DOI: 10.26599/FSHW.2026.9251209

Contents lists available at SciOpen

Food Science and Human Wellness

journal homepage: https://www.sciopen.com/journal/2097-0765

China Food Publishing Co.

Goat milk-derived *Lactobacillus salivarius* extracellular vesicles mitigate LPS-induced intestinal inflammatory injury in mice

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ABSTRACT: Milk-derived *Lactobacillus* plays an important role in the adjuvant therapy of intestinal inflammatory injury, but the potential mechanisms of its involvement in the inflammatory regulation are still unclear. Recent studies have suggested that probiotic-derived extracellular vesicles are considered novel regulators in intestinal inflammatory injury. In the current study, we first isolated a strain of *Lactobacillus salivarius* with inflammatory regulation from raw goat milk. Subsequently, the preventive effect of *Lactobacillus salivarius*-derived extracellular vesicles on lipopolysaccharide-induced intestinal inflammation in mice was analyzed. Our results indicated that *Lactobacillus salivarius*-derived extracellular vesicles alleviated lipopolysaccharide-induced disruption of intestinal epithelial barrier function, activation of inflammatory signaling, and imbalance of gut microbiota in mice. Mechanistically, let-7 in *Lactobacillus salivarius*-derived extracellular vesicles targeted regulating MAPK8 gene expression in intestinal epithelium and mediating inflammatory signaling regulation. In summary, our results revealed the potential application value of milk-derived *Lactobacillus salivarius*-derived extracellular vesicles in intestinal inflammatory regulation.

Key words: milk-derived probiotics, *Lactobacillus salivarius*, extracellular vesicles, inflammatory regulation

1. Introduction

With rapid shifts in dietary patterns, lifestyles, and societal structures, the incidence of inflammatory conditions affecting the gut is rising globally [1]. A critical consequence of intestinal inflammatory injury is heightened intestinal permeability [2]. This compromised barrier allows for the translocation of gut-derived bacteria and their products, such as lipopolysaccharide (LPS), into the systemic circulation, triggering systemic inflammation [3]. Emerging evidence indicated that microbiota-based interventions (fecal microbiota transplantation, antibiotics treatment, and specific probiotic supplementation) can relieve intestinal inflammation and restore intestinal barrier function [4, 5]. However, an incomplete understanding of the microbial community information and their functional modules limits the development of microbial-based

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Received 14 July 2025
Received in revised from 12 October 2025
Accepted 19 March 2026

therapies [6]. Thus, exploring effective, safe, and stable microbial regulation methods is crucial for alleviating intestinal inflammation and maintaining intestinal barrier function.

Milk-derived probiotics have gained considerable attention due to their diversity, safety profile, and manufacturing feasibility [7]. Isolating and exploiting these strains is a potential mean to promote the development of intestinal inflammation treatment technology [8]. Milk-derived *Lactobacillus* play a critical role in gut homeostasis regulation [9]. Administration of *Lactobacillus rhamnosus* [10], *Lactobacillus gasseri* [11], and *Lactobacillus reuteri* [12] has shown therapeutic benefits in intestinal injury regulation [13]. However, the colonization of exogenous strains in the gut still full of challenges [14].

Emerging evidence indicates that the function of *Lactobacillus* is associated with their bioactive derivatives [15]. These bacterial metabolic components, also known as postbiotics, are new classes of intestinal homeostasis regulators [16-18]. Among them, extracellular vesicles (EVs) released by *Lactobacillus* display unique immunomodulatory properties [19-21]. Compared with live bacteria, these functional EVs component rich in protein, lipids, and nucleic acids has a more stable, safe, and efficient effect [22, 23]. Thus, more information needs to be revealed to enrich the mechanism of *Lactobacillus* EVs.

Dairy products serve as important sources of probiotic *Lactobacillus* strains [24]. Among these, goat milk has received distinct attention due to its unique nutritional profile and gut health benefits [25]. Given the distinct microbial compositions across different milk types, exploring the specific *Lactobacillus* strains present in goat milk becomes particularly significant. Emerging evidence indicated that *Lactobacillus salivarius* (*L.s*) and its derived EVs play a crucial role in modulating gut function [26, 27]. Here, we isolated an anti-inflammatory *L.s* strain from raw goat milk and investigated the role of its EVs in LPS-induced intestinal injury. Our findings emphasize a mechanism of *L.s*-derived EVs (*L.s*-EVs) in modulating epithelial barrier function integrity, regulating inflammatory damage, and maintaining gut microbiota homeostasis, wherein miRNA delivery from *L.s*-EVs to intestinal epithelium is critical, thus providing a new perspective of probiotic-derived EVs for treating intestinal inflammatory injury regulation.

2. Materials and methods

2.1 Isolation and identification of goat milk-derived L.s

Raw goat milk was immediately collected from Saanen dairy goats at 2 d postpartum on a commercial farm in Guangzhou, China. Isolation was carried out following the previously reported protocol with minor modifications [28]. Briefly, 100 μL of raw goat milk was inoculated into 10 mL De Man, Rogosa and Sharp (MRS) broth and incubated at 37°C for 12 h. The culture was then transferred to modified MRS broth adjusted to pH 3.0 and incubated for a further 3 h to enrich acid-tolerant *Lactobacillus*. Aliquots of the enriched culture were spread onto MRS agar plates and incubated at 37°C for 24 h. Individual colonies with typical *Lactobacillus* morphology were sub-cultured twice on fresh MRS agar and grown in MRS broth (37°C, 24 h) to obtain pure cultures.

Microbial DNA was extracted with a commercially available kit (DP302; Tiangen Biotech Co. Ltd.) according to the manufacturer's instructions. The 16S rRNA gene was amplified using universal primers 27F

(5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGYTACCTTGTTACGACTT-3′). PCR products were observed on a 1% (w/v) agarose gel run at 120 V for 20 min. The specificities of the amplified bands were purified and sequenced on an Illumina NovaSeq platform (Illumina, USA). Then, the sequence homology was analyzed against the GenBank database via BLAST. The suspected strains of *Lactobacillus* were examined by Gram staining. To further identify the species of the isolated strains, 16S rRNA gene were aligned against the NCBI 16S rRNA database (identity threshold > 95 %). Phylogenetic analysis was conducted in MEGA v11 using the neighbour-joining method with 1,000 bootstrap replications. On the basis of sequence similarity and phylogenetic placement, one isolate strain was identified as *L.s* from raw goat milk.

2.2 Agar well diffusion assay

The inhibitory effect of the cell-free supernatant (CFS) from *L.s* was determined by the Oxford cup diffusion assay [22]. *L.s* (1×10^8 CFU/mL) was grown in MRS broth at 37°C for 24 h. The culture was centrifuged at 3,000 × g for 30 min at 4°C, and the supernatant was collected and filtered through a 0.22 μm sterile filter. Indicator strains (1×10^8 CFU/mL) were spread evenly on Luria Bertani agar plates. Sterile Oxford cups were placed on the agar surface and filled with 150 μL of either CFS or sterile MRS broth (as negative control). After incubation at 37°C for 24 h, inhibition zones were recorded to evaluate antimicrobial activity.

2.3 Isolation and characterization of L.s-EVs

L.s-EVs were isolated following the protocol of previous reports with minor modifications [29]. *L.s* cultures (24 h, 37°C) were centrifuged at 5,000 × g for 30 min at 4°C. The supernatant was filtered through a 0.22 μm sterile filter and concentrated three times in a 100 kDa ultrafiltration device at 3,000 × g for 30 min per cycle. The concentrate was then ultracentrifuged at 150,000 × g for 2 h at 4°C. Pellets containing vesicles were gently resuspended in sterile PBS to obtain the *L.s*-EVs suspension, which was stored at -80°C. Particle size distribution and concentration were determined by nanoparticle tracking analysis, and vesicle morphology was examined by transmission electron microscopy [30].

2.4 Animals and treatments

All procedures were approved by the Animal Care and Use Committee of South China Agricultural University and complied with its institutional guidelines (2025f245). Thirty-six healthy 8-week-old male C57BL/6J mice (body weight 20.88 ± 1.07 g) were obtained from the Guangdong Laboratory Animal Center (Guangzhou, China). Animals were housed under controlled conditions (24 ± 1°C, 65 ± 5% relative humidity) with a 12 h light/12 h dark cycle and given free access to standard chow and water. After a 1-week acclimation, the mice were randomly assigned to two independent experiments. In experiment 1, 18 mice were randomly divided into 3 groups, including the control group, the LPS group, and the LPS + *L.s* group, with 6 mice in each group. In experiment 2, the remaining mice were randomly divided into the control group, LPS group, and LPS + *L.s*-EVs group, with 6 mice in each group. The mice in the control group received seven days of PBS (200 μL/day) by gavage followed by two consecutive days of intraperitoneal injection of PBS

(200 μL/day). The mice in the LPS group received seven days of PBS (200 μL/day) by gavage followed by two consecutive days of intraperitoneal injection of LPS (2.5 mg/kg/day) (O111:B4, Sigma-Aldrich). The mice in the *L.s* group received seven days of *L.s* (1×10^8 CFU/mL) (200 μL/day) by gavage followed by two consecutive days of intraperitoneal injection of LPS (2.5 mg/kg/day). The mice in the LPS + *L.s*-EVs group received seven days of *L.s*-EVs (1 mg/kg/day) by gavage followed by two consecutive days of intraperitoneal injection of LPS (2.5 mg/kg/day). After the experiment, serum, intestinal tissues, and fecal samples were collected for subsequent analysis of the relevant parameters.

2.5 Histology and immunofluorescence

Small intestine tissue (mid-jejunum) was collected, fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 5 μm. Haematoxylin-eosin (H&E) staining was used to evaluate LPS-induced epithelial injury. For immunofluorescence, frozen sections (7 μm) were incubated overnight at 4°C with primary antibodies against ZO-1 (511417, zen-bio), Cluadin-1 (343203, zen-bio), TNF-α (GB11188, Servicebio) and IL-6 (GB11117, Servicebio), followed by FITC- or Cy3-conjugated secondary antibodies. Nuclei were counter-stained with DAPI and visualised under a fluorescence microscope.

2.6 Cell culture and treatment

MODE-K cells (Shanghai Yaji Biotechnology, China) were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells (2×10^5 cells/well) were seeded in 12-well plates. At 80% confluence they were stimulated with *L.s*-EVs (20 μg/mL) for 48 h, then challenged with LPS (10 μg/mL) for a further 12 h to establish an inflammatory-injury model. Cells were harvested for subsequent analyses.

2.7 Internalisation of L.s-EVs

L.s-EVs were labelled with PKH67 according to the manufacturer's instructions (BB-441112, Bestbio) and added to MODE-K cells for 12 h. After staining the nuclei with DAPI, the localization of fluorescent *L.s*-EVs within the cells was examined by fluorescence microscopy.

2.8 Enzyme-linked immunosorbent assay (ELISA)

Serum concentrations of TNF-α, IL-6 and IL-1β were determined with commercial ELISA kits following the manufacturers' protocols (MEIMIAN, China).

2.9 Quantitative Real-time PCR (qRT-PCR)

Total RNA was extracted with TRIzol. For mRNA analysis, RNA was reverse-transcribed with a commercial kit, and qRT-PCR was performed using SYBR Green chemistry on a CFX96 Real-Time System (Bio-Rad, USA). For miRNA analysis, reverse transcription was carried out with the stem-loop method. Relative expression was normalized to GAPDH (mRNA) or U6 (miRNA) and calculated with the $2^{-\Delta\Delta Ct}$ method. Primer sequences are listed in Table 1.

2.10 Western blotting

Proteins were extracted with RIPA buffer and quantified with a BCA assay. Western blotting was performed as described previously [31]. Band intensities were quantified with ImageJ, using GAPDH and β-Tubulin as the internal reference. Primary antibodies included ZO-1 (511417, zen-bio), Claudin-1 (343203, zen-bio), Occludin (502601, zen-bio), p65 (380172, zen-bio), p-p65 (310013, zen-bio), IKK (530546, zen-bio), p-IKK (381289, zen-bio), TLR4 (505258, zen-bio), GAPDH (AP0063, Bioworld), and β-Tubulin (AP0060, Bioworld) (Tbale S1).

2.11 miRNA sequencing and bioinformatics

miRNAs in *L.s*-EVs were isolated and libraries prepared with the TruSeq Small RNA Sample Prep Kit. Single-end sequencing (1 × 50 bp) was carried out on an Illumina HiSeq 2000/2500 platform. Target genes were predicted from the intersection of miRanda (Miranda-Energy < -10) and TargetScan (score > 50) results, followed by KEGG and GO enrichment analyses.

2.12 16S rRNA gene sequencing

Faecal microbiota profiling was performed as previously reported [31]. The V4 region of the bacterial 16S rRNA gene was amplified with primers 515F/806R, purified, and sequenced on a NovaSeq 6000 platform (PE 250). Taxonomic assignment used the SILVA_138.1 database via the Mothur pipeline.

2.13 Dual-luciferase assay

The fragments of wild-type (WT) or mutant-type (MUT) MAPK8 untranslated region (UTR) were loaded into the pmirGLO luciferase vectors (TSINGKE, Beijing, China). The WT or MUT luciferase reporter vectors were co-transfected into 293T cells with either let-7 mimics or negative-control mimic using Lipofectamine 2000 (Thermo Fisher Scientific, USA). After 48 h of treatment, luciferase activity was measured by a Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. miRNA mimics sequences are listed in Table 1.

Table 1. Sequences for qRT-PCR and cell transfection

Gene	Sequences (5'-3')
mmu-GAPDH	F: TGGCCTTCCGTGTTCCTAC R: GAGTTGCTGTTGAAGTCGCA
mmu-U6	F: CTCGCTTCGGCAGCACA R: AACGCTTCACGAATTTGCGT
mmu-TNF-α	F: GGTGCCTATGTCTCAGCCTCTT R: GCCATAGAACTGATGAGAGGGAG
mmu-IL-6	F: TACCACTTCACAAGTCGGAGGC R: CTGCAAGTGCATCATCGTTGTTC
mmu-IL-1β	F: TGGACCTTCCAGGATGAGGACA R: GTTCATCTCGGAGCCTGTAGTG
mmu-ZO-1	F: GACACCAAAGCATGTGAGCC R: GGTAAGGCATTCCTGCTGGT
mmu-Occludin	F: GCGCACGTGTGTGTGTTATT R: ATCATTGCCTTTCCTGGGGG
mmu-Claudin-1	F: CCCGATGACCTTTCTGGACG R: TTAGCAGCACAGCCACTCAG
mmu-MAPK8	F: AAACAGGCCTAAATACGCTGGA R: GACGGCTGCCCTCTTATGAC
let-7	F: GCGCGCGCGGAGGAGA

	R: AGTGCAGGGTCCGAGGTATT
RT-let-7 (for reverse transcription)	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTTCTC
let-7 mimics	Sense: UGAGGUAGUAGGUUGUAUAGUU Antisense: AACUAUACAACCUACUACCUCA
NC-mimics	Sense: UUCUCCGAACGUGUCACGUTT Antisense: ACGUGACACGUUCGGAGAATT

2.14 Statistical analysis

Statistical analyses were conducted with SPSS 22.0. Data are presented as mean ± SEM. Normality and homogeneity of variance were assessed with the Shapiro-Wilk and Levene tests, respectively. Differences among groups were evaluated by one-way ANOVA followed by Tukey's HSD post-hoc test. $P < 0.05$ was considered significant differences.

3. Results

3.1 Goat milk-derived L.s alleviate LPS-induced intestinal injury

The bacterial strain initially harvested from raw goat milk was purified on MRS agar and subjected to phenotypic identification. The purified colonies were white, smooth-edged, and moist. Moreover, the gram staining of these colonies represented typical morphology of *Lactobacillus* (short rods, Gram-positive) (Figure 1A). Subsequently, the probiotic potential of the isolate strain was evaluated. The CFS of isolated strain indicated inhibition effect to growth indicator bacteria (*Escherichia coli* and *Salmonella enterica*) (Figure 1B). To further clarify the taxonomic distributions of isolated strain, the bacterial 16S rRNA gene was amplified with species-specific primers (Figure 1C) and retrieved the associated sequences from GenBank database to construct a phylogenetic tree (Figure 1D). In these cases, *L.s* was eventually determined. For the *in vivo* assay, the protective effect of *L.s* against intestinal inflammatory injury was evaluated (Figure 1E). In the current study, *L.s* alleviated the LPS-induced body weight loss ($P < 0.05$) (Figure 1F), prevented intestinal villus injury including villus shortening and epithelial vacuolization (Figure 1G and Figure S1A), and significantly reduced epithelial inflammatory cytokine expression ($P < 0.05$) (Figure 1H). These findings implied that raw goat milk-derived *L.s* is a potential probiotic with anti-inflammatory effects.

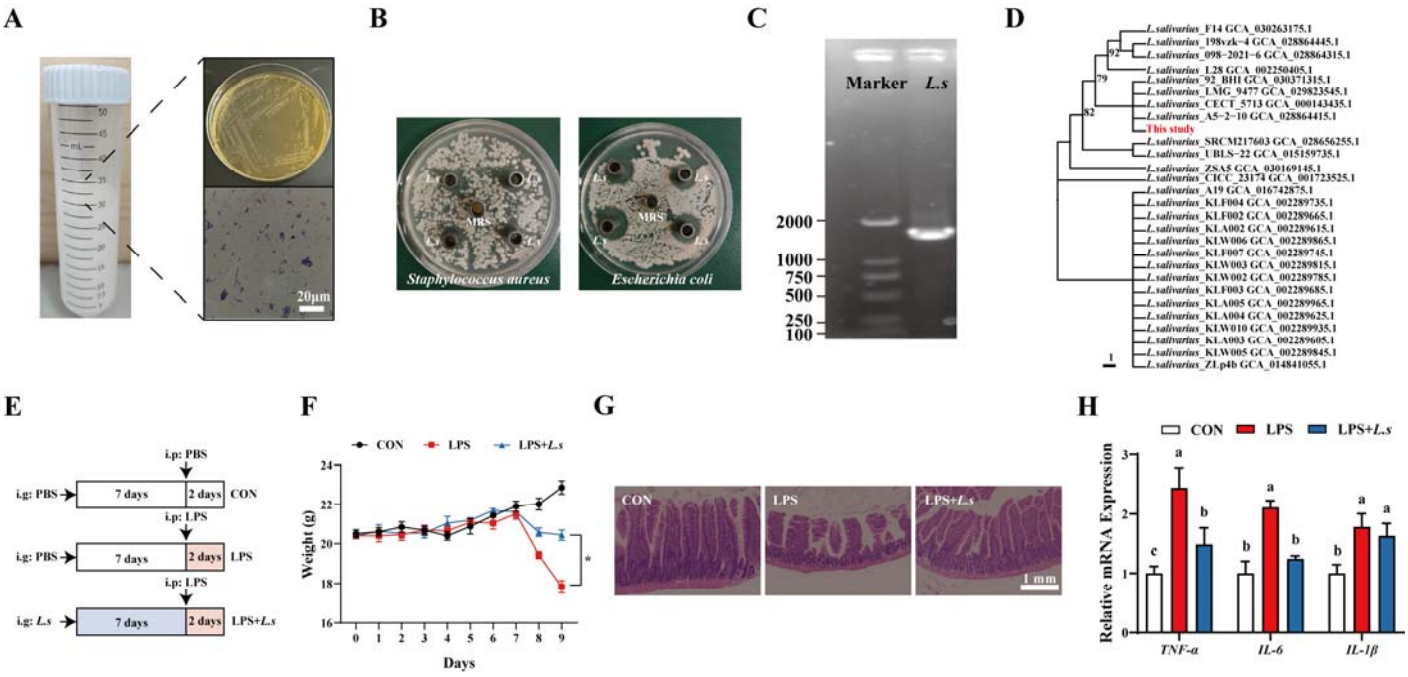


Figure 1. Raw goat milk-derived *L.s* alleviated LPS-induced intestinal damage in mice (n = 6). (A) Isolation and identification of raw goat milk-derived *L.s*. (B) Antimicrobial activity testing of *L.s*. (C) The 16S rDNA amplification products of *L.s*. (D) Phylogenetic tree constructed based on GenBank database. (E) Flowchart of animal experiments. (F) Mice body weight; (G) HE staining of mice intestinal tissue (scale bar = 1 mm). (H) Gene expression of TNF-α, IL-1β, and IL-6 in mice intestinal epithelial tissue. All data are presented as mean ± SEM. Different letters indicate significant differences ($P < 0.05$).

3.2 L.s-EVs restore LPS-induced intestinal barrier dysfunction

To determine whether *L.s*-EVs participate in the regulation of intestinal injury, the fermentation supernatant of *L.s* cultures was collected for EVs isolation and characterization. Transmission electron microscopy revealed cup-shaped vesicles with a double-layer membrane structure. Moreover, nanoparticle-tracking analysis showed the mean particle size of *L.s*-EVs was 151.3 ± 66.1 nm and the particle concentration was 2.5×10^{11} particles/mL (Figure 2A). Subsequently, we examined the protective function of *L.s*-EVs against inflammatory intestinal injury (Figure 2B). Our results indicated that *L.s*-EVs administration had no effect on the body weight of mice under conventional conditions ($P > 0.05$), while significantly reversed the weight loss of mice treated with LPS ($P < 0.05$) (Figure 2C). The intestinal morphology of mice showed that *L.s*-EVs alleviated LPS-induced shortening of the intestine ($P < 0.05$) (Figure 2D). Furthermore, *L.s*-EVs reversed LPS induced damage to intestinal epithelial villi (Figure 2E and Figure S1B). Notably, the epithelial damage is related to the disorder of barrier function. In the current study, we found that LPS caused disruption of intestinal epithelial barrier function, while *L.s*-EVs increased the expression of barrier-related genes and proteins ($P < 0.05$) (Figure 2F and 2G). Immunofluorescence further demonstrated that *L.s*-EVs restored the localization expression of Claudin-1 and ZO-1 in the intestinal epithelium (Figure 2H and Figure S1C). These results emphasized the potential role of *L.s*-EVs in intestinal injury repair.

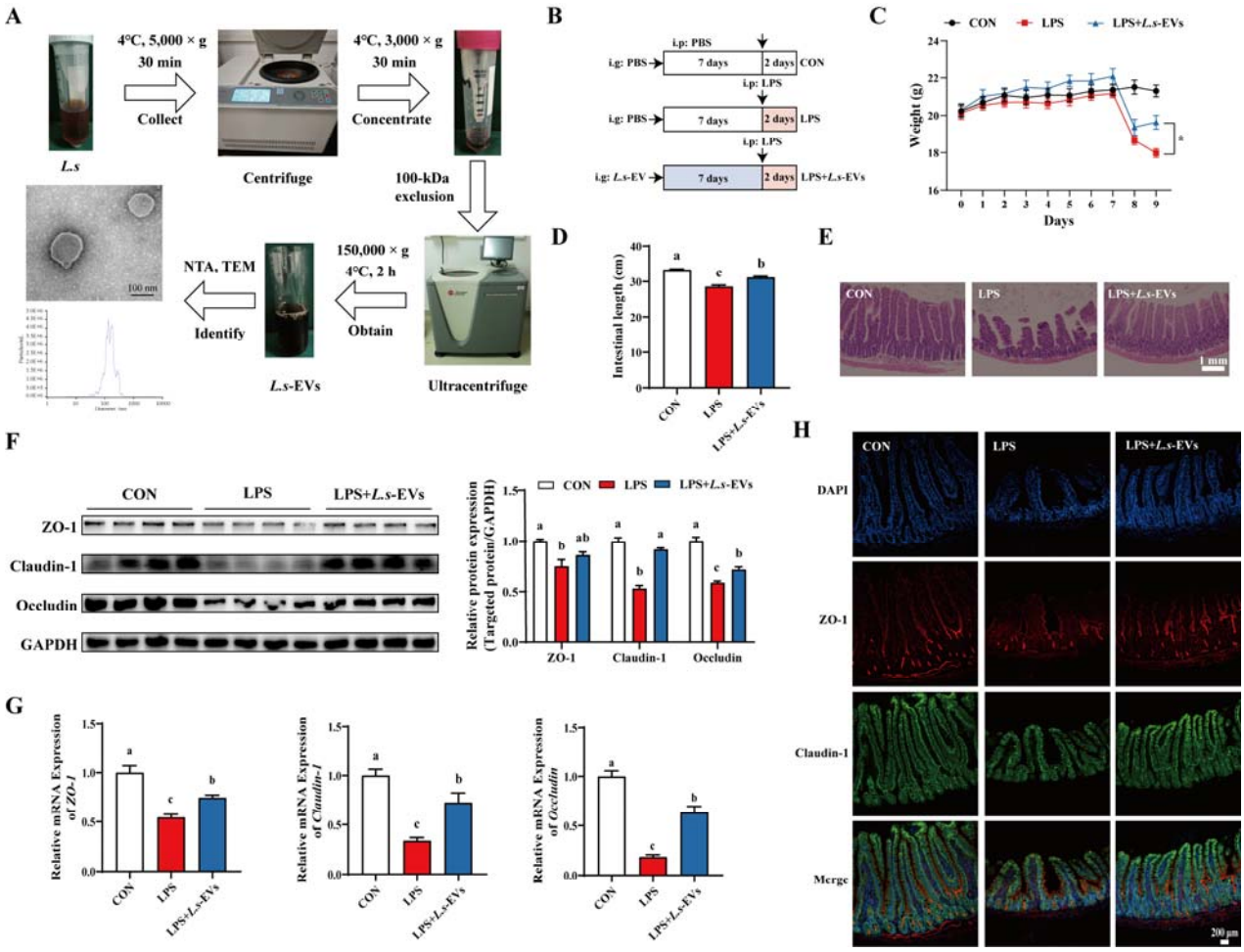


Figure 2. *L.s*-EVs participate in the LPS-induced intestinal epithelial damage repair (n = 6). (A) Isolation and identification of *L.s*-EVs. (B) Experimental workflow diagram. (C) Mice body weight. (D) Mice small intestine length. (E) HE staining of mice intestinal tissue (scale bar = 1 mm). (F) Expression of intestinal epithelial barrier-related proteins (n=4). (G) Expression of intestinal epithelial barrier-related genes. (H) Immunofluorescence of Claudin-1 and ZO-1 in intestinal epithelium (scale bar = 200 μm). All data are presented as mean ± SEM. Different letters indicate significant differences ($P < 0.05$).

3.3 L.s-EVs modulate intestinal inflammatory signalling

The expression of inflammatory signals induced by LPS in the intestine is the main cause of barrier dysfunction. Thus, the serum levels of TNF-α, IL-1β, and IL-6 were measured. Compared with the control group, LPS stimulation significantly increased serum concentrations of TNF-α, IL-6, and IL-1β, whereas *L.s*-EVs treatment significantly reversed this effect (Figure 3A). Moreover, a similar trend was seen for the gene expression of TNF-α, IL-6, and IL-1β in the intestine of mice treated with *L.s*-EVs (Figure 3B). To explore the underlying mechanism, we examined the activity of TLR4/NF-κB pathway. As expected, LPS exposure significantly up-regulated epithelial TLR4 expression and promoted the phosphorylation of IKK and p65, thereby driving the transcription of pro-inflammatory genes, while *L.s*-EVs administration effectively reversed these molecular events ($P < 0.05$) (Figure 3C). Immunofluorescence analysis further demonstrated that *L.s*-EVs attenuated the LPS-stimulated expression of TNF-α and IL-6 in intestinal epithelium (Figure 3D and Figure S1D). These results emphasized the potential role of *L.s*-EVs in regulating intestinal inflammatory signaling.

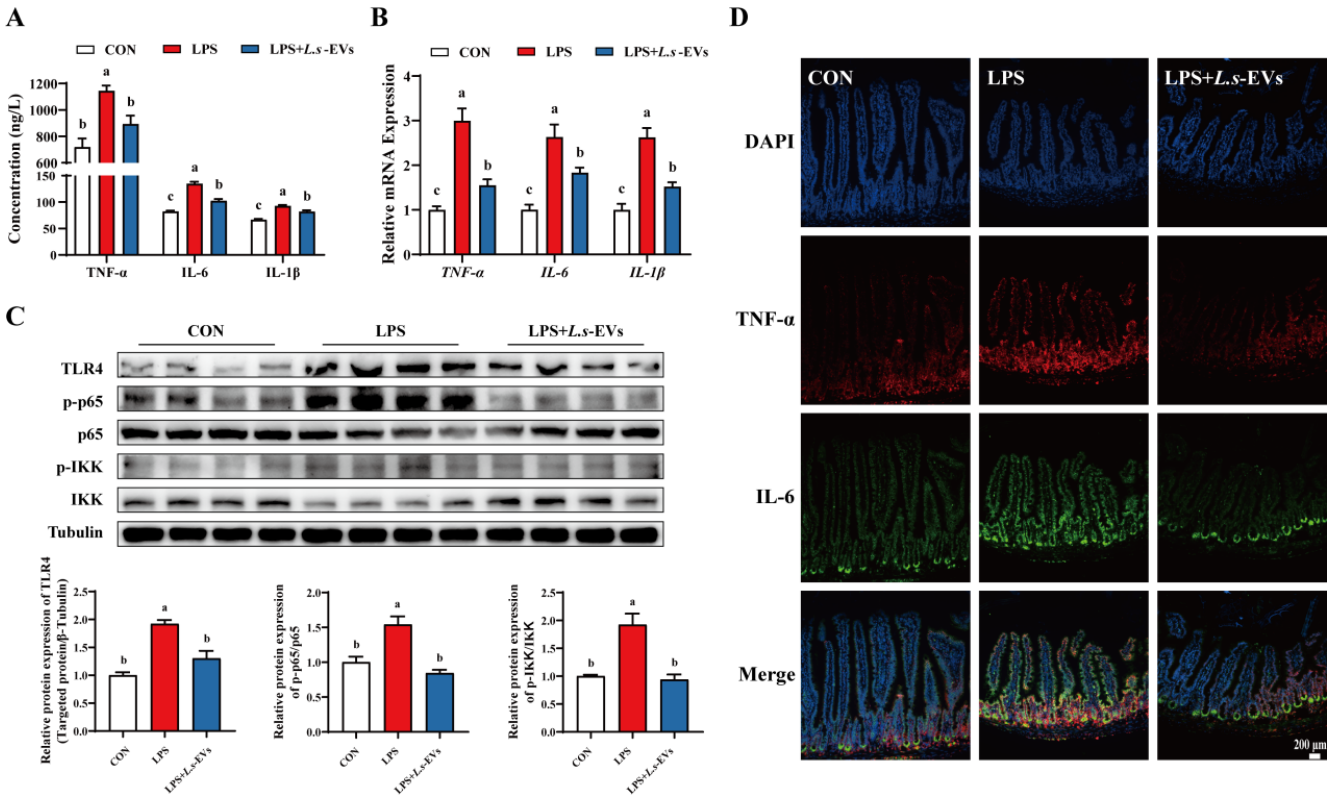


Figure 3. *L.s*-EVs participate in the regulation of intestinal epithelial inflammatory signaling through the TLR4/NF-κB pathway (n = 6). (A) Levels of TNF-α, IL-6, and IL-1β in mice serum. (B) The mRNA expression levels of TNF-α, IL-6, and IL-1β in intestinal epithelial tissue. (C) Changes in TLR4/NF-κB signaling protein expression in intestinal epithelial tissue (n=6). (D) Immunofluorescence of TNF-α and IL-6 in intestinal epithelial tissue (scale bar = 200 μm). All data are presented as mean ± SEM. Different letters indicate significant differences ($P < 0.05$).

3.4 L.s-EVs reshape the host gut microbiota

To explore the impact of *L.s*-EVs on gut microbial composition, 16S rRNA gene amplicon sequencing of faecal samples was performed. Principal-coordinate analysis (PCoA) based on the Bray-Curtis distances showed a clear segregation of the LPS group from both the control and *L.s*-EVs groups, indicating successful model construction. PCoA1 and PCoA2 accounted for 39.61 % and 25.04 % of the total variance, respectively (Figure 4A). At the genus level, *Lactobacillus*, *Streptococcus*, *Candidatus Saccharimonas*, *Dubosiella*, and *Desulfovibrio* were the five most abundant of all samples (Figure 4B). Compared with the control group, LPS treatment significantly decreased the abundance of *Lactobacillus*, *Lachnoclostridium*, *Enterorhabdus*, and *Desulfovibrio* ($P < 0.05$). In contrast, *L.s*-EVs treatment significantly increased the relative abundance of *Lactobacillus* ($P < 0.05$) (Figure 4C). Functional prediction of the microbial composition indicated that metabolic pathways constituted the principal functional category affected (Figure 4D). Consistently, LEfSe analysis (LDA > 4) highlighted *Lactobacillus* as the biomarker most strongly associated with *L.s*-EVs treatment ($P < 0.05$) (Figure 4E).

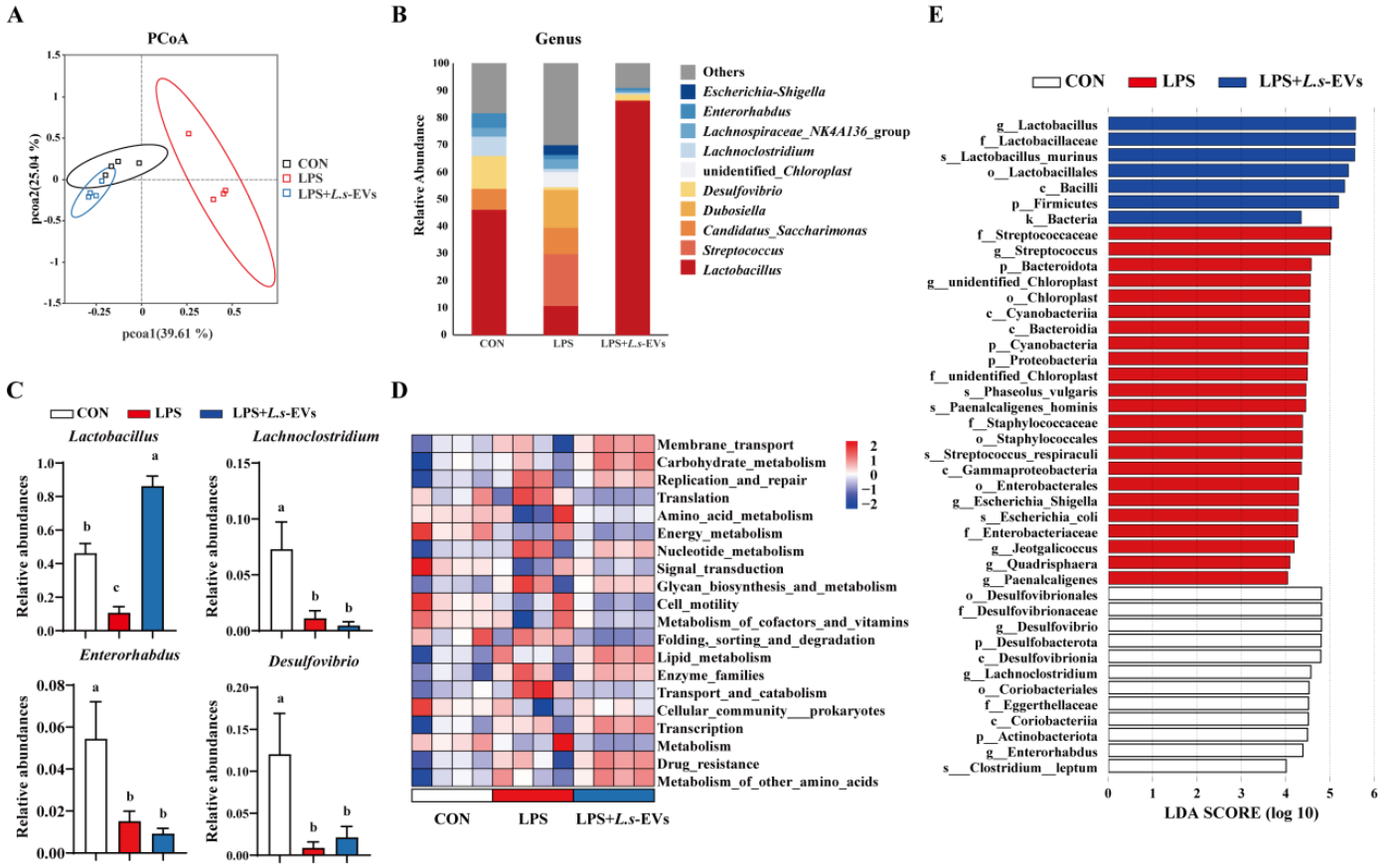


Figure 4. Effects of *L.s*-EVs on the gut microbiota of mice (n = 4). (A) PCoA plot based on Bray-Curtis distances. (B) Differentially microorganisms at the genus level. (C) Relative abundance of differentially microorganisms. (D) Functional enrichment analysis of differentially microorganisms. (E) LDA scores (threshold > 4). All data are expressed as mean ± SEM. Different letters indicate significant differences ($P < 0.05$).

3.5 miRNA profiles of L.s and L.s-EVs

The biological activity of *L.s*-EVs is closely linked to the cargo they carry. Therefore, we compared the composition of miRNAs in *L.s* and *L.s*-EVs via miRNA sequencing analysis. A total of 41 miRNAs were identified in *L.s*, whereas 12 miRNAs were detected in *L.s*-EVs; nine of these were common to both sources (Figure 5A). Functional enrichment of the shared miRNAs was performed with KEGG and GO enrichment analysis. The KEGG enrichment analysis indicated that the differentially distributed miRNAs predominantly targeted genes involved in the MAPK pathway, AGE-RAGE signaling pathway, Phosphatidylinositol signaling system, FoxO signalling, and ECM-receptor interaction (Figure 5B). The GO enrichment analysis revealed that these miRNAs were mainly associated with biological processes (such as transcriptional regulation and signal transduction), cellular components (including the cytoplasm, plasma membrane, and nucleus), and molecular functions (related to protein, metal-ion and nucleotide binding as well as transferase activity) (Figure 5C). Among the nine shared miRNAs, seven highly expressed species were selected for further analysis, among which let-7 exhibited the highest abundance in both *L.s* and *L.s*-EVs (Figure 5D and 5E).

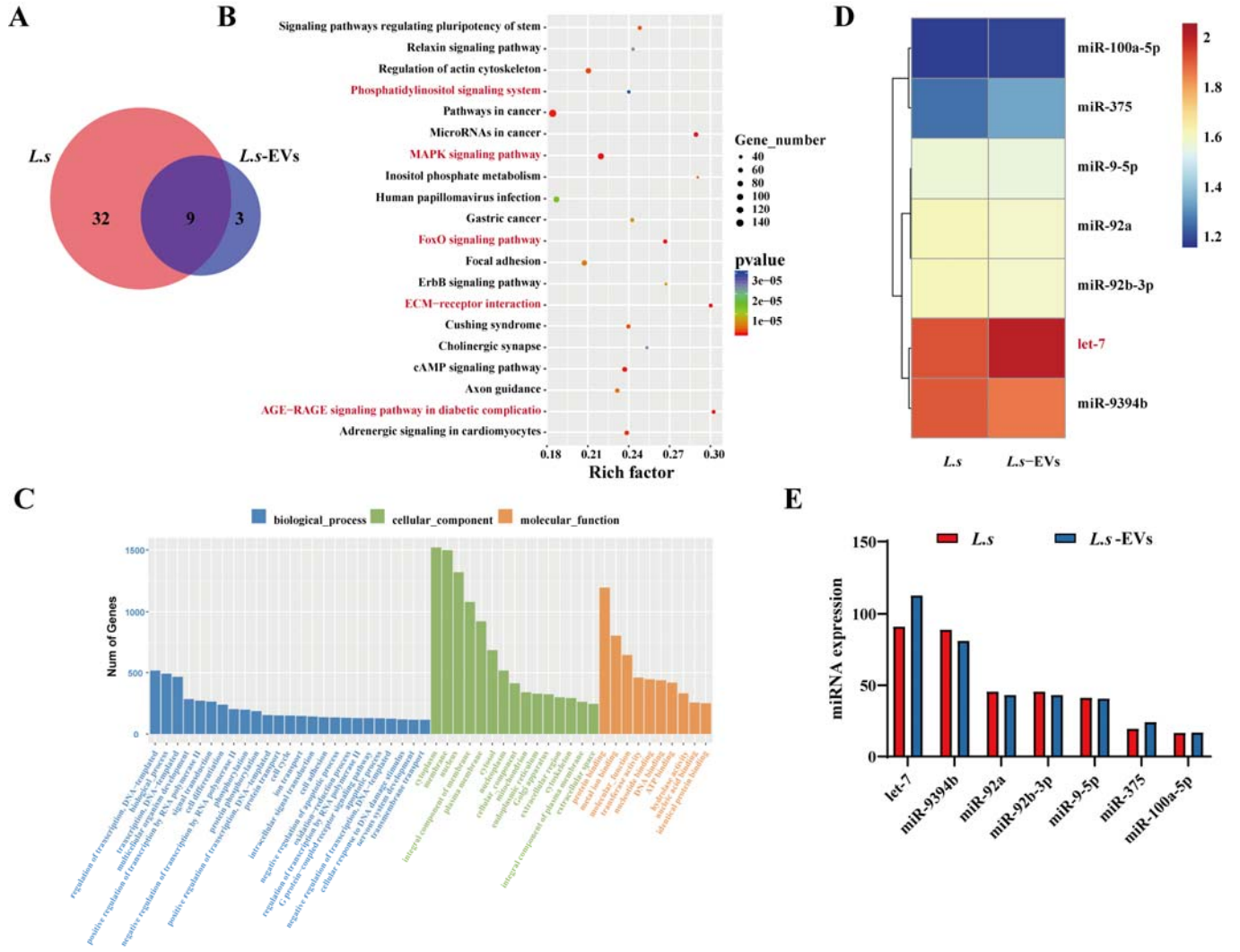


Figure 5 Analysis of miRNA profiles in *L.s* and *L.s*-EVs. (A) Venn diagram. (B) KEGG enrichment analysis of shared miRNAs between *L.s* and *L.s*-EVs. (C) GO enrichment analysis of shared miRNAs between *L.s* and *L.s*-EVs. (D) Heatmap of highly expressed miRNAs in shared miRNAs between *L.s* and *L.s*-EVs. (E) Expression of seven key miRNAs in shared miRNAs.

3.6 L.s-EVs mitigate LPS-induced intestinal injury by delivering let-7

Efficient uptake of EVs by epithelial cells is a foundation for their regulatory activity. In the current study, PKH67-labelled *L.s*-EVs were effectively internalized by MODE-K cells, as observed by fluorescence microscope (Figure 6A). Consistent with our *in vivo* observations, *L.s*-EVs treatment reversed the LPS-induced up-regulation of TNF-α, IL-6, and IL-1β gene expression in MODE-K cells (Figure 6B). Afterward, the contribution of *L.s*-EVs derived miRNAs to anti-inflammatory effect was assessed. Considering the high expression abundance of let-7 in both *L.s* and *L.s*-EVs, we further predicted the inflammatory regulation-related genes that target let-7, based on TargetScan and miRanda. Dual-luciferase reporter assays confirmed that MAPK8 is a direct target of let-7 (Figure 6C). Quantitative analyses in MODE-K cells revealed that LPS exposure suppressed let-7 expression and increased MAPK8 gene expression, while *L.s*-EVs administration restored let-7 expression and concomitantly reduced MAPK8 gene expression ($P < 0.05$) (Figure 6D and 6E). These results indicate that *L.s*-EVs convey let-7 to epithelial cells, downregulating MAPK8 and inhibiting LPS-driven inflammatory signaling.

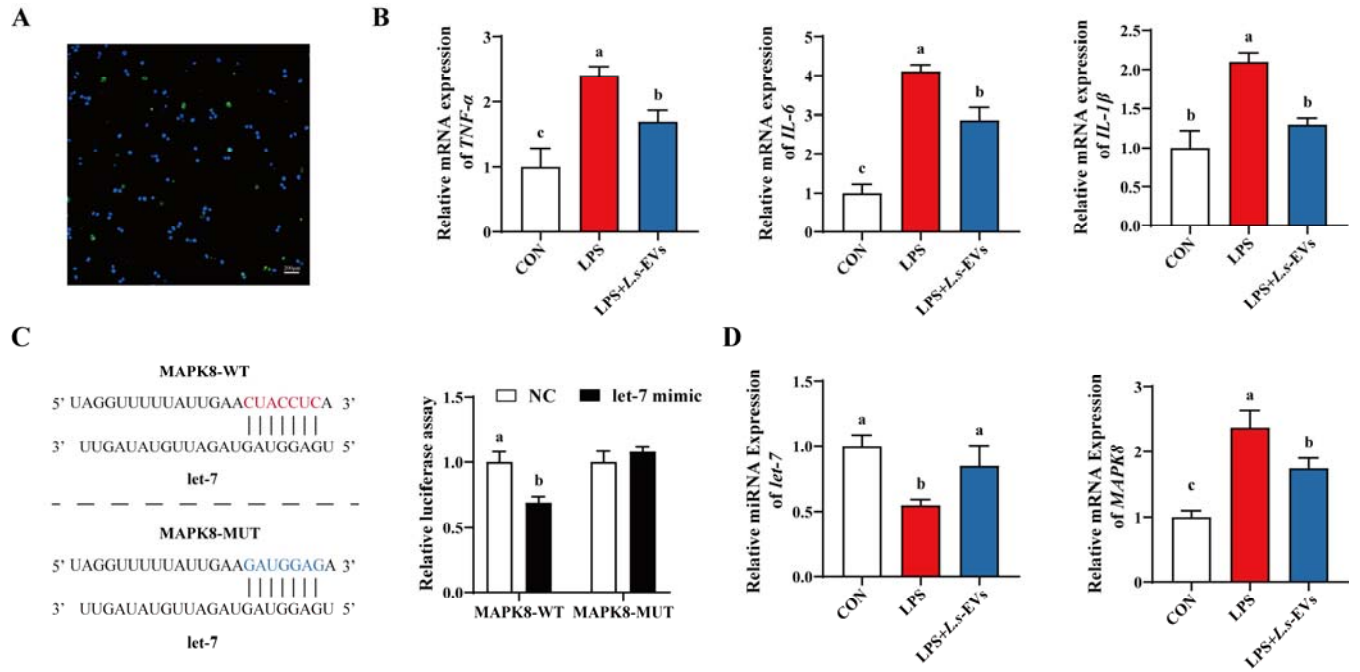


Figure 6 *L.s*-EVs regulate intestinal inflammation by delivering let-7 (n = 6). (A) Internalization of PKH67-labeled *L.s*-EVs in MODE-K cells. (B) The gene expression of TNF-α, IL-1β, and IL-6 in MODE-K cells. (C) Dual luciferase assay of let-7 and MAPK8. (D) The gene expression of let-7 and MAPK8 in MODE-K cells. All data are expressed as mean ± SEM. Different letters indicate significant differences ($P < 0.05$).

4. Discussion

During inflammatory injury of the intestine, the change of microbial community and the disorder of metabolic signals are commonly observed [32]. Various clinical and experimental studies demonstrated that the abundance of *Lactobacillus* spp. is associated with intestinal inflammatory injury [33]. Accordingly, supplementation with *Lactobacillus*-derived microbial preparations is considered an adjuvant strategy for intestinal inflammatory injury regulation [13]. However, administering conventional model strains neglects inter-individual differences in disease development and causes inconsistent therapeutic outcomes [34]. Thus, exploring naturally occurring strains with personalized adaptation potential has become a promising strategic direction.

Foodborne *Lactobacillus* is more abundant and generally considered safe, healthy, and functional [35]. The development of foodborne probiotics enriched the applicability of *Lactobacillus* in maintaining intestinal health [36]. Dairy products, particularly raw milk and artisanal cheeses, are important sources of foodborne *Lactobacillus* [37]. Recent reports indicate that milk-derived *Lactobacillus* participates in systemic metabolic regulation [38]. Given the potential value of milk-derived *Lactobacillus*, we isolated a *L.s* strain from local raw goat milk and evaluated its probiotic properties. Consistent with previous reports, the isolated *L.s* inhibited the proliferation of pathogenic microorganisms and exhibited ideal probiotic properties [39]. Moreover, exogenous administration of *L.s* contributed to epithelial barrier integrity and modulation of inflammatory signaling [40]. Although *L.s* is typically considered a probiotic strain with potential for development in the gastrointestinal tract of animals [41], other studies including ours showed the presence of

L.s in dairy products [42, 43]. These findings indicate that milk-derived *L.s* has potential application value in the supplementation of probiotics.

The regulatory capacity of gut microbiota is often mediated by their secreted functional derivatives [15]. Among these, bacterial-derived EVs have emerged as key postbiotics involved in intestinal metabolic regulation [20]. Recent studies have showed that *Lactobacillus* EVs can replicate many of the probiotic effects of their parental strains, especially in the inflammatory injury regulation [44]. Notably, *Lactobacillus*-derived EVs play critical role in intestinal immune modulation [45], intestinal barrier function maintenance [46], and gut microbiota homeostasis regulation [47]. *Lactobacillus johnsonii*-derived EVs reshape gut microbiota and restore intestinal immunity via taurine [48]. Emerging evidence indicated that *L.s*-EVs play important roles in inflammatory response regulation [49]. Moreover, *L.s*-EVs regulated intestinal function and microbiota in patients with non-alcoholic fatty liver disease [27]. Similar to live bacteria, in the current study, *L.s*-EVs modulated the LPS-induced TLR4/NF-κB pathway and remodeled the gut microbiota [50, 51]. Notably, the gut microbiota reshaping caused by *L.s*-EVs appears to be linked to changes in vesicular cargo [52]. Emerging evidence indicated that the protein of *Lactobacillus*-derived EVs participated in the regulation of intestinal homeostasis [44]. *Lactobacillus johnsonii*-derived EVs carrying GAPDH also reported in against ulcerative colitis [54]. Moreover, Sdp-SH3b2 domain contained in *Lactobacillus johnsonii* participated in the resistance against enterovirus infection [53]. Thus, individual components of *Lactobacillus*-derived EVs may have limited function in regulating intestinal inflammatory injury.

As a key mediator for communication between gut microbiota and host metabolism, miRNAs have drawn particular attention [55]. The function of *Lactobacillus* in maintaining intestinal homeostasis is related to the remodeling of miRNA profiles in intestinal epithelium [56]. Recent studies have emphasized the cross-kingdom regulatory function of bacterial EVs-encapsulated miRNAs [29]. Our profiling of shared miRNA profiles in *L.s* and *L.s*-EVs also revealed that let-7, a negative regulatory factor of inflammatory injury, may be a key miRNA delivered to the intestine in *L.s*-EVs [57]. Bioinformatic prediction followed by dual-luciferase assays confirmed that let-7 directly targets the 3′-UTR of MAPK8, to inhibit the activation of inflammatory signaling pathways [58]. Thus, engineering expression regulation of let-7 in *L.s* and *L.s*-EVs may be a potential application technology for alleviating intestinal inflammatory injury.

5. Conclusions

In summary, we successfully isolated a probiotic, *L.s*, from raw goat milk. Subsequently, *L.s*-EVs were collected, purified, and characterized. Our results indicate that *L.s*-EVs alleviate LPS-induced epithelial injury, inflammation, and gut microbiota dysbiosis. Mechanistically, *L.s*-EVs-encapsulated let-7 suppressed intestinal MAPK8 expression, leading to attenuation of inflammatory responses.

Funding

This work was supported by the Natural Science Foundation of China Program (32573160, 32072812, and 32072714), Key-Area Research and Development Program of Guangdong Province (2025B0202080002),

the National Key Research and Development Program of China (2022YFD1300904), and the Project of Guangdong Provincial Nature Science Foundation (2025A1515010952 and 2023A151502511). All procedures were approved by the Animal Care and Use Committee of South China Agricultural University and complied with its institutional guidelines (2025f245).

Availability of data and materials

The 16S rRNA sequence data have been deposited in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/) under accession numbers PRJNA1270972. The miRNA-seq data have been deposited in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/) under accession numbers PRJNA1272273.

Declarations

Ethics approval and consent to participate

All animal studies carried out under the protocols of the Animal Experimentation and Ethics Committee at South China Agricultural University have received approval.

Consent for publication

All authors participating in this research gave their full consent to publish the findings.

Competing interests

The authors confirm they have no financial conflicts of interest.

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