



Characterisation of kefir-derived lactic acid bacteria and their extracellular vesicles

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ABSTRACT

The signalling effects of microbial biofilms and extracellular polysaccharides (EPS) determine the stability of microbial communities. As messenger molecules, extracellular vesicles (EVs) mediate the interactions of microbial populations by carrying cargo. Therefore, this study took biofilm and EPS as screening factors, preliminarily explored the basic characteristics of strains with strong biofilm formation ability and high EPS production. The results showed that the EVs concentration of three lactic acid bacteria (KLK29, KLH2 and KEF14) were high. The concentration of their EVs was proportional to the EPS production, indicating that EVs were directly involved in the formation and regulation of EPS, but it did not mean that EVs could directly affect other biological characteristics of the strains. This study offers crucial information about kefir-derived strains and their EVs, and provides a preliminary research basis for EVs-mediated expression of probiotic characteristics of strains in kefir.

The contributions of the authors are described here (The authors are sorted by article order).

1. Introduction

Originating first in the North Caucasus region of Russia, kefir is a low alcohol, acidic, sparkling milk drink prepared by fermenting kefir grains with fresh milk (Azizi et al., 2021). Kefir has a taste different from other fermented milk drinks. It is termed "Champagne Yogurt" and has a richer nutritional value. It possesses many beneficial properties such as maintenance of intestinal homeostasis, anti-inflammatory, obesity control, and immune regulation (Baars et al., 2023; Pereira et al., 2024; Tiss et al., 2020; Zhong et al., 2020). The flavor characteristics and health values are a result of the complex microbial environment naturally formed in kefir grains. During microbial interaction and self-metabolism, they produce nutrients, such as polysaccharides, that gradually accumulate into the extracellular matrix (ECM) along with other substances, which then result in the formation of a biofilm wrapping a structured microbial cell group. This cell group can rapidly adapt to the extracellular microenvironment. Kefir grains from different sources have varying microbial compositions, but they are primarily classified into three types based on the microbial types present: lactic acid bacteria (LAB), yeast, and acetic acid bacteria. Metabolites

(glucose, galactose, etc.) produced by LAB can serve as the energy source for yeast and acetic acid bacteria, and yeast produces growth factors (vitamins, amino acids, etc.) that are crucial for LAB and acetic acid bacteria in growth and reproduction. CO₂, ethanol, lactic acid, and other substances produced during this process also inhibit the production of spoilage microorganisms, and so, a natural and stable functional microbial system exists in kefir (Han et al., 2018; Rahimzadeh et al., 2015).

LAB such as *Lactobacillus helveticus*, *Lactococcus lactis*, *Leuconostoc mesenteroides*, *Lentilactobacillus kefir*, and *Lentilactobacillus sunkii* are dominant (80%) in kefir's complex microbial system (Han et al., 2018). Interestingly, related studies have explored the characteristics of LAB from different kefir sources more than those of other microorganisms. This may be because LAB are more representative in kefir, with their proportion being higher. Moreover, kefir-derived LAB provide relatively greater protection to the human body and various benefits; therefore, they have more development value (Kang et al., 2020). Of note, the probiotic characteristics of kefir-derived LAB are also attributable to the bioactive substances they produce during fermentation and metabolism in kefir. Extracellular polysaccharides (EPS) produced by kefir-derived LAB have strong probiotic functions (antibacterial, antitumor, etc. (Jenong et al., 2017; Yamane et al., 2018)) and physical properties (stability, emulsification, gelation, etc. (Li et al., 2023; Wang and Bai, 2022)), and therefore, they have a wide application prospect. Studies

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have confirmed that one of the abilities of LAB is to use EPS produced by LAB as a crucial component of extracellular polymer precursors for biofilm production (Hooshdar et al., 2020). This is especially evident in kefir's fermentation system. Therefore, exploring EPS and biofilms produced by microorganisms, specifically LAB, in the kefir system is helpful for studying the action mechanism of kefir grains. Current research on kefir-derived LAB has mostly focused on analyzing a certain characteristic of a certain strain, while studies focusing on the screening criteria and relatively comprehensive characterization of kefir-derived LAB are not thorough. This has led to the lack of a relevant theoretical basis for studying the mechanism underlying microenvironment formation in kefir.

Extracellular vesicles (EVs) are spherical nanoparticles composed of lipid bilayers of transmembrane proteins. They are released by cells and carry the bioactive components of their parents (Welsh et al., 2024). As an intercellular communication tool with diverse biological functions, EVs are usually used as a major important carrier for transporting bioactive components, so they have a great potential and application value in biopharmaceuticals, stem cell regeneration medicine, tumor diagnosis and other fields (Bell et al., 2016; Molina-Tijeras et al., 2019). In recent years, EVs have gradually been actively used in the food field as they offer new possibilities for the targeted delivery of food compounds according to their structural properties (Reiner and Somoza, 2019). The currently available sources of EVs samples are predominantly animal biological fluids (Colja et al., 2023; Hao et al., 2024), but few studies have obtained EVs from fermented food. Morales et al. (2021) expected EVs to be a vital player in the mechanism underlying microbe-targeted interaction in fermented foods.

In this study, based on the uniqueness of kefir grains formation, LAB were screened from kefir on the basis of the EPS yield and the biofilm formation ability, and their characteristics were analyzed comprehensively. Then, EVs of the screened strains were extracted and their morphology, size, and protein content were evaluated. The correlation between the strain characteristics and strain EVs was comprehensively analyzed. This study offers a preliminary basis for EVs-mediated expression of microbial probiotic characteristics in kefir and provides a theoretical support for the complex microbial interaction in kefir.

2. Materials and methods

2.1. Isolation of LAB from kefir

Kefir grains used was from pastoral families in Xilin Gol League, Inner Mongolia, China. According to Mohammed and Çon (2021), the preparation of kefir was 5% (w/v) inoculated and cultured at 24 °C for 18 h. An appropriate amount of kefir was evenly spread into De Man Rogosa Sharpe (MRS) medium and cultured at 37 °C for 24–48 h. A single colony was selected for multiple streaking culture. DNA from the strains was extracted using the TianAMP Bacteria DNA Kit (IANGEN Biotechnology, Beijing, China). The primer 27F (5'-agagttgatcttctcag-3')/1492R (5'-ggttacctgttaggactt-3'), synthesized by Sangon Biotech Co., Ltd., (Shanghai, China) was selected for PCR amplification. PCR reaction setup: 2 × Taq PCR MasterMix, 10 µL; 27F, 1 µL; 1492R, 1 µL; Template DNA, 1 µL; ddH₂O, 7 µL. The sequence of each isolate was compared with those available on the National Center for Biotechnology Information database. The isolate was identified on the basis of the highest basic local comparison search tool (BLAST) score.

2.2. Determination of EPS yield and biofilm formation ability of LAB

The bacterial solution was inoculated (inoculation rate: 3% (v/v)) in the MRS medium, and the follow-up experiment was conducted after the bacterial amount was adjusted to OD_{600nm} = 1 (Cell count: approximately 1 × 10⁹ CFU/mL).

The yield of EPS in LAB was determined using the phenol-sulfuric

acid method. The bacterial liquid was centrifuged at 3000×g for 10 min, and the supernatant was retained. Then, 4 times of absolute ethanol was added, and alcohol precipitation was conducted at 4 °C for 12 h. The solution was centrifuged at 7600×g for 10 min, and the supernatant was discarded. Then, 10 mL distilled water was added to the residue, shaken, poured into the 3500 Da dialysis bag, and stored for 12 h (Yin et al., 2013). Then, 500 µL phenol solution was added to the 500 µL sample solution, followed by the addition of 2.5 mL concentrated sulfuric acid. The mixture was allowed to stand for 10 min, and absorbance was measured at OD_{490nm}.

According to Crémet et al. (2013), the biofilm formation ability of LAB was determined through crystal violet staining. The bacterial liquid was added to a 96-well cell culture plate (200 µL/well) and cultured at 37 °C for 24 h. The upper fermentation liquid was discarded, and the cell culture plate was washed with sterile PBS to wash off the floating bacteria that formed no film. Then, 10% methanol solution was added to the plates to fix the cells for 15 min. After methanol was discarded, the cells were dyed with 0.1% crystal violet dye solution for 20 min, and the waste dye solution was washed with sterilized PBS for three times. Subsequently, 95% ethanol solution was added for 10 min to decolorize the cells. The absorbance was measured at OD_{560nm} and OD_{620nm}. The ability to form a specific biofilm was expressed as SBF (Han et al., 2018).

$$SBF = (AB - CW) / G.$$

AB—OD_{560nm} of adherent bacteria.

CW—OD_{560nm} of the medium.

G—OD_{620nm} of bacteria.

2.3. Characteristics of LAB

2.3.1. Adhesion

Auto-aggregation was determined according to Ocana and Nader-Macias (2002). The cultured bacterial solution was centrifuged at 3000×g for 15 min and the lower layer was collected. The content of the lower layer was washed twice with PBS and resuspended in the same buffer to attain absorbance values of 0.9–1.0 at 600 nm (A₀). Then, 4 mL of the suspension was removed, shaken for 10 s, and allowed to stand at room temperature. Subsequently, 0.1 mL of the supernatant was collected at 1, 3, and 5 h, transferred to a test tube containing 3.9 mL PBS buffer, vortexed, and mixed. The absorbance at 600 nm was determined (A_t). Auto-aggregation was calculated as follows:

$$\text{Auto-aggregation (\%)} = (1 - A_t/A_0) \times 100\%$$

Hydrophobicity was determined according to the methods of Zou et al. (2023) with appropriate modifications. The cultured bacterial liquid was centrifuged at 3000×g for 15 min. After the cells were washed with PBS twice, they were resuspended to attain the absorbance value of 0.9–1.0 (A₀) at 600 nm. Then, 1 mL xylene was added to 3 mL of the LAB suspension, mixed thoroughly, and left to stand for 30 min at room temperature so that the organic and aqueous phases can be completely separated. The absorbance value of the water phase was measured at 600 nm by using PBS buffer as control (A_t). Hydrophobicity was calculated as follows:

$$\text{Hydrophobicity (\%)} = [(A_0 - A_t)/A_0] \times 100\%$$

2.3.2. Acid and bile salt tolerance of LAB

The bacteria were inoculated (inoculum size: 3% (v/v)) in acidic MRS medium (pH = 3) and bile salt (MeilunBio, Shanghai, China) -containing MRS medium (0.3% (w/v) bile salts) and incubated at 37 °C for 3 h. The samples were collected and streaked after 0 and 3 h, respectively, and the LAB viable counts were measured.

2.3.3. Determination of bacteriostatic capacity of LAB

The bacteriostatic capacity of LAB was determined using the agar

diffusion method after referring to [Zhang et al. \(2024\)](#). Fermentation broth (FB) and cell-free supernatant (CFS) (3000×g, 10 min) of LAB were prepared. The plates were primed with nutrient agar (NA) and inserted into Oxford cups. Pathogenic bacteria (*Escherichia coli* NBRC3301 and *Salmonella typhimurium* NBRC12529 from the Biological Resource Center of Japan, *Staphylococcus aureus* IID1617 from the Tokyo Medical University) stored in the laboratory were activated, added to NA, poured into the plates, and then removed from the Oxford cups after complete solidification. Subsequently, 100 µL each of FB and CFS of LAB was added to the wells (H₁). The plates were incubated at 37 °C for 48 h. We used 100 µL of 1 mg/mL polymyxin B as a positive control (H₂). The diameter of inhibition for each well was measured. The relative inhibition rate (I) of the strains was calculated as follows:

$$I = H_1/H_2 \times 100\%$$

2.3.4. Safety determination of LAB

Phenotypic resistance typing was performed using the Kirby Bauer standard disc diffusion protocol to determine antibiotic susceptibility patterns ([Haryani et al., 2023](#)). Nine different antibiotic-sensitive papers (cefazolin, penicillin, ampicillin, erythromycin, gentamicin, amikacin, chloramphenicol, ciprofloxacin, and norfloxacin) were selected for testing the resistance of LAB to these antibiotics. Fresh FS of LAB was first evenly spread on Müller-Hinton agar (MHA). After the surface of MHA was dry, the antibiotic-sensitive papers were placed, and the plates were incubated at 37 °C for 24–48 h. The diameter of inhibition around the antibiotic-sensitive papers was observed, and the sensitivity was determined according to the CLSI standards ([Clinical and Laboratory Standards Institute. M100S, 2016](#)).

The hemolytic experiments were conducted according to [Argyri et al. \(2013\)](#). The activated LAB were spot-coated on Columbia Blood Agar Base and cultured at 37 °C for 24 h to observe whether hemolysis occurred. *S. aureus* IID1617 was used as a positive control strain.

2.4. Characterization of EVs from LAB

2.4.1. Extraction of EVs

The extraction steps are shown in [Fig. 6a](#). The FB of LAB was centrifuged at 4 °C, 10,000×g for 30 min to remove cell debris, and the supernatant was collected. Large floating impurities were filtered using a 0.45 µm filter membrane. The concentrate was concentrated 25-fold through ultrafiltration by using a 100 KD ultrafiltration tube and transferred to a centrifuge tube. A small amount of PBS was blown into the inner tube of the ultrafiltration tube to collect missed materials. Crude extracts of EVs were obtained through filtration by using the 0.22 µm filter membrane. Finally, purified EVs were obtained through ultra-high speed centrifugation (4 °C, 120,000×g, 90 min) (Hitachi, Japan), and the EVs precipitate was resuspended, purified with 100 µL pre-cooled PBS, and stored at −80 °C ([Chio et al., 2019](#); [Kang et al., 2020](#); [Seo et al., 2018](#)).

2.4.2. Structural characterization of EVs

The morphology of the EVs was observed through transmission electron microscopy (TEM). The EVs (10 µL) were placed on a copper grid for 5–10 min, and excess liquid was absorbed using a filter paper. The samples were blotted and air-dried, then stained with 2% uranyl acetate (10 µL) and observed using the HT7700 transmission electron microscope (Hitachi, Japan) ([Chen et al., 2018](#)).

The number and size of EVs were measured through nanoparticle tracking analysis (NTA) by using a ZetaView_Particle Metrix (Particle Metrix, PMX-120, Germany). Before use, the instrument was calibrated using 100 nm polystyrene beads (Thermo Fisher Scientific, Fremont, CA, USA). Nanoparticle concentrations (grains/mL) were determined using NTA software. Measurements were performed using the batch method

included in the NTA software ([Hwang et al., 2023](#)).

2.4.3. Protein content of EVs

The Modified BCA Protein Assay Kit (Sangon Biological Engineering Technology & Services Co, Shanghai, China) was employed to determine the total protein concentration of EVs in LAB.

2.5. Statistical analysis

All experimental data were expressed as the mean value ± standard deviation. The experiment included three groups of replicates. Student's-test was used for conducting statistical analysis. The data were statistically analyzed using SPSS 26.0. MEGA was used to draw the phylogenetic tree, and GraphPad Prism 9.5.1 was used to draw the histogram.

3. Results

3.1. Identification of LAB in kefir

In this study, using BLAST, the sequence information of strains isolated from kefir were compared for homology and a phylogenetic tree was constructed ([Fig. 1b](#)). In total four LAB species were isolated, namely *Lactobacillus delbrueckii*, *L. kefir*, *L. helveticus*, and *Enterococcus faecium* ([Supplementary Table 1](#)). Of these, *L. helveticus* were the most abundant, followed by *E. faecium* and *L. kefir*. On exploring the microbial diversity of kefir, [Li and Bai \(2024\)](#) found that *L. kefir*, *Lactobacillus kefiranoferiens* and *L. lactis* were the main kefir dominant strains in some countries and regions, and kefir also contained *L. plantarum*, *L. helveticus* and *E. faecalis*. Therefore, different kefir grains sources and diverse preparation processes lead to certain variations in the LAB species of kefir.

3.2. Yield of EPS and biofilm formation ability of LAB

Because of their high EPS production and high biofilm formation capacity, some LAB are protected from the external environment. These properties also confer diverse probiotic and pro-technological characteristics to LAB. This type of LAB participate in kefir fermentation, and their EPS and biofilm play a major role in the stable symbiotic environment of microorganisms ([Caggianiello et al., 2016](#)). They also improve the quality, flavor, and nutritional characteristics of fermented milk ([Li et al., 2023](#)). Therefore, we explored the biofilm formation and EPS production of kefir-derived LAB.

SBF >1.10 indicates a strong biofilm formation ability. SBF between 0.70 and 1.09 indicates a moderate ability, that between 0.35 and 0.69 indicates a weak ability; and that <0.35 indicates no biofilm formation ability ([Han et al., 2018](#)). In the present study, the 20 strains isolated were screened to obtain high-quality LAB. LAB with SBF of <0.69 were excluded, and 15 strains were finally obtained. These LAB were of three types: *L. helveticus*, *L. kefir*, and *E. faecium*. As shown in [Table 1](#), EPS production is higher in the 15 LAB strains (32.72–269.60 µg/mL). Among them, three *L. kefir* strains (KLK29, KLK57, and KLK43) were largely different in EPS production and biofilm formation ability. Among the more abundant *L. helveticus* strains, 63% exhibited high EPS production and strong biofilm formation capacity (KLH2, KLH22, KLH11, KLH56 and KLH8). The *E. faecium* strains were consistent in their biofilm-forming ability, but their EPS-producing ability varied (e. g., KEF14 and KEF9). The biofilm-forming ability was correlated with the LAB species, whereas the EPS-producing ability may largely stem from the inherent characteristics of LAB and is irregular. In this study, the best performing strains (KLK29, KLH2, and KEF14) were selected from the three LAB classes based on the EPS-producing and biofilm-forming abilities for subsequent experiments.

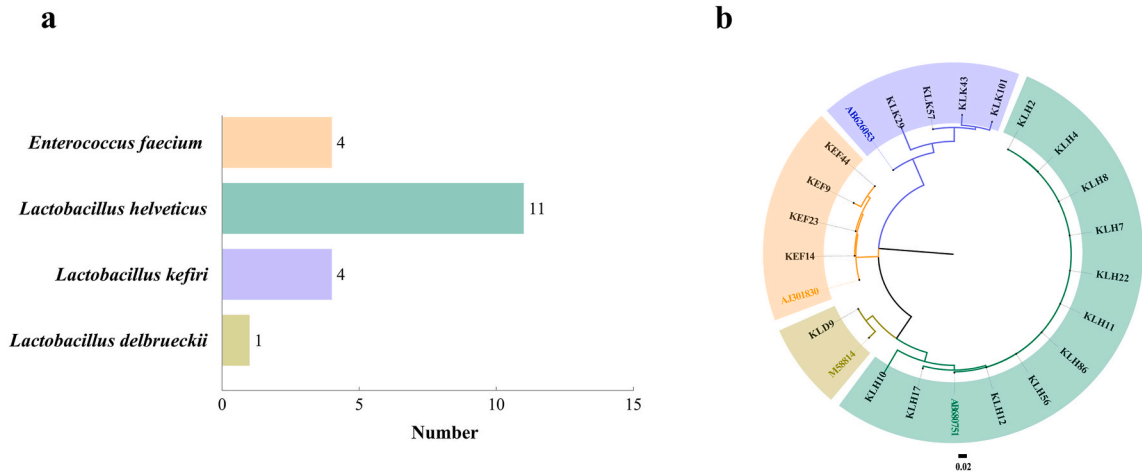


Fig. 1. Identification of LAB in kefir.

Table 1
EPS yield and biofilm formation ability of LAB.

No.	Strain number	EPS yield/ $\mu\text{g/mL}$	SBF
1	KLK29	269.60 ± 3.95	S
2	KLH2	210.73 ± 22.85	S
3	KLH22	206.76 ± 20.89	S
4	KLH11	198.11 ± 24.32	S
5	KEF14	191.44 ± 29.25	S
6	KLH56	150.94 ± 14.43	S
7	KEF44	139.74 ± 17.62	S
8	KEF23	134.70 ± 30.92	S
9	KLH8	108.46 ± 22.14	S
10	KLH7	91.16 ± 27.07	M
11	KLH10	70.73 ± 27.20	M
12	KLH86	68.18 ± 5.56	M
13	KEF9	54.84 ± 17.87	S
14	KLK57	42.22 ± 24.09	S
15	KLK43	37.72 ± 10.77	M

Note: S in SBF denotes strong, M denotes moderate. Strain number KLK denotes *L. kefir*, KLH denotes *L. helveticus*, and KEF denotes *E. faecium*.

3.3. Analysis of LAB characteristics

3.3.1. Adhesion of LAB

Auto-aggregation is a phenomenon wherein a bacterium rapidly multiplies in large numbers, leading to the formations of aggregates. Auto-aggregation of a strain is positively correlated with its effective colonization and adhesion in the gastrointestinal tract (Hao et al., 2020). As shown in Fig. 2a, all three strains exhibited strong auto-aggregation

in the following order: KEF14 > KLH2 > KLK29. The auto-aggregation ability can reach 78.92–84.38% in 1 h culture. At the same time, as the culture time increased, the auto-aggregation of the three strains exhibited a slow increase trend. Hydrophobicity revealed that cell surface substances prefer non-aqueous environments, which initially attach to intestinal epithelial cells because of their hydrophobicities (Collado et al., 2007). The hydrophobicities of KLK29, KLH2, and KEF14 to xylene were 46.93%, 64.22%, and 64.33%, respectively (Fig. 2b).

Microbial auto-aggregation and hydrophobicity are critical indicators of microbial adhesion capacity. Therefore, based on these two indicators, KEF14 can be preliminarily judged to possess the highest adhesion capacity, followed by KLH2 and KLK29. All these three strains were high adhesion strains. Interestingly, LAB adhesion in this study was not proportional to EPS production, possibly because of the structural diversity of EPS in different strains, where the branching or ordering of EPS structures influences their molecular interactions, and thus the adhesion ability of the strains (Li et al., 2023). In addition, adhesion mechanisms such as non-specific physical contact between cell surfaces and contact between specific adhesins and receptors are extremely complex. Adhesins are mainly some proteins, polysaccharides, lipoteichoic acid and other diversified molecules on the surface of LAB, which may directly or indirectly participate in the adhesion process of LAB. Of note, EPS production by the bacterium is not only for bacterial adhesion but also in response to a change in an environmental factor or the expression of a function. However, when the biosynthesis of peptidoglycan is unbalanced or hydrophobic molecules are inserted into the outer membrane to cause cell membrane disorder, the outer membrane bubbles and vesicles are produced. EVs has the ability to diffuse, cross

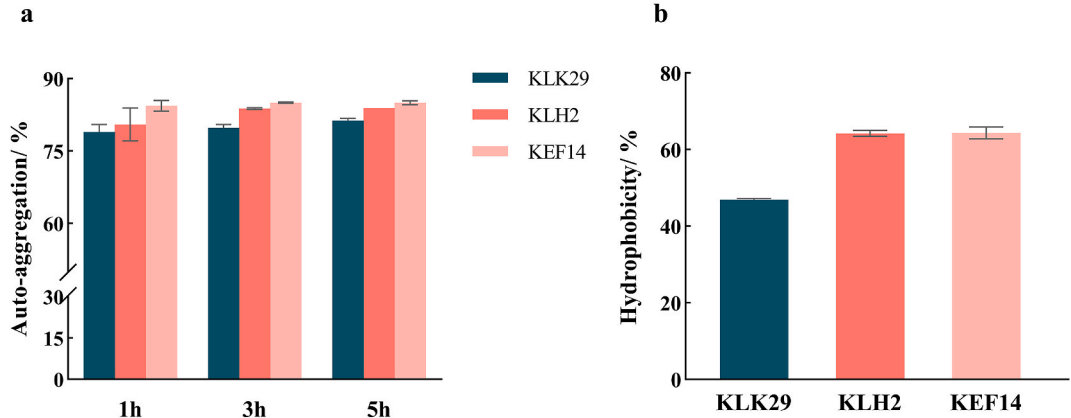


Fig. 2. Adhesion ability of LAB.

the mucus layer, migrate directly to other tissues and interact with host cells (Ruiz et al., 2023). Therefore, EVs-mediated probiotics of LAB have become the focus of attention. Based on the probiotic properties of the strains, EVs deliver signaling molecules, thereby modulating the production of an appropriate response. A complex and diverse relationship exists between EPS production in LAB and the adhesion ability of the strain. This adhesion ability cannot be directly judged based on the ability of LAB to produce EPS. Thus, exploring the probiotic properties of the strain and its EVs is particularly crucial.

3.3.2. Acid resistance and bile salt resistance of LAB

The pH of gastric acid in human gastric juice is usually 3.0, and bile salts in the small intestine range from about 0.3%. The passage of food through the stomach and small intestine is usually within 4 h (Emma et al., 2022). In this study, the gastric acidic environment of pH 3 and the small intestine environment containing 0.3% bile salts were used to treat the strains for 0 and 3 h, respectively, so as to simulate the colonization of the strains in human gastrointestinal fluids.

The results are presented in Fig. 3. Compared with 0 h, the viable counts of all strains under the acidic environment at 3 h exhibited different degrees of decreasing trend. The viable counts of KLK29 (7.81–7.55 lg CFU/mL) significantly decreased ($p < 0.01$). In the presence of 0.3% bile salts, a greater difference was noted in the strain viable counts. Compared with 0 h, the viable counts of KLK29 (0–3 h: 8.09–7.41 lg CFU/mL) exhibited a highly significant decreasing trend ($p < 0.001$). By contrast, the viable counts of KEF14 (0–3 h: 7.39–7.55 lg CFU/mL) displayed a significant increasing trend ($p < 0.01$). This may be attributable to the ability of the strain to induce adaptive tolerance in extreme environments and stimulate stress responses to protect its growth and proliferation. By contrast, KLH2 displayed an extremely poor growth capacity (2.52 lg CFU/mL) at 0 h and a complete inability to adapt to the bile salt environment at 3 h, resulting in cessation of growth. In summary, among the three strains, only 1 strain (KLH2) could not exert its effects after entering the human intestine. The other two strains (KLK29 and KEF14) were able to tolerate the gastrointestinal environment and might exhibit their functional properties in the human intestinal tract.

3.3.3. Bacteriostatic capacity of LAB

The bacteriostatic activity of LAB is a crucial player preventing the infection and invasion of pathogenic bacteria. To investigate the bacteriostatic activity of LAB and the metabolic substances or extracellular substances they produce, the FB and CFS of LAB were used in the bacteriostatic assay against *E. coli* NBRC3301, *S. aureus* IID1617, and *S. typhimurium* NBRC12529. As shown in Table 2, KLH2 exhibited the strongest bacteriostatic ability (73.07–83.02%) against the three pathogens. KLK29 (48.44–51.53%) and KEF14 (48.18–52.77%) exhibited a certain degree of bacteriostatic effect against the three pathogens. KLH2 exhibited a strong bacteriostatic ability possibly because more

antibacterial metabolites such as bacteriostatic peptides and bacteriocins were accumulated during its own reproduction, which inhibited the growth and reproduction of pathogenic bacteria. However, specific reasons need to be further verified and analyzed. Additionally, F-KLK29 and F-KEF14 exhibited the highest inhibitory activity against *Escherichia coli* NBRC3301, with inhibition rates of 51.23% and 52.66%, respectively. F-KLH2 exhibited the highest inhibitory activity against *S. typhimurium* NBRC12529, with a bacteriostatic rate of 79.33%. The significance analysis of FB/CFS of the same LAB showed that there were significant differences in the antibacterial properties of F-KLK29 and F-KLH2 against pathogens in FB ($p < 0.05$), and significant differences in the antibacterial properties of C-KLH2 against pathogens in CFS ($p < 0.05$). This result reflects the potential broad-spectrum nature of KLH2 against pathogens. There were significant differences ($p < 0.05$) in the bacteriostatic ability of FB and CFS of each LAB under the same pathogen, except for the non-significant inhibitory ability of F-KEF14 and F-KLK29 against *S. aureus* IID1617. This phenomenon suggests that physiological characteristics or metabolites of strains can cause differences in their bacteriostatic mechanisms against the same pathogenic bacteria.

According to the difference analysis of the bacteriostatic ability of FB and CFS (Fig. 4), the bacteriostatic ability of FB and CFS of KLK29 was significantly different ($p < 0.05$). The FB and CFS of KLH2 differed significantly in their bacteriostatic ability against the three pathogens ($p < 0.001$). The FB and CFS of KEF14 only differed significantly in their bacteriostatic ability against *S. typhimurium* NBRC12529 ($p < 0.05$). Thus, the content in the CFS of KLK29 and KLH2 exhibited strong bacteriostatic ability against common food-borne pathogens. By contrast, the bacteriostatic ability of the CFS of KEF14 only manifested against specific pathogens. Of note, the bacteriostatic rate of CFS of the three LAB strains was higher than that of FB. CFS contains metabolites produced by LAB or their secreted extracellular substances (organic acids, fatty acids, and EVs wrapped with nutrients such as polysaccharides and proteins) (Mohammed and Çon, 2021). These inclusions may be a crucial part of the antagonistic effect of the strain on pathogens.

3.3.4. Safety evaluation of LAB

To evaluate the safety of the three LAB strains, their antibiotic resistance and hemolysis abilities were assessed. Table 3 presents the results of the susceptibility of the three strains to nine antibiotics. All the three strains were resistant to ciprofloxacin and norfloxacin of quinolones. *E. faecium* was earlier considered a symbiotic bacterium harmless to humans in the early stage, but with its long-term evolution, virulence genes appeared, which were believed to be related to human infection. The drug resistance of KEF14 was significantly higher than that of other strains in this study. Similar results were reported by Mohammed and Çon (2021). KLK29 was significantly sensitive to five antibiotic classes, cephalosporins (CZO), β -lactams (PEN, AMP), macrolides (E), aminoglycosides (G, AMI), and phenicols (CHL). KLH2 was sensitive to five antibiotic types: cephalosporins (CZO), β -lactams (PEN, AMP), macrolides (E), aminoglycosides (AMI), and phenicols (CHL). In fact, antibiotic resistance itself is not a safety concern, and when a risk of drug resistance transfer exists, it may be dangerous to human health. Therefore, if the beneficial nature of KEF14 must be confirmed, whether it carries any drug resistance determinants that may be transferred must be further verified. Drago et al. (2016) proposed that LAB exhibit variable resistance to ciprofloxacin. Based on these results, we speculated that KLK29 and KLH2, although resistant to ciprofloxacin and norfloxacin, may not confer resistance genes to fluoroquinolones. The reason for the difference in the susceptibility of the two LAB strains to aminoglycosides may stem from the differences in their biological properties or may be related to the differences in the action mechanism of the antibiotics. Overall, KLK29 and KLH2 were susceptible to various antibiotics, whereas KEF14 exhibited resistance. Therefore, KEF14 must still be selected with caution, and its safety can be confirmed through in-depth validation of

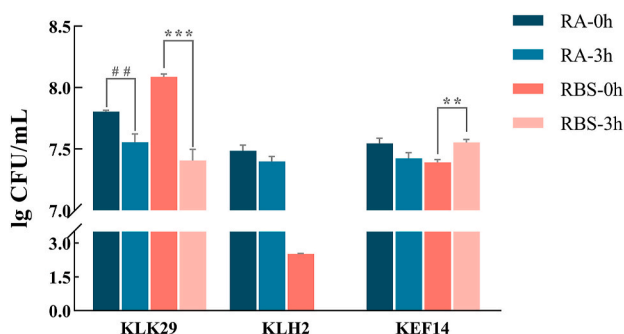


Fig. 3. Tolerance of LAB to acid and bile salts. RA indicates acid resistance, RBS indicates bile salt resistance. # indicates significance in an acidic environment, * indicates significance in a bile salt environment.

Table 2
Bacteriostatic capacity of LAB.

Strains	<i>E. coli</i> NBRC3301		<i>S. aureus</i> IID1617		<i>S.typhimurium</i> NBRC12529	
	BD/mm	RBR/%	BD/mm	RBR/%	BD/mm	RBR/%
F-KLK29	11.63 ± 0.01	51.23 ^{Ca}	11.43 ± 0.09	48.44 ^{Bc}	11.47 ± 0.04	49.09 ^{Bb}
F-KLH2	17.59 ± 0.11	77.46 ^{Ab}	17.25 ± 0.03	73.07 ^{Ac}	18.54 ± 0.09	79.33 ^{Aa}
F-KEF14	11.96 ± 0.02	52.66 ^{Ba}	11.46 ± 0.15	48.57 ^{Bb}	11.26 ± 0.04	48.18 ^{Cb}
C-KLK29	11.68 ± 0.11	51.53 ^{Ca}	12.11 ± 0.03	51.31 ^{Bb}	11.89 ± 0.06	50.86 ^{Bc}
C-SKLH2	19.03 ± 0.04	83.82 ^{Aa}	18.92 ± 0.10	80.16 ^{Aa}	19.15 ± 0.03	81.93 ^{Ab}
C-SKEF14	11.99 ± 0.06	52.77 ^{Ba}	11.74 ± 0.08	49.74 ^{Cb}	11.51 ± 0.04	49.27 ^{Cb}

Note: BD indicates the bacteriostatic diameter, RBR denotes the relative bacteriostatic rate. Upper case letters represent the significance of different LAB (FB/CSF) under the same pathogen; lower case letters represent the significance of different pathogens under the same lactic acid bacteria (FB/CSF). FB/CSF represents significant analysis of LAB fermentation broth and LAB supernatant respectively.

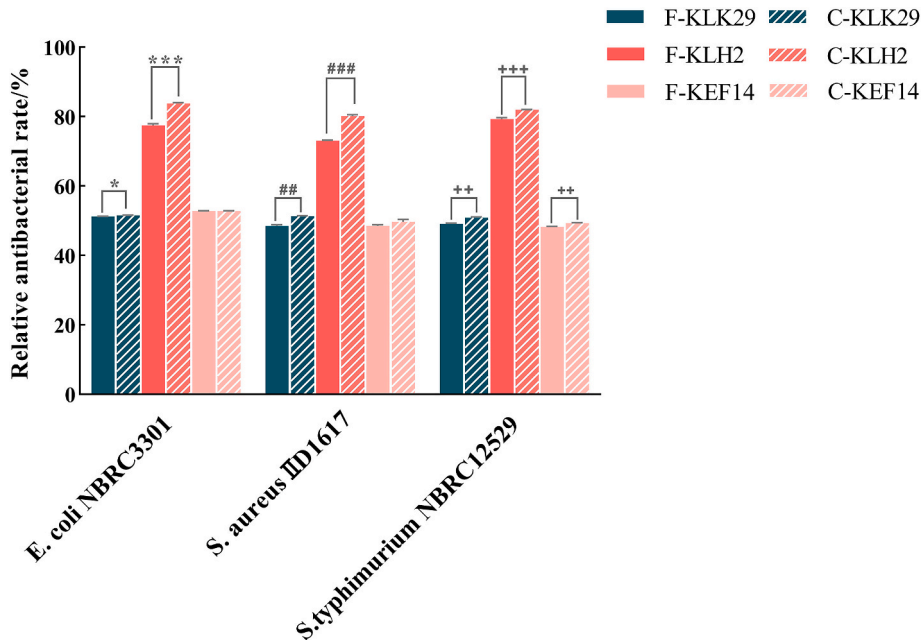


Fig. 4. Bacteriostatic capacity of LAB. F indicates FB, C indicates CFS. *, #, and + indicate the significant inhibitory effects of FB and CFS on *E. coli* NBRC3301, *S. aureus* IID1617, and *S. typhimurium* 12529, respectively. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Table 3
Antibiotic resistance of LAB.

Strains	Antibiotic Susceptibility									
	Cephalosporins		β -lactams		Macrolides	Aminoglycosides		Phenicol	Quinolones	
	CZO		PEN	AMP	E	G	AMI	CHL	CIP	NFX
KLK29	S		S	S	S	S	S	S	R	R
KLH2	S		S	S	S	S	R	S	R	R
KEF14	R		R	R	I	I	R	I	R	R

Note: S, sensitive; I, intermediate; R, resistant. CZO, Cefazolin (30 μ g); PEN, penicillin (10 μ g); AMP, ampicillin (10 μ g); E, erythromycin (15 μ g); G, gentamicin (10 μ g); AMI, amikacin (30 μ g); CHL, chloramphenicol (30 μ g); CIP, ciprofloxacin (5 μ g); NFX, norfloxacin (10 μ g).

its resistance determinants.

Hemolytic bacteria are severely harmful to humans, and so, hemolysis is a vital link in the in vitro evaluation of LAB safety. In this study, *S. aureus* IID1617 was used as a control, and these bacteria displayed β hemolysis. A 2–4 mm well-defined and completely transparent hemolysis ring was observed around the strains. None of the three strains exhibited hemolysis (Fig. 5).

3.4. Analysis of characteristics of EVs in LAB

Referring to the recommendations of the International Society for

Extracellular Vesicles (Welsh et al., 2024), the characteristics of EVs of the three strains were analyzed for morphology, size, and protein content. The purified EVs were observed through TEM (Fig. 6b). The EVs of all three LAB strains had a complete structure (inclusions wrapped by a lipid bilayer). This suggests that our method of extracting EVs has a reference value. Among them, the EVs of KLK29 had semi-concave spherical structures, whereas those of KLH2 and KEF14 had typical circular shapes. Fig. 6c illustrates the particle size/concentration distribution of EVs after dilution, in which the EVs concentration in KLK29 was 6.8×10^{11} particles/mL, with an average particle size of 173 nm and a peak at 158 nm. The EVs concentration in KLH2 was 6.6×10^{11}

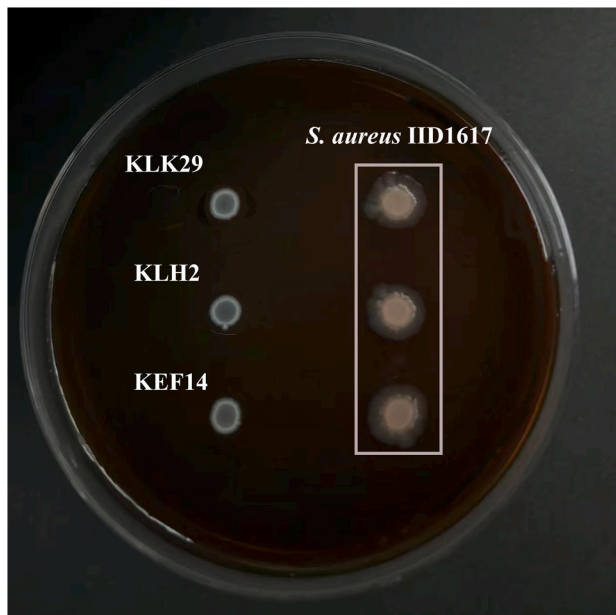


Fig. 5. Hemolysis of LAB.

particles/mL, with an average particle size of 149 nm and a peak at 143 nm. The EVs concentration in KEF14 was 1.1×10^{10} particles/mL, with an average particle size of 171 nm and a peak at 163 nm. The EVs concentration in the three strains was high, and their sizes were consistent with the criteria for the EVs range (Welsh et al., 2024). Fig. 6d presents the protein content of the EVs in the three strains. The EVs protein content in KLK29, KLH2, and KEF14 was 21.71, 18.54, and 14.81 $\mu\text{g}/\mu\text{L}$, respectively.

Seo et al. (2018) reported that the concentration of EVs derived from *L. kefir* KCTC 3611 in kefir was 7.73×10^7 particles/mL. The concentration of EVs extracted from *Lactobacillus crispatus* BCRC 14618 was 10^{10} particles/mL (Wang et al., 2022) and the concentration of EVs extracted from *L. druckerii* was 10^7 particles/mL (Han et al., 2023). Liang et al. (2019) reported the EVs protein content of 7.3 $\mu\text{g}/\text{mL}$ in *S. typhimurium* CCVCC2226 and Han et al. (2021) noted the EVs protein content of 5.1 $\mu\text{g}/\text{mL}$ in *Bacillus subtilis*. Therefore, on comparison, we noted that the concentration and protein content of EVs from the three strains were at a high level. Meanwhile, the surface morphology, EVs concentration, and EVs protein content in the three strains were different. Of them, the EVs concentration and EVs protein content of rod-shaped bacteria (KLK29, KLH2) were higher than that of the cocci (KEF14). A comparative analysis of EVs of different LAB types is currently lacking. The phenomenon noted in this study may depend on the cell morphology of LAB. More sites may be present on the surface of rod-shaped bacteria to release EVs, whereas the surface area of cocci is relatively small, which results in the release of less EVs. At the same time, different strains have distinct metabolic activities, which may also affect the release of EVs. Notably, the concentration of EVs and the protein content of EVs in the three strains were positively correlated with their EPS production. This phenomenon may be a crucial indication that LAB maintain the stability of their microenvironment.

4. Discussion

KLK29, KLH2, and KEF14 all exhibited strong biofilm formation ability, and their EVs levels were high. Microbial biofilm is a crucial player in microbial survival, adaptation, and interaction. Signal transmission is among the vital functions of biofilm of LAB. This means that biofilm-related molecules participate in signal transmission and interaction among cells (Baeza et al., 2021). Therefore, the biofilm is closely related to EVs. Specifically, specific proteins in biofilm may influence

the assembly and release of EVs (Garrido et al., 2021). Quorum sensing signaling molecules secreted by EVs regulate bacterial growth and behavior according to cell community density and induce microbial growth in biofilm (Jeong et al., 2024). EVs are also crucial for biofilm formation as they transport components such as EPS, proteins, and eDNA (Garrido et al., 2021). Baeza and Mercade (2021) demonstrated that biofilm formation is dependent on EVs integrity. Being one of the biofilm components, EPS is crucial for biofilm formation and stability. EPS provides structural support for LAB biofilms, thus offering a stable platform for information transfer, and involve in different stages of biofilm formation, thereby aiding cells in adhering to surfaces, contributing to cell aggregation and biofilm maturation, and protecting cells from environmental changes. Changes in EPS synthesis-associated genes affect biofilm formation (Caggianiello et al., 2016). And the chemical structure, relative amount and charge, abiotic surface properties, and surrounding environment may influence the role of EPS in biofilm formation (Neville and O'Toole, 2010). EPS can protect cells from damage. This protection may be derived from EVs as a means to facilitate the maintenance of EVs integrity and protect bioactive components within EVs (Sun et al., 2023). In addition, elevated EPS production may lead to imbalances in peptidoglycan biosynthesis or the insertion of hydrophobic molecules into the outer membrane, resulting in cellular disruption. This disruption can trigger the formation of membrane buds and eventually the release of vesicles, which release signalling molecules to ensure communication between cells (Raghu and Valerie, 2020). EVs contain various proteins, among which adhesion proteins are extremely abundant. These adhesion proteins can bind to EPS and participate in the targeted transport of EVs. Adhesion proteins in EVs and EPS can also offer adhesion substrates to enable strains to attach to specific surfaces and form biofilm structures (Zarnowski et al., 2022). In summary, high EPS production may contribute to EVs release, thereby increasing the concentration of EVs and guaranteeing the output of EVs. The goods transported by EVs have an interaction relationship with EPS. Therefore, the coexistence of high-quality EVs and EPS can effectively promote synergies in the microbial community.

KLK29 had the highest EPS yield, EVs concentration and EVs protein content. Therefore, KLK29 may play a more crucial role in LAB survival and inter-community interactions than the other two strains. KLK29 may serve as an ideal model for exploring information transfer in microbial communities, signal exchange, and synergistic roles in kefir so as to investigate more profoundly the interactions of biofilms, EPS, and EVs in microbial community interactions. Among the different properties of KLH2, antimicrobial properties were particularly prominent. This strain also had a strong ability to produce EPS. Considering the high EVs levels, we believe that the strong antimicrobial properties of KLH2 were correlated with the proteins released from its EVs. Because the EVs of KLH2 possibly release related antimicrobial proteins, such as antimicrobial peptides, the strain can defend itself against external hazards and have an advantage in a competitive microbial population (Wagner et al., 2018). Thus, KLH2 has a strong correlation with its EVs, which may affect the stability and function of microbial system. Although KLH2 could not colonize the intestinal tract, it can still be developed for use in other fields such as biopharmaceuticals and agricultural production. Drug resistance was the most obvious characteristic of KEF14, and drug resistance in *E. faecium* is known to be a manifestation of adaptive evolution. A correlation may exist between its drug resistance and high EVs levels. On the one hand, the drug-resistant strain is exposed to stress in the environment. Therefore, it produces a stress response mechanism, that is, it produces bioactive molecules, which are secreted by EVs, in response to external stresses, and this behavior is effective in sustaining the strain's viability (Paganelli et al., 2013). On the other hand, the resistance property may be a result of the EVs-mediated delivery of resistance genes by the strain. The release of these cargo proteins may help in maintaining the strain's survival and its ability to adapt to the environment (Paganelli et al., 2013). Based on KEF14 properties, the strain may have potential biomedical applications such as infection

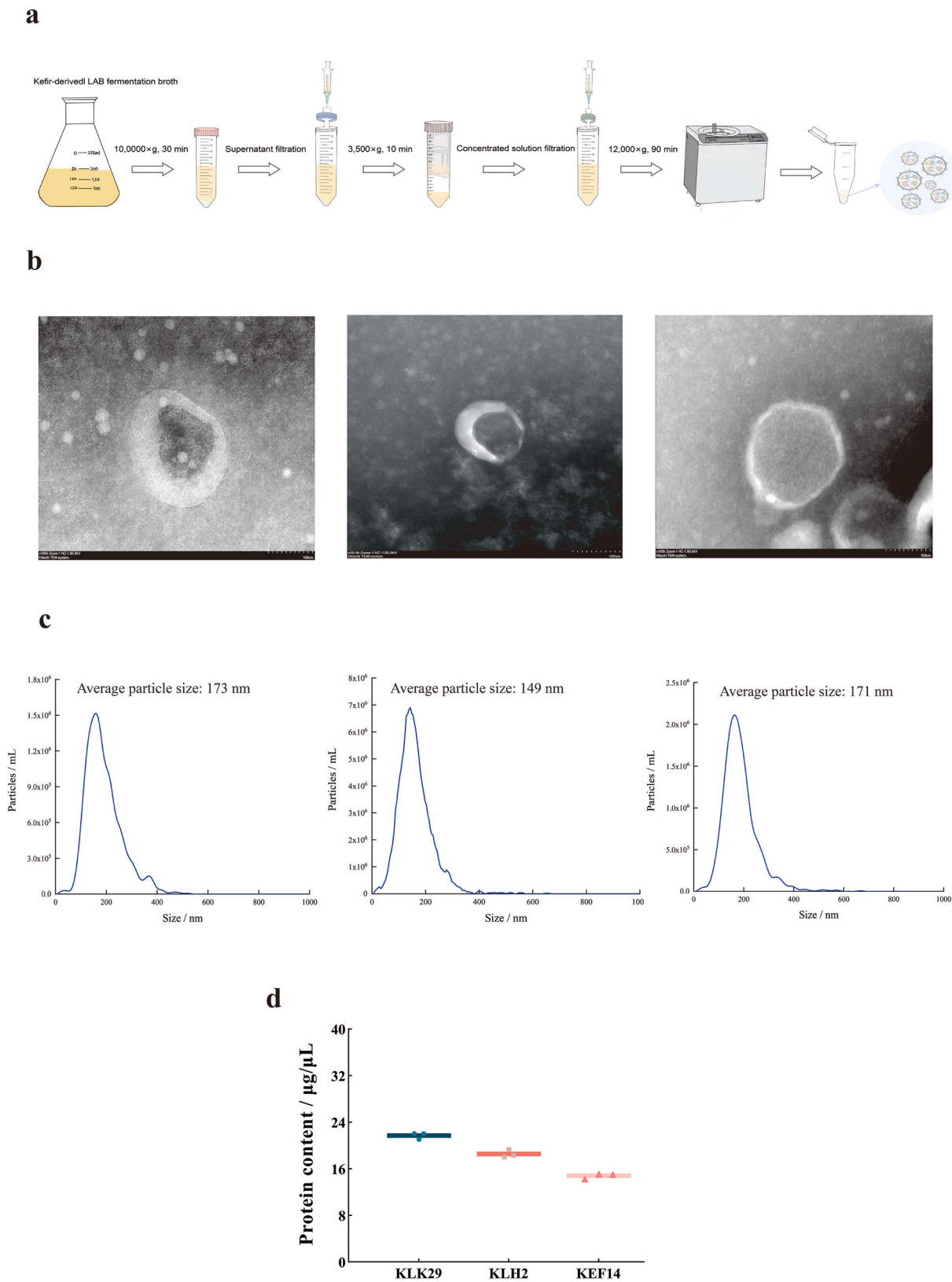


Fig. 6. Characterization of EVs of LAB. (a) The step of EVs extraction from the three strains. (b) The transmission electron micrograph of the EVs of the strains, scale bar: 100 nm. (c) The particle size/concentration distribution of the EVs of the strain. (d) represent, from left to right, the relevant characterization of EVs of KLK29, KLH2, and KEF14.

control, diagnostics, and drug delivery. Notably, drug-resistant strains still carry certain risks when used in food, and therefore, further risk assessment and safety management measures are required to ensure the strain's safety and efficacy. It is worth noting that the three strains of lactic acid bacteria, from the perspective of high EPS production characteristics, still possess good application prospects and market potential. Studies have shown that EPS plays an important role in the potential probiotics of LAB, and at the same time, EPS produced by LAB can also significantly improve the structural organization and rheological properties of fermented milk (Bai and Liu, 2019, Bai and Wang, 2022).

Overall, the concentration of EVs in the three strains was proportional to their EPS production, suggesting that high EPS production may induce the formation of EVs, and the cargo proteins of EVs may directly participate in the formation and regulation of EPS. However, the relationship between high concentrations of EVs and other biological characteristics of the strains remains unclear, which may be due to the heterogeneity of EVs, the complexity of their biosynthesis, and the diversity of their functions. To gain a more accurate understanding of the strains' EVs and the regulatory mechanisms of EVs in microbial interactions, this area of research remains a significant challenge and a hot topic, warranting further investigation.

5. Conclusion

In this study, based on the screening criteria of biofilm formation and EPS production, we screened out the strains that contributed greatly to maintaining the steady state of kefir: KLK29, KLH2 and KEF14. The basic characteristics and EVs structure of the strains were analyzed. This study indicated that all three strains of LAB had high EPS production and strong biofilm formation ability, and EVs produced were complete in structure and high in concentration. The characteristics of different strains were different, but their EPS production was positively correlated with EVs concentration. Among them, KLK29 was considered as an ideal model to explore cell signal transduction. This study offers information crucial for the development of kefir-derived LAB and their EVs. It provides more possibilities for EVs-mediated expression of probiotic characteristics of LAB as well as theoretical support for the complex microbial interaction in kefir.

CRedit authorship contribution statement

Puyu Li: Author 1 (First Author), Conceptualization, Data curation, Methodology, Visualization, Formal analysis, Writing – original draft, Writing – review & editing. **Ying Bai:** Author 2 (Corresponding Author), Conceptualization, Funding acquisition, Project administration, Writing – review & editing. **Shiwei Li:** Author 3, Methodology, Validation, Resources, Writing – review & editing. **Zhiying Zhang:** Author 4, Methodology, Validation, Visualization, Writing – review & editing.

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Declaration of competing interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.crfs.2024.100925>.

Data availability

I have shared the link to my data/code at the Attach File step

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