99% confluence was achieved. The EMEM was replaced with 200 μL of EMEM (with HI_FBS and P/S) containing 50%, 25%, 12.5%, and 6.25% postbiotics and incubated at 37 °C for 24 h in a 5% CO_2 atmosphere. Finally, 20 μL of MTT solution was added to the well and incubated at 37 °C for 4 h in a 5% CO_2 atmosphere. After removing the MTT solution, the blue formazan crystals were solubilized with 150 μL of dimethylsulfoxide (Sigma-Aldrich). The absorbance was measured at 595 nm using a Multiskan FC (Thermo Fisher Scientific, Shanghai, China).

Cell viability (%) was calculated as follows: (Absorbance of cells with postbiotics/Absorbance of cells without postbiotics) × 100 1

2.5. Isolation and identification of bovine mastitis pathogens

The bovine mastitis pathogens were isolated from bovine mastitis milk with SCC ≥200,000 per mL collected from dairy farms in South Korea from 2017 to 2020. Bovine mastitis milk was inoculated on blood agar and incubated at 37 °C for 24 h. After incubation, colonies were identified using the matrix assisted laser desorption ionization–time of flight mass spectrometer (bioMérieux), according to the manufacturer's instructions (Kim et al., 2023). In brief, each bacterial colony was cultivated on tryptic soy agar (Difco) at 37 °C for 24 h. Subsequently, each colony was transferred onto a polished steel MSP 96 target BC (Bruker Daltonik, Bremen, Germany) using a sterile toothpick. To crystalize bacterial components, 1 μL of saturated α-cyano-4-hydroxy-cinnamic acid matrix solution in 50% acetonitrile-2.5% trifluoroacetic acid (Bruker Daltonik) was overlaid on each well and air dried at 25 °C. Main spectrum profiles of isolates were acquired using the microflex LT mass spectrometer (Bruker Daltonik) with default main spectrum profiles identification standard settings (linear positive mode, 2000 to 20,000 Da).

2.6. Anti-microbial and anti-biofilm assays on bovine mastitis pathogens

All experiments using pathogens were approved by the Konkuk University Institute Biosafety Committee (KUIBC-2023-19). To measure the anti-microbial activity, we prepared 100 μL of each *S. aureus, E. faecalis, P. aeruginosa,* and *E. coli* strain at 1×10^8 colony-forming units (CFU)/mL in nutrient broth (Sigma-Aldrich). Next, 100 μL of each pathogen and 100 μL of each postbiotics were added to 96-well plates (SPL Life Sciences), and absorbance was measured at 595 nm after incubation at 37 °C for 24 h.

The anti-biofilm activity of postbiotics treatment against pathogens was based on a method described by Kim, Youn, et al. (2022) with some modifications. After dispensing pathogens and postbiotics into a 96-well plate (SPL Life Sciences) as described above, the 96-well plate was incubated at 37 °C for 24 h. Afterward, the culture was removed and gently washed thrice with phosphate-buffered saline (PBS; Sigma-Aldrich). The adherent biofilm layer was stained with 200 μL of 0.1% crystal violet (Sigma-Aldrich) at 25 °C for 20 min. After washing the crystal violet thrice with PBS (Sigma-Aldrich), 200 μL of 99% ethanol (Sigma-Aldrich) was added to the well, and the absorbance was measured at 595 nm using a Multiskan FC (Thermo Fisher Scientific).

2.7. Mechanisms of anti-biofilm activity of postbiotics treatment

2.7.1. Hydrophobicity and auto-aggregation of bovine mastitis pathogens

The hydrophobicity and auto-aggregation of pathogens were evaluated according to the methods proposed by Youn et al. (2022), with some modifications. Briefly, 5 mL each of *S. aureus, E. faecalis, P. aeruginosa,* and *E. coli* strain, adjusted to 1×10^8 CFU/mL, was mixed with an equal volume of postbiotics, and incubated at 37 °C for 24 h. The non-treatment, comprising each pathogen adjusted to 1×10^8 CFU/mL and an equal volume of MRS broth (Difco), was used as a control. After the culture was centrifuged at 7280×*g* at 4 °C for 10 min, the resulting pellet was resuspended in PBS (Sigma-Aldrich) at 1×10^8 CFU/mL ($OD_{initial}$). Afterward, 2 mL of the suspension was added to 0.5 mL of chloroform (Sigma-Aldrich) and vortexed for 2 min. The mixture was placed upright for 15 min to ensure complete separation of the two phases. Subsequently, the absorbance of the aqueous phase ($OD_{treatment}$) was measured at 595 nm using a Multiskan FC (Thermo Fisher Scientific).

To assess auto-aggregation, 5 mL each of *S. aureus, E. faecalis, P. aeruginosa,* and *E. coli* strain, adjusted to 1×10^8 CFU/mL, was mixed with an equal volume of postbiotics and incubated at 37 °C for 24 h. After the culture underwent an additional incubation at 4 °C for 24 h, the absorbance of the supernatant ($OD_{treatment}$) and the suspension ($OD_{initial}$) was measured at 595 nm using a Multiskan FC (Thermo Fisher Scientific).

Hydrophobicity and Auto-aggregation (%) = (1 - $OD_{treatment}$ /$OD_{initial}$) × 1002

2.7.2. EPS production of bovine mastitis pathogens

The EPS production of pathogens following postbiotics treatment was measured using the phenol-sulfuric acid method with some modifications (Adeosun, Baloyi, & Cosa, 2022). Briefly, 5 mL each of *S. aureus, E. faecalis, P. aeruginosa,* and *E. coli* strain, adjusted to 1×10^8 CFU/mL, was mixed with an equal volume of postbiotics and incubated at 37 °C for 24 h. Thereafter, the culture was centrifuged at 7280×*g* for

10 min and filtered using a 0.2-μm syringe filter (Millipore). Subsequently, 1 mL of the filtered supernatant was added to 3 mL of 99% ethanol (Sigma-Aldrich) and incubated at 4 °C for 24 h. The culture was then centrifuged at 7280×*g* for 10 min, and the supernatant was removed. The resulting pellet was dissolved in 1 mL of distilled water. The suspension was sequentially added to 1 mL of 5% phenol (Sigma-Aldrich) and 5 mL of 98% sulfuric acid (Daejung Chemicals & Metals). The expressed color was measured at 595 nm using a Multiskan FC (Thermo Fisher Scientific).

EPS production (%) = (Absorbance of pathogens with postbiotics/Absorbance of pathogens without postbiotics) × 100 3

2.7.3. RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

The mRNA gene expression related to hydrophobicity, autoaggregation, and EPS production of pathogens was assessed using RT-qPCR (Supplementary Data 1). RNA was extracted from pathogens treated with postbiotics using TRIzol (Life Technologies, Carlsbad, CA, USA) and a PureLink™ RNA Mini Kit (Life Technologies). cDNA was prepared using a PrimeScript RT Reagent kit (Takara Bio Inc., Shiga, Japan). The reaction was performed with an initial denaturation at 95 °C for 60 s, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 40 s, using SYBR Premix Ex Taq (Takara Bio Inc.) on an ABI 7500 system (Applied Biosystems, Foster City, CA, USA). The differences in mRNA expression were calculated using the $2^{-\Delta\Delta CT}$ method, with 16S rRNA serving as the reference gene for normalization (Huang, Fei, Yu, Gou, & Huang, 2014).

2.8. Statistical analysis

All experiments were repeated in triplicate, and data are presented as the mean ± standard deviation. All data were analyzed by analysis of variance (ANOVA) with Tukey's *post hoc* test using SPSS software (version 25.0; SPSS Inc., Chicago, IL, USA). Statistical significance was defined as $P < 0.05$. All graphs were constructed using GraphPad Prism 8.0.2 (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Biochemical activities of isolated LAB

The LAB from kefir and normal raw milk were identified as *Lentilactobacillus kefiri* LK1 and *Enterococcus faecium* EFM2, respectively. The 16S rRNA gene sequences of *L. kefiri* LK1 and *E. faecium* EFM2 have been deposited in GenBank under accession numbers OR647326 and OR647328, respectively. Table 1 shows the biochemical activities of *L. kefiri* LK1 and *E. faecium* EFM2, obtained using the VITEK 2 system. Both L. *kefiri* LK1 and *E. faecium* EFM2 showed positive results for D-glucose, 5-bromo-4-chloro-3-indoxyl-beta-glucoside, beta-galactopyranoside indoxyl, phenylalanine arylamidase, and tyrosine arylamidase. However, only *L. kefiri* LK1 tested positive for 5-bromo-4-chloro-3-indoxyl-alpha-galactoside, alpha-arabinose, arbutin, L-proline arylamidase, Ala-Phe-Pro arylamidase, and phenylphosphonate. In contrast, only *E. faecium* EFM2 was positive for D-galactose, D-cellobiose, D-maltose, beta-mannosidase, maltotriose, esculin hydrolysis, L-arabinose, leucine arylamidase, L-pyrrolydonyl-arylamidase, and arginine.

3.2. Organic acid-producing ability of isolated LAB

Table 2 presents the organic acids produced by *L. kefiri* LK1 and *E. faecium* EFM2. *L. kefiri* LK1 postbiotics (POS_LK1) exhibited significantly higher lactic acid production (40.59 ± 1.71 mM) than that of *E. faecium* EFM2 postbiotics (POS_EFM2; 30.62 ± 0.28 mM) ($P < 0.05$).

Table 1
Results of biochemical analysis of *Lentilactobacillus kefiri* LK1 and *Enterococcus faecium* EFM2 using the VITEK 2 system.

Biochemical test	*Lentilactobacillus kefiri* LK1	*Enterococcus faecium* EFM2
D-glucose	+	+
5-bromo-4-chloro-3-indoxyl-beta-glucoside	+	+
Beta-galactopyranoside indoxyl	+	+
Phenylalanine arylamidase	+	+
Tyrosine arylamidase	+	+
5-bromo-4-chloro-3-indoxyl-alpha-galactoside	+	-
Alpha-arabinose	+	-
Arbutin	+	-
D-galactose	–	+
D-cellobiose	–	+
D-maltose	–	+
Beta-mannosidase	–	+
Maltotriose	–	+
Esculin hydrolysis	–	+
L-arabinose	–	+
Leucine arylamidase	–	+
L-proline arylamidase	+	–
L-pyrrolydonyl-arylamidase	–	+
Ala-Phe-Pro arylamidase	+	–
Arginine	–	+
Phenylphosphonate	+	–
Positive/total tests (%)	11/36 (30.56%)	15/36 (41.67%)

Negative results for both *Lentilactobacillus kefiri* LK1 and *Enterococcus faecium* EFM2 are not shown.

Table 2
Organic acid concentrations in postbiotics.

Treatment	Lactic acid (mM)	Acetic acid (mM)	Citric acid (mM)
MRS broth	2.38 ± 0.13^{c}	77.31 ± 5.41^{a}	9.27 ± 0.07^{a}
POS_LK1	40.59 ± 1.71^{a}	74.48 ± 4.40^{a}	7.96 ± 0.05^{c}
POS_EFM2	30.62 ± 0.28^{b}	74.93 ± 4.49^{a}	9.02 ± 0.07^{b}

All data are expressed as mean ± standard deviation ($n = 3$).
Different letters indicate statistical significance ($P < 0.05$, ANOVA, Tukey's test).
MRS: De Man, Rogosa, and Sharpe; POS_LK1: *Lentilactobacillus kefiri* LK1 postbiotics; POS_EFM2: *Enterococcus faecium* EFM2 postbiotics.

Moreover, *L. kefiri* LK1 and *E. faecium* EFM2 produced neither acetic nor citric acid.

3.3. Cytotoxicity of postbiotics treatment

Fig. 2 depicts the cytotoxicity of postbiotics treatment on Caco-2 cells. According to ISO 10993-5 guidelines, cell viability >80% is considered to indicate no cytotoxicity; cell viabilities within 80–60%, 60–40%, and below 40% are considered to indicate weak, moderate, and strong cytotoxicity, respectively (ISO, 2009). Treatment with POS_LK1 at 50% concentration showed weak cytotoxicity (62.71 ± 0.63%), whereas treatment at 25%, 12.5%, and 6.25% concentrations showed no cytotoxicity (82.52 ± 1.75%, 98.70 ± 1.05%, and 99.11 ± 0.15%, respectively) ($P < 0.05$). Similarly, treatment with POS_EFM2 at 50% concentration exhibited moderate cytotoxicity at 47.89 ± 2.91%, whereas treatment at 25%, 12.5%, and 6.25% concentrations exhibited no cytotoxicity (81.49 ± 1.04%, 84.85 ± 1.84%, and 85.84 ± 4.79%, respectively) ($P < 0.05$). Considering these results, both postbiotics with no cytotoxicity at 25% concentration were used for subsequent experiments.

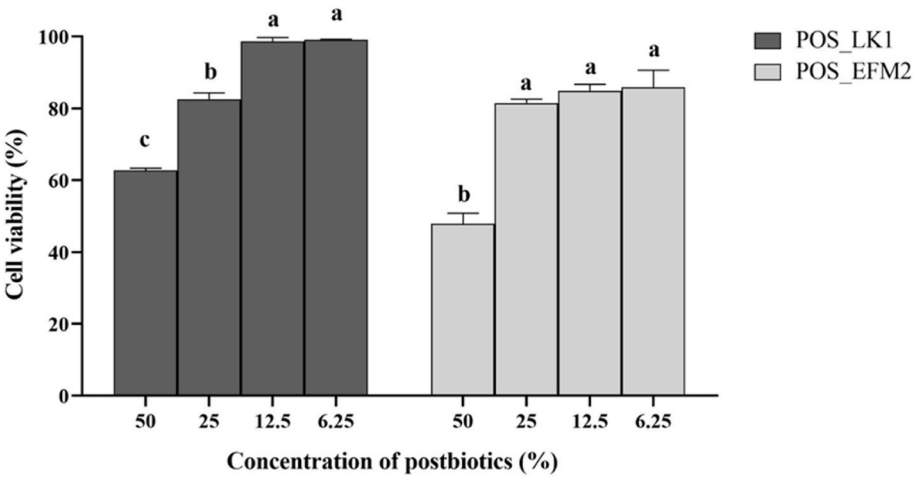

Fig. 2. Colorectal adenocarcinoma (Caco-2) cell viability with postbiotics at 50%, 25%, 12.5%, and 6.25% concentrations.
All data are expressed as mean ± standard deviation. Letters above the bars indicate statistical significance within the same treatment ($P < 0.05$, ANOVA, Tukey's test). POS_LK1: *Lentilactobacillus kefiri* LK1 postbiotics; POS_EFM2: *Enterococcus faecium* EFM2 postbiotics.

3.4. Anti-microbial and anti-biofilm activities of postbiotics against bovine mastitis pathogens

Pathogens, including *S. aureus, E. faecalis, P. aeruginosa,* and *E. coli* strains, were isolated from bovine mastitis milk (Supplementary Data 2). The growth and biofilm formation of all pathogens were significantly inhibited after treatment with POS_LK1 and POS_EFM2 at a 25% concentration, as compared with those of non-treatment (Figs. 3 and 4; $P < 0.05$).

3.5. Modulation of hydrophobicity, auto-aggregation, and EPS production of bovine mastitis pathogens

The phenotypic modulation of hydrophobicity, auto-aggregation, and EPS production of pathogens with postbiotics at 25% concentration are shown in Table 3. The hydrophobicity, auto-aggregation, and EPS production of pathogens were significantly reduced by POS_LK1 and POS_EFM2 treatments compared with those of the non-treatment ($P < 0.05$). Notably, the auto-aggregation and EPS production of *E. faecalis* after POS_LK1 treatment (8.65 ± 3.11% and 43.00 ± 5.98%, respectively) were more inhibited than those in the POS_EFM2 treatment (60.99 ± 5.08% and 69.94 ± 2.28%, respectively) ($P < 0.05$).

3.6. Modulation of biofilm-related genes in bovine mastitis pathogens

The genotypic modulations considering mRNA gene expression for hydrophobicity, auto-aggregation, and EPS production in pathogens are presented in Figs. 5 and 6. Expression of all genes related to biofilm formation in Gram-positive bacteria, such as *S. aureus* and *E. faecalis* strains, was significantly modulated by POS_LK1 and POS_EFM2 treatments, in comparison with the non-treatment ($P < 0.05$; Fig. 5). Among the Gram-negative bacteria, *wspA* and *pelA* expression in *P. aeruginosa* strains was significantly downregulated by POS_LK1 treatment (0.18 ± 0.09 and 0.24 ± 0.18, respectively), compared with their expression in the POS_EFM2 treatment (0.63 ± 0.03 and 0.85 ± 0.13, respectively) ($P < 0.05$; Fig. 6B and C). In the *E. coli* strains, *csgA*, *lsrA*, and *wcaF* expression was significantly downregulated by the POS_LK1 and POS_EFM2 treatments, in comparison with the non-treatment ($P < 0.05$; Fig. 6D–F).

4. Discussion

Various bacteria, viruses, mycoplasma, yeasts, and algae have been identified as causing bovine mastitis, with bacterial infections accounting for 95% of the cases (Zigo et al., 2021). Therefore, controlling bovine mastitis pathogens could reduce the incidence of bovine mastitis and facilitate the supply of safe dairy products for humans (Oliver,

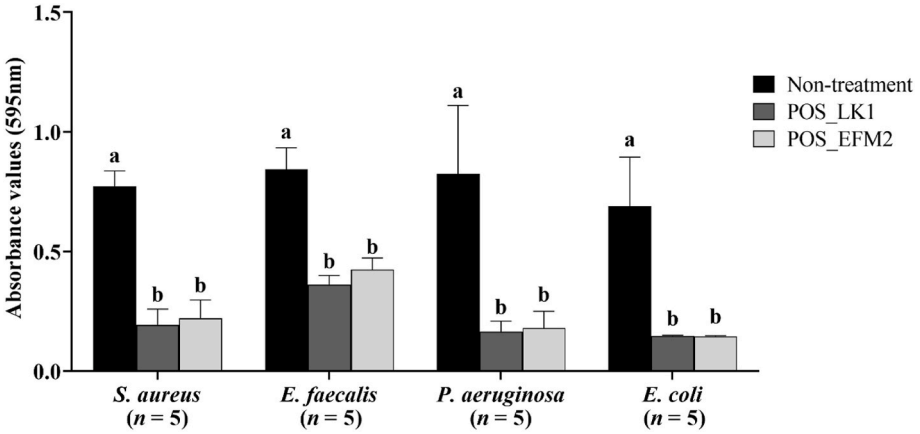

Fig. 3. Anti-microbial activity of postbiotics at 25% concentration against bovine mastitis pathogens.
All data are expressed as mean ± standard deviation ($n = 5$). Letters above the bars indicate statistical significance within the same pathogen ($P < 0.05$, ANOVA, Tukey's test). POS_LK1: *Lentilactobacillus kefiri* LK1 postbiotics; POS_EFM2: *Enterococcus faecium* EFM2 postbiotics.

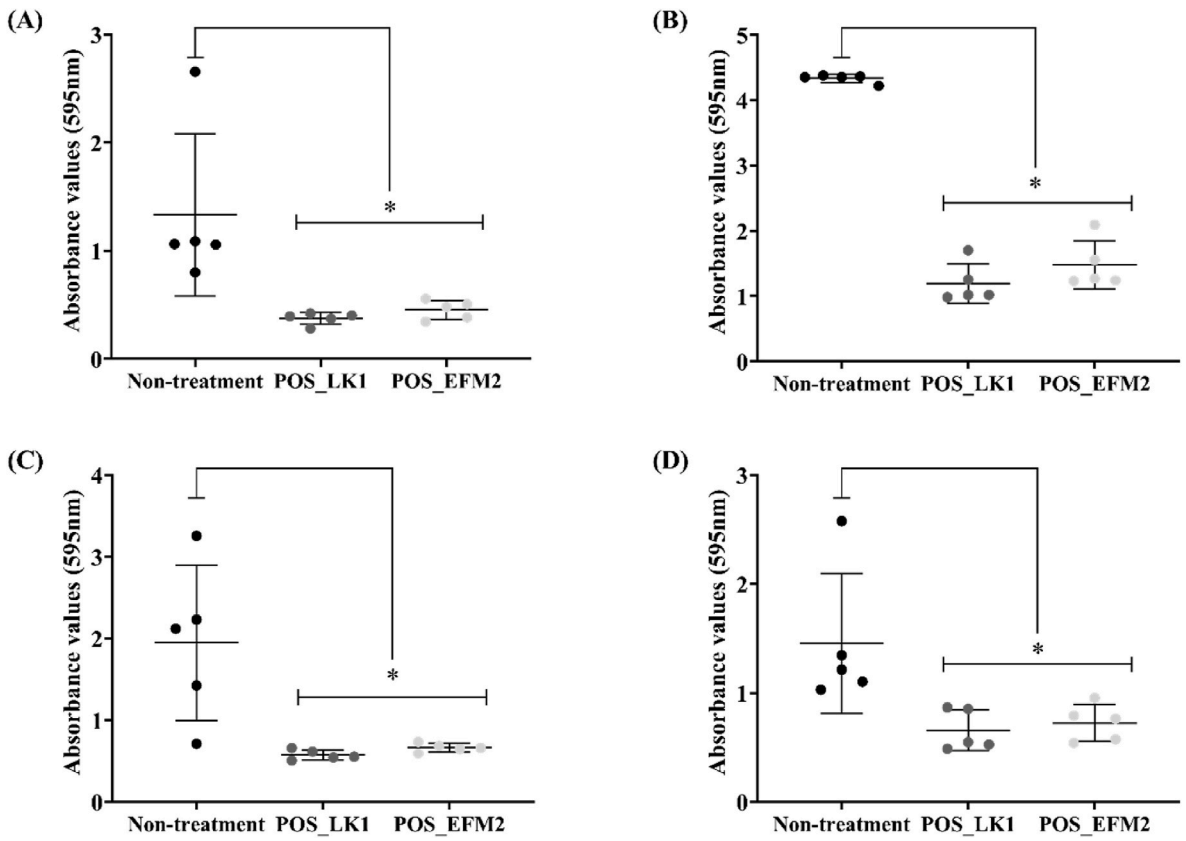

Fig. 4. Anti-biofilm activity of postbiotics at 25% concentration against (A) *Staphylococcus aureus* ($n = 5$), (B) *Enterococcus faecalis* ($n = 5$), (C) *Pseudomonas aeruginosa* ($n = 5$), and (D) *Escherichia coli* ($n = 5$).
All data are expressed as mean ± standard deviation ($n = 5$). * indicate statistical significance ($P < 0.05$, ANOVA, Tukey's test). POS_LK1: *Lentilactobacillus kefiri* LK1 postbiotics; POS_EFM2: *Enterococcus faecium* EFM2 postbiotics.

Table 3
Hydrophobicity, auto-aggregation, and exopolysaccharide (EPS) production by bovine mastitis pathogens after treatment with postbiotics at 25% concentration.

Anti-biofilm mechanisms (%)		*S. aureus* ($n = 5$)	*E. faecalis* ($n = 5$)	*P. aeruginosa* ($n = 5$)	*E. coli* ($n = 5$)
Hydrophobicity	Non-treatment	59.04 ± 2.91^{a}	63.21 ± 1.34^{a}	51.61 ± 8.87^{a}	58.35 ± 11.81^{a}
	POS_LK1	13.76 ± 10.28^{b}	24.20 ± 5.08^{b}	24.60 ± 3.68^{b}	22.82 ± 2.94^{b}
	POS_EFM2	13.88 ± 6.36^{b}	26.05 ± 8.16^{b}	24.27 ± 3.17^{b}	25.31 ± 5.05^{b}
Auto-aggregation	Non-treatment	81.59 ± 4.05^{a}	85.32 ± 0.63^{a}	65.03 ± 9.96^{a}	76.85 ± 6.82^{a}
	POS_LK1	12.58 ± 4.50^{b}	8.65 ± 3.11^{c}	16.82 ± 9.35^{b}	16.23 ± 9.64^{b}
	POS_EFM2	21.27 ± 7.34^{b}	60.99 ± 5.08^{b}	16.96 ± 8.67^{b}	14.33 ± 9.73^{b}
EPS production	Non-treatment	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
	POS_LK1	34.63 ± 18.18^{b}	43.00 ± 5.98^{c}	40.67 ± 10.70^{b}	36.17 ± 18.47^{b}
	POS_EFM2	54.67 ± 13.28^{b}	69.94 ± 2.28^{b}	42.23 ± 8.32^{b}	36.59 ± 15.22^{b}

All data are expressed as mean ± standard deviation ($n = 5$).
Different letters indicate statistical significance ($P < 0.05$, ANOVA, Tukey's test).
EPS: Exopolysaccharide; POS_LK1: *Lentilactobacillus kefiri* LK1 postbiotics; POS_EFM2: *Enterococcus faecium* EFM2 postbiotics.

Jayarao, & Almeida, 2005). The present study revealed the anti-microbial and anti-biofilm activities of postbiotics derived from *L. kefiri* LK1 and *E. faecium* EFM2 against bovine mastitis pathogens by controlling all stages of biofilm formation. Furthermore, our results showed that POS_LK1, containing high lactic acid concentrations, notably reduced the auto-aggregation and EPS production of *E. faecalis* and *P. aeruginosa* by metabolizing different carbohydrates than those metabolized by POS_EFM2.

Since postbiotics can contain anti-microbial substances owing to metabolic processes of LAB, understanding the metabolic characteristics that define the anti-microbial and anti-biofilm mechanisms of POS_LK1 and POS_EFM2 is crucial (Sevin et al., 2021). Biochemical activity assessment indicated that *L. kefiri* LK1 and *E. faecium* EFM2 could utilize glucose, mannose, sucrose, and lactose to produce lactic acid through the glycolytic or pentose phosphoketolase pathways (Abedi & Hashemi, 2020). Notably, *L. kefiri* LK1 produced more lactic acid by breaking down a different type of carbohydrate, despite having a 30.56% positive rate in biochemical activities relative to *E. faecium* EFM2 (41.67%). *L. kefiri* LK1 that is positive for 5-bromo-4-chloro-3-indoxyl-alpha-galactoside can break down melibiose into galactose and glucose using alpha-galactosidase, ultimately producing lactic acid (Anisha, 2017). Furthermore, LAB assimilate arabinose, which *L. kefiri* LK1 can utilize, resulting in a higher concentration of lactic acid than that of *E. faecium* EFM2, which utilizes maltose (Gobbetti, Lavermicocca, Minervini, De Angelis, & Corsetti, 2000). Arabinose can induce increases in the growth rate, acidification rate, and organic acid production exhibited by LAB (Gobbetti et al., 2000). Consistent with these results, a previous study found that *Lactobacillus acidophilus* could not utilize pentoses including

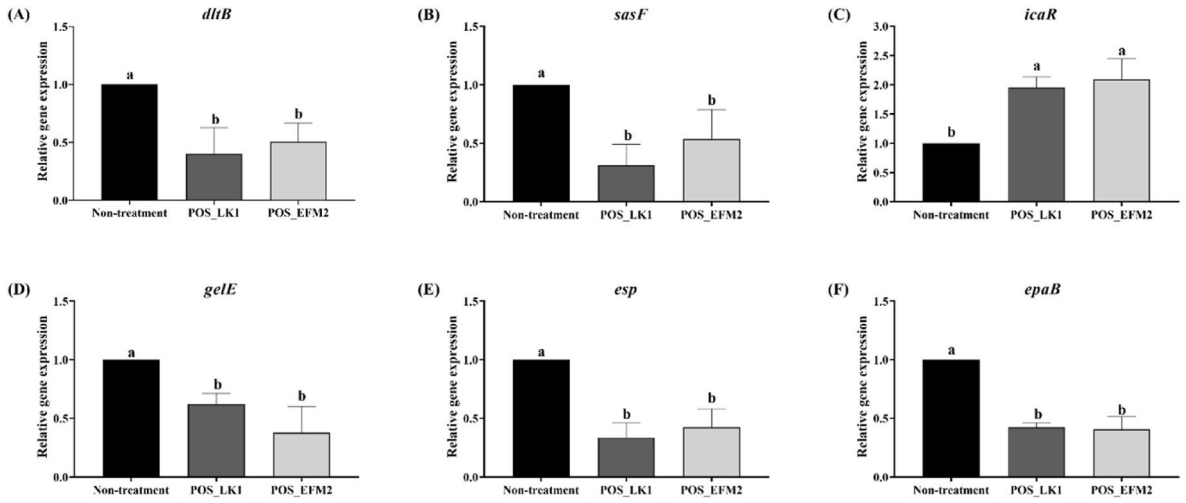

Fig. 5. Expression of genes related to hydrophobicity, auto-aggregation, and exopolysaccharide production of Gram-positive bovine mastitis pathogens following treatment with postbiotics at 25% concentration. (A–C) *Staphylococcus aureus* ($n = 5$); (D–F) *Enterococcus faecalis* ($n = 5$).
All data are expressed as mean ± standard deviation ($n = 5$). Letters above the bars indicate statistical significance ($P < 0.05$, ANOVA, Tukey's test). POS_LK1: *Lentilactobacillus kefiri* LK1 postbiotics; POS_EFM2: *Enterococcus faecium* EFM2 postbiotics.

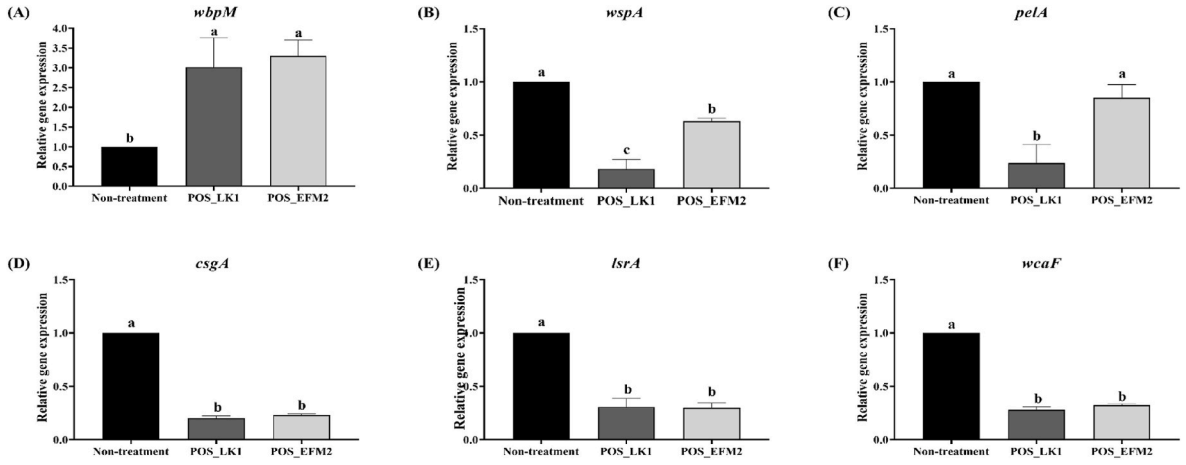

Fig. 6. Expression of genes related to hydrophobicity, auto-aggregation, and exopolysaccharide production of Gram-negative bovine mastitis pathogens following treatment with postbiotics at 25% concentration. (A–C) *Pseudomonas aeruginosa* ($n = 5$); (D–F) *Escherichia coli* ($n = 5$).
All data are expressed as mean ± standard deviation ($n = 5$). Letters above the bars indicate statistical significance ($P < 0.05$, ANOVA, Tukey's test). POS_LK1: *Lentilactobacillus kefiri* LK1 postbiotics; POS_EFM2: *Enterococcus faecium* EFM2 postbiotics.

arabinose and produced lactic acid at approximately 5.72 mM, which is lower than that produced by *L. kefiri* LK1 (Özcelik, Kuley, & Özogul, 2016).

The anti-microbial activity of postbiotics varies depending on the type of LAB, culture conditions (including media, temperature, and time), and anti-microbial substrate type and concentration (Abedi & Hashemi, 2020). In contrast to our results, a previous study reported that *L. kefiri* Kef-m15 postbiotics isolated from kefir had no anti-microbial effect against *S. aureus* and *E. coli*, which could be attributed to differences in LAB strains and culture conditions (30 °C for 72 h; Tan et al., 2022). Another study revealed that *E. faecium* TW15, TW20, and TW22 postbiotics isolated from normal raw milk inhibited *S. aureus* by secreting enterocins A and P, while having no anti-microbial effects against *E. coli* and *P. aeruginosa* (Rivas, Castro, Vallejo, Marguet, & Campos, 2012). Among various anti-microbial substances, lactic acid provides adverse environments for the growth of pathogens by lowering the pH and enhancing the attachment of proteinaceous anti-microbial molecules (Gut, Vasiljevic, Yeager, & Donkor, 2021). Furthermore, lactic acid releases protons through the cell membrane of bacteria; dissipation of the protons prevents energy production, resulting in anti-microbial and anti-biofilm activities (Gut et al., 2021). In our findings, the broad-spectrum anti-microbial and anti-biofilm activities of POS_LK1 and POS_EFM2 were induced by lactic acid. Further research is required to establish the optimal culture conditions for lactic acid production, to cost-effectively utilize postbiotics. Additionally, specific applications for their use at the farm level, without compromising the flavor of food, should be identified.

Pathogens initially adhere to substrates through high cell surface hydrophobicity, ultimately leading to udder infection and severe bovine mastitis through auto-aggregation, EPS production, biofilm formation, and host invasion (Cheng & Han, 2020; Doyle, 2000). Cell surface hydrophobicity is affected by outer membrane proteins and lipids, surface fibrils, and the teichoic acid of bacteria (Krasowska & Sigler, 2014). Lactic acid disrupts the outer membrane of pathogens, facilitating the penetration of other anti-microbial substances and altering the integrity and hydrophobicity of the cell surface (Alakomi et al., 2000; Gut et al., 2021). Considering biochemical activities, *L. kefiri* LK1, which tested positive for arbutin, effectively inhibited the auto-aggregation and EPS

production of *E. faecalis* relative to *E. faecium* EFM2. Arbutin is decomposed into glucose and hydroquinone. Hydroquinone reduces the biofilm formation of pathogens by destroying cell membranes and regulating virulence based on phenotypic and genotypic expression (Kim, Lee, Kim, Tan, & Lee, 2022; Ma et al., 2019).

Pathogens regulate biofilm formation through quorum sensing, a process that modulates gene expression in response to variations in bacterial cell population density (Dwivedi & Sehgal, 2022). In quorum sensing, bacteria produce signaling molecules known as auto-inducers, whose accumulation leads to the differential expression of virulence genes related to biofilm formation (Dwivedi & Sehgal, 2022). For *S. aureus* strains, the increased gene expression level of *dltB* enhances hydrophobicity and induces initial adhesion by reducing the negative charge of the cell envelope (Huang et al., 2014). This is achieved through the conversion of D-alanine into teichoic acid, a major component of the surface of Gram-positive bacteria (Huang et al., 2014). Matrix-producing cells within bacterial biofilms are primarily located in the core of the biofilm structure to maintain their rigidity and overall integrity (Beitelshees, Hill, Jones, & Pfeifer, 2018). These cells are responsible for producing EPS composed mainly of nucleic acids, lipids, and proteins (Beitelshees et al., 2018). Additionally, EPS contributes to maintaining biofilm by promoting intercellular communication and serving as protection against extreme environments (Beitelshees et al., 2018). In *S. aureus*, EPS production is controlled by the *icaR* (regulatory) and *icaADBC* (biosynthesis) genes, with elevated *icaR* expression inhibiting biofilm formation by downregulating *icaADBC* expression (Liu et al., 2017). Several studies have demonstrated that biofilm formation in *S. aureus* is inhibited by downregulating *sasF* gene expression and upregulating *icaR* gene expression, leading to reduced adhesion and inhibition of EPS production (Liu et al., 2017; Smith et al., 2010). Moreover, our previous study indicated that the presence of *gelE* and *esp* genes in *E. faecalis* is related to strong biofilm formation due to the consequent presence of greater hydrophobicity and adherence ability (Kim, Youn, et al., 2022; Toledo-Arana et al., 2001). A *gelE* expression is involved in gelatinase production, which enhances cell surface hydrophobicity by cleaving the substrates of hydrophobic residues (Kim, Youn, et al., 2022; Wang et al., 2011). The *ebpB* gene of *E. faecalis* is regarded as a virulence gene that promotes inflammation and biofilm formation by participating in EPS production, bacterial aggregation, and adhesion (Liu et al., 2020). POS_LK1 and POS_EFM2 influenced the regulation of major genes involved in biofilm formation, effectively modulating phenotypes associated with biofilm formation.

The upregulation of the *wbpM* gene in *P. aeruginosa* induces the synthesis of O-antigen, a major lipopolysaccharide element in Gram-negative bacteria related to the decrease in cell surface hydrophobicity (Azimi et al., 2021). Notably, POS_LK1 downregulated *wspA* and *pelA* genes more significantly than POS_EFM2, leading to the suppression of cyclic-di-GMP (Seder, Bakar, & Rayyan, 2021). The cyclic-di-GMP is a bacterial second messenger involved in biofilm formation and increased cyclic-di-GMP levels stimulate adherence, aggregation, and EPS production (Seder et al., 2021). For *E. coli* strains, the expression of *csgA* increases cell surface hydrophobicity by facilitating the formation of curli fibers, while *lsrA* encodes for the autoinducer-2 molecule used for quorum sensing (Boyer et al., 2007; Wasfi, Elkhatib, & Khairalla, 2016). Furthermore, the *wcaF* gene, the expression of which is involved in the biosynthesis of colanic acid among the EPS constituents of *E. coli*, has been used as a parameter for evaluating anti-biofilm activity (Yu et al., 2018). POS_LK1 inhibited bacterial sensing (quorum sensing and cyclic-di-GMP) that induces phenotypic and genotypic changes during biofilm maturation and can thus be utilized as a potential anti-biofilm agent. Overall, the results of the present study offer novel insights into the mechanisms by which POS_LK1 prevents bovine mastitis and may facilitate its reduced incidence and chronicity.

The limitation of the present study is the absence of methods and results for using POS_LK1 as a disinfectant and treatment for bovine udders or farm environments where pathogens form biofilms. In another study, postbiotics from *Bacillus subtilis* C and *L. acidophilus* were applied to the teat apex areas of cows by massage movements, and housing areas (including surfaces of floors, walls, feeders, drinkers, and iron partitions) were treated with these compounds using a sprayer (Zhumakayeva, Zhubatkanova, Asauova, Tokayeva, & Kemeshov, 2023). Treatment with postbiotics inhibited the pathogenic growth such as *P. aeruginosa*, *S. aureus*, and *Candida* spp., and alleviated bovine mastitis (Zhumakayeva et al., 2023). Therefore, further research is necessary to investigate the preventive or therapeutic effects of POS_LK1 treatments on the teat apex areas in bovine mastitis models, as well as in various environments, such as bedding, milking equipment, and workers' hands.

5. Conclusions

The present study demonstrated that POS_LK1 and POS_EFM2 exhibit anti-microbial and anti-biofilm activities, modulating biofilm-related phenotypic and genotypic mechanisms, such as hydrophobicity, auto-aggregation, and EPS production. Notably, POS_LK1, characterized by a high lactic acid content, demonstrated more significant suppression of auto-aggregation and EPS production in *E. faecalis* and downregulation of *wspA* and *pelA* gene expression in *P. aeruginosa*, in comparison with POS_EFM2. These findings emphasize the postbiotics produced by *L. kefiri* LK1, isolated from kefir, as a potent anti-biofilm agent against bovine mastitis pathogens. Our data contribute to sustainable livestock farming based on novel development strategies for alternative or complementary treatment in bovine mastitis management.

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CRediT authorship contribution statement

Hyeon-Jin Kim: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Hye-Young Youn:** Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft, Writing – review & editing. **Jin-San Moon:** Writing – review & editing. **Hyunsook Kim:** Writing – review & editing. **Kun-Ho Seo:** Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2023.115699.

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