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Proteomic study of extracellular vesicles from kefir-derived lactic acid bacteria and their role in inflammation alleviation

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ABSTRACT: Kefir is a traditional fermented dairy beverage rich in lactic acid bacteria (LAB) with probiotic potential. Extracellular vesicles (EVs) produced by LAB carry diverse bioactive components and play key roles in inter-strain communication and functional regulation. However, the roles and mechanisms of microbial EVs in fermented foods remain to be systematically elucidated. Here, we characterized the proteomic features and anti-inflammatory effects of EVs from kefir-derived LAB-*Lentilactobacillus kefiri* KLK29 (KLK29EVs) and *Enterococcus faecium* KEF14 (KEF14EVs). The two EV proteomes were dominated by cytosol-associated proteins and were enriched in functional modules related to carbohydrate metabolism, transmembrane transport, signal transduction, and environmental response. Several proteins were associated with inflammation regulation. At non-cytotoxic doses (KLK29EVs, 5 μg/mL; KEF14EVs, 1 μg/mL), both EV preparations downregulated pro-inflammatory cytokines, upregulated anti-inflammatory cytokines, and suppressed NF-κB activation. This study provides important information on the functions and mechanistic insights of fermented-food-derived LAB EVs and offers a theoretical basis for their potential application in functional food development.

Keywords: kefir, lactic acid bacteria, extracellular vesicles, proteomics, inflammation modulation

1. Introduction

Kefir is a low-alcoholic fermented milk beverage with high nutritional value. It is produced by fermenting fresh milk with kefir grains and has shown potential efficacy in the prevention and adjunct treatment of gastrointestinal and metabolic disorders, particularly inflammatory diseases such as colitis, gastritis, neuroinflammation, hypertension, and diabetes[1-4]. The functional benefits of kefir are largely attributed to its complex extracellular polymeric matrix and symbiotic microbial community. Within the kefir fermentation system, lactic acid bacteria (LAB), yeasts, and acetic acid bacteria co-establish a dominant community, form a biofilm-like matrix, and generate diverse bioactive components, including exopolysaccharides and extracellular vesicles (EVs)[5-7].

LAB are the predominant and metabolically most active members of the kefir microbial ecosystem[8]. Kefir-derived LAB generally exhibit favorable probiotic properties and safety profiles[9]. Among these, *Lentilactobacillus kefiri* is a key species in kefir; its surface S-layer facilitates adhesion and host

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interactions. Orally administered *L. kefiri* can colonize the intestine, improve gut microbial balance, and shows anti-inflammatory potential[10-14]. In addition to bacilli, kefir also contains cocci. *Enterococcus* isolates recovered from kefir have exhibited good safety profiles and potential roles in the modulation of inflammation[15]. Studies further indicate that *Enterococcus faecium* can colonize mucosal surfaces, enhance the mucus barrier, inhibit pathogens via bacteriocins or specific proteins, and participate in the regulation of inflammatory responses[16-17]. Accordingly, *L. kefiri* and *E. faecium* represent probiotic candidates worthy of further investigation.

EVs are lipid bilayer-enclosed nanoparticles and are widely present in diverse biological systems. Their native membranes enable the packaging and transport of diverse cargoes, including proteins, lipids, polysaccharides, and nucleic acids[18]. These cargoes act as key mediators of intercellular communication, allowing EVs to participate in a broad spectrum of pathophysiological processes. To date, the functions and applications of mammalian EVs have been extensively explored-for example, in neurodegeneration, immune regulation, and tissue repair [19–23]. By contrast, systematic evidence regarding the composition, functions, and underlying mechanisms of EVs released by microbes in food fermentation systems remains limited. Against this backdrop, we investigated kefir-derived LAB, systematically profiled the proteomes of their EVs and performed functional enrichment analyses, and evaluated anti-inflammatory effects in LPS-stimulated macrophages, with a specific focus on the NF-κB signaling pathway. This work provides foundational data on the functional characteristics of fermented-food–associated microbial EVs and provides a rationale for their potential applications in functional food development and cross-disciplinary biomedicine.

2. Materials and methods

2.1 Proteomics

2.1.1 Sample preparation

Samples were extracted using 1% sodium deoxycholate (SDC) in 100 mM tris-HCl (pH 8.5), sonicated, and centrifuged at 12,000 × *g* for 5 min. Protein concentrations were determined using a BCA assay. Equal amounts of protein were diluted to the same volume using the extraction buffer. Proteins were reduced and alkylated with tris(2-carboxyethyl)phosphine hydrochloride and chloroacetamide at 60℃ for 30 min. SDC was diluted to <0.5% before trypsin digestion (enzyme-to-protein ratio 1:50, w/w) at 37℃ overnight. Digestion was terminated with trifluoroacetic acid to adjust the pH to 6.0, followed by centrifugation at 12,000 × g for 5 min. The supernatant was collected, desalted using a self-packed SDB column, dried under vacuum, and stored at -20 °C.

2.1.2 Mass spectrometry analysis

Peptides were analyzed using a timsTOF Pro (Bruker, USA) TIMS-QTOF mass spectrometer coupled to an UltiMate 3000 RSLCnano system (Thermo, USA) with a CaptiveSpray nano source (Bruker, USA). The peptides were first loaded onto a C18 trap column (75 μm × 2 cm, 3 μm, 100 Å, Thermo, USA) and

then separated using a C18 analytical column (75 μm × 15 cm, 1.7 μm, 100 Å, IonOpticks, Australia) at a flow rate of 300 nL/min with mobile phases A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). Data were acquired in the PASEF mode with the following settings: 1,500 V capillary voltage, m/z range of 100-1700, ion mobility range of 0.75-1.4 Vs/cm², and a cycle time of 1.16 s (1 MS + 10 PASEF MS/MS). Accumulation/ramp time were 100 ms. Precursors with charge states ≥2 and intensity >1000 were selected, with a target intensity of 10,000 and a dynamic exclusion time of 0.4 min. Quadrupole isolation width was set to 2.0 Da at m/z 700 and 3.0 Da at m/z 800. Collision energy ranged from 59 eV at $1/K_0$ = 1.6 Vs/cm^2 to 20 eV at $1/K_0$ = 0.6. Vs/cm^2.

2.1.3 Database retrieval

Raw data were processed using MaxQuant (v2.1.2.0) with the Andromeda search engine against UniProt databases for *E. faecium* (3055 entries) and *L. kefiri* (2359 entries). Each identified protein contains at least one unique peptide. The false discovery rate (FDR) was controlled at 1% at the PSM, peptide, and protein levels using a target - decoy approach. Search parameters included variable modifications: oxidation (M, +15.994915), acetylation (protein N-term, +42.010565) and deamidation (N/Q, +0.984016); fixed modification: carbamidomethylation (C, +57.021464); enzyme: trypsin/P with up to 2 missed cleavages; precursor mass tolerance: 20 ppm (first search), 10 ppm (main search); and fragment mass tolerance: 25 ppm. Proteins indistinguishable based on unique peptides were merged by MaxQuant into one group. Label-free quantification was performed using the MaxLFQ algorithm, with “match between runs” enabled. All proteomics data have been deposited in PRIDE (PXD069374).

2.2 Cell culture and treatment

RAW264.7 cells (CL-0190, Procell, China) were expanded and cultured to the logarithmic growth phase, seeded at a density of 0.8 × 10⁴ cells/well in 96-well plates, and incubated at 37 °C with 5% CO_2 for 24 h. After removing the medium, the cells were treated for another 24 h under four conditions: control, LPS only, and LPS with EVs.

2.3 Cell viability and nitric oxide determination

To determine optimal EVs concentrations for anti-inflammatory studies, RAW264.7 cells were treated with 1 μg/mL LPS (L2880, Sigma, USA) in combination with KLK29EVs (0.1, 1, 5, 10, and 20 μg/mL) or KEF14EVs (0.1, 0.5, 1, 5, and 10 μg/mL). Cell viability was assessed using the CCK-8 assay (40037, Biyuntian, China) by adding 10 μL of reagent per well, followed by incubation at 37℃ with 5% CO_2 for 1 h. Absorbance at 450 nm was measured using a microplate reader (MK3, Thermo, USA). Cell viability was expressed as a percentage of the untreated control group (set as 100%). Nitric oxide (NO) levels were determined using a NO detection kit (S0021, Biyuntian, China).

2.4 Flow cytometry

Following 24 h treatments, cells were washed with PBS, treated with (1) fresh DMEM/F-12 medium culture (L310KJ, BasalMedia, China), (2) 1 μg/mL LPS, (3) LPS + 5 μg/mL KLK29EVs, and (4) LPS + 1

μg/mL KEF14EVs. Cells were harvested using EDTA-free trypsin, washed with precooled PBS, and resuspended in 500 μL PBS. They were incubated with 5 μL CD86 antibody (12-0862-82, Ebioscience, USA) for 30 min at 37℃ in the dark, washed with PBS, resuspended in 500 μL PBS, and analyzed using a flow cytometer (CytoFLEX, Beckman Coulter, USA).

2.5 In vitro tracing of cells

After 24 h of culture, all cells were washed with PBS. EVs were labeled with 100 μM PKH67 (UR52303, Umibio, China) for 10 min at 37℃ in the dark. Excess dye was removed by washing twice with PBS. Nuclei were counterstained with Hoechst 33342 (1:1000; C1025, Biyuntian, China) and incubated at 37℃ in the dark for 5 min. After the cells had absorbed the dye solution, they were washed twice with PBS. Uptake was visualized using a confocal microscope (FV3000, Olympus, Japan).

2.6 Cytokine assay

Cytokine levels in cell supernatants were quantified using the Bio-plex Pro Mouse Cytokine GRP I panel (M60009, RDPD, USA) on a Luminex 200 System (Luminex Corporation, USA), following the manufacturer's instructions for liquid-phase suspension chip analysis.

2.7 Western blot

RAW264.7 cells were treated with LPS and/or EVs for 24 h. Cells were lysed in RIPA buffer with PMSF, and protein concentrations were determined using the BCA assay kit (BL521A, Biosharp, China). Equal amounts of protein (30 μg) were separated through SDS-PAGE (Bio-Rad Mini-PROTEAN 3 cell, USA) and transferred to PVDF membranes (Millipore HATF00010, Germany). Membranes were blocked with 5% BSA (A9647, Biosharp, China) and incubated overnight at 4°C with the following primary antibodies: rabbit anti-iNOS (bs-0162R, Bioss, USA), rabbit anti-MTCO2 (bs-10431R, Bioss, USA), rabbit anti-NF-κB p65 (bs-0465R, Bioss, USA), rabbit anti-IκBα (bs-1287R, Bioss, USA), and rabbit anti-phospho-IκBα (Ser32 + Ser36) (bs-2513R, Bioss, USA). After the membranes were washed, they were incubated with HRP-conjugated secondary antibodies, and signals were visualized using an ECL detection system (ChemiScope 5300 Pro, China).

2.8 Data analysis

Proteomic bioinformatics analyses were conducted in the R statistical programming environment. Functional annotations were performed using Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), EggNOG, Pfam, UniProt subcellular localization databases. All in vitro experiments were performed in triplicate. Data are presented as mean ± standard deviation (SD). Statistical significance was determined using Tukey’s HSD test. Graphs were generated with GraphPad Prism 9.5.1.

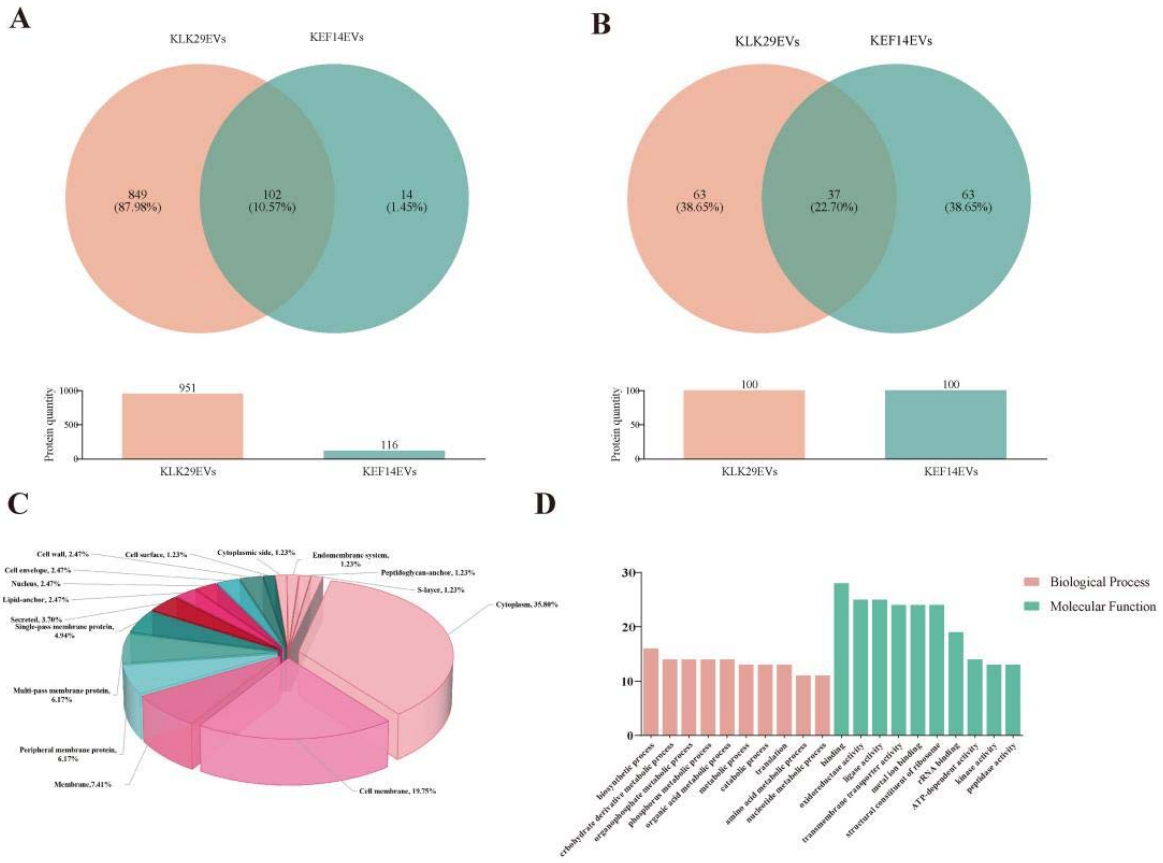
3. Results

3.1 Selection of LAB of kefir origin and isolation of their EVs

L. kefiri KLK29 and *E. faecium* KEF14 were selected as representative strains due to their critical roles in the formation of the kefir extracellular matrix and their potential functional characteristics[9]. EVs of strains were isolate using a combination of ultrafiltration and ultracentrifugation, following the guidelines for EV characterization established by the International Society for Extracellular Vesicles[23]. The detailed procedures and initial results of EV isolation have been described in a separate publication[9]. In short, these results confirm the successful acquisition of high-quality EVs from both KLK29 and KEF14.

3.2 Proteomic analysis of EVs derived from kefir LAB

To gain a deeper understanding of the functional potential of EVs of KLK29 and KEF14, label-free quantitative proteomic analysis was performed. A total of 951 proteins were identified in KLK29EVs and 116 proteins in KEF14EVs, as shown in Fig 1A. The substantial difference in protein quantity may be attributed to the relatively small genome size of *E. faecium* and its conservative EVs secretion mechanism[24]. Additionally, the broad distribution of *L. kefiri* in dairy environments and the extensive annotation of its proteins in public databases may have enhanced mass spectrometry identification efficiency, contributing to the higher number of identifiable proteins in KLK29EVs. To facilitate objective comparative analysis, the top 100 most abundant proteins (based on Log2 intensity) from each EV sample were selected. Of these, 37 proteins were shared between both samples, while 63 were specific to each (Fig 1B; Table S1).



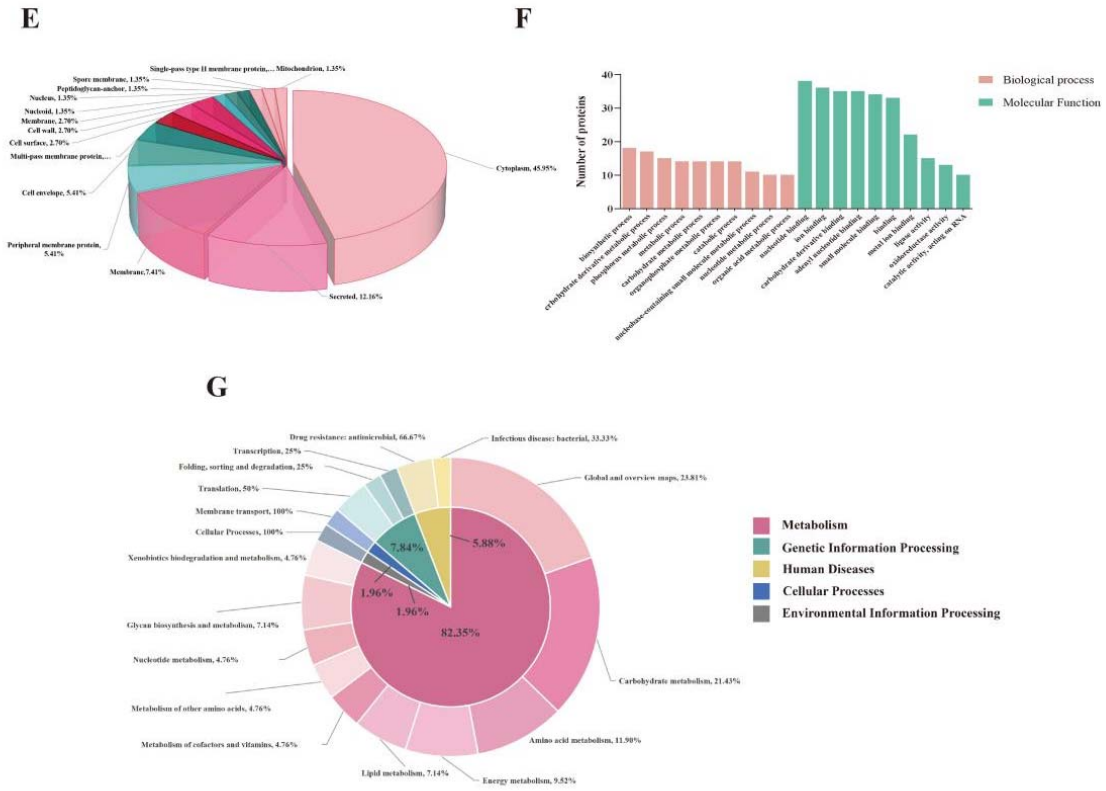


Fig. 1. Proteomic analysis of KLK29EVs and KEF14EVs. Venn diagram of all proteins identified in KLK29EVs and KEF14EVs (A). Venn diagram of the top 100 most abundant proteins in KLK29EVs and KEF14EVs (B). Subcellular localization of KLK29EV protein (C). GO annotation bar chart of the KLK29EV protein (annotated to the top 10 items in BP and MF rankings) (D). Subcellular localization of the KEF14EV protein (E). GO annotation bar chart of the KEF14EV protein (annotated to the top 10 items in BP and MF rankings) (F). The KEGG pathway diagram co-annotated with KLK29EVs and KEF14EVs (G). The inner circle represents the level 1 of KEGG, and the outer circle represents its level 2.

Functional annotation of EVs proteins. Bioinformatic analysis revealed that the subcellular localization (SL) of KLK29EV and KEF14EV proteins was primarily cytoplasmic, accounting for 35.8% and 45.95%, respectively (Figure 1A and 1C). This observation is consistent with previous findings on EVs of LAB by Lee et al.[25]. EV formation in gram-positive bacteria originates from plasma membrane budding, resulting in the selective encapsulation of cytoplasmic proteins[26]. The second most enriched localizations were the cell membrane (19.75%) for KLK29EVs and secreted proteins (12.16%) for KEF14EVs. This suggests that KLK29EVs incorporate more membrane components, which may enhance their structural stability and facilitate intercellular communication[26]. By contrast, KEF14EVs are enriched in secreted proteins, potentially improving their adaptability to environmental changes and their interactions with the host[22].

In the GO molecular function (MF) category (Figure 1B and 1D; Table S2), 91 KLK29EV proteins were annotated by the GO database, mainly associated with nucleotide binding (GO:0009116, 30.77%), small molecule binding (GO: 0098796, 27.47%), adenyl nucleotide binding (GO:0140097, 27.47%), ion binding (GO:0003924, 26.37%), and carbohydrate derivative binding (GO:0051082, 26.37%). For KEF14EVs, 89 proteins were annotated in the MF category, with notable functions including nucleotide binding (42.70%), ion binding (40.45%), carbohydrate derivative binding (39.33%), adenyl nucleotide binding (39.33%), and small molecule binding (38.20%). In the biological process (BP) category, 71 KLK29EV proteins were

annotated by the GO database, primarily associated with the biosynthetic process (GO:0009058, 22.54%), carbohydrate derivative metabolic process (GO: 1901135, 19.72%), organophosphate metabolic process (GO:0019637, 19.72%), phosphorus metabolic process (GO:0006793, 19.72%), and organic acid metabolic process (GO: 0006082, 19.72%). In comparison, 74 KEF14EV proteins were annotated, with major roles in the biosynthetic process (24.32%), carbohydrate derivative metabolic process (22.97%), phosphorus metabolic process (20.27%), metabolic process (GO:0008152, 18.92%), and carbohydrate metabolic process (GO:0005975, 18.92%). Both EV proteomes were enriched for carbohydrate-metabolism functions, suggesting a role in supplying precursors and energy for extracellular polysaccharide (EPS) biosynthesis.

KEGG annotation identified 64 pathways for KLK29EVs and 59 for KEF14EVs, with 51 shared (Fig. 1E; Table S3). At the level 1, Metabolism dominated (82.35%). Level 2 included: Global and overview maps (23.81%), Carbohydrate metabolism (21.43%), Amino acid metabolism (11.90%), Energy metabolism (9.52%), Lipid metabolism (7.14%), Glycan biosynthesis and metabolism (7.14%), Metabolism of cofactors and vitamins (4.76%), Metabolism of other amino acids (4.76%), Nucleotide metabolism (4.76%), Xenobiotic biodegradation and metabolism (4.76%). Importantly, both proteomes included QS (map02024) and ABC transporters (map02010), implicating cell - cell signaling and EV cargo transport.

In conclusion, KLK29EVs and KEF14EVs were enriched in pathways related to transport, signaling, and carbohydrate metabolism, which are potentially associated with inflammation regulation. This suggests that they may play a role in modulating host inflammation and highlights the need for further mechanistic studies.

3.3 EVs of kefir-derived LAB alleviate inflammation

3.3.1 Effects of varying EV concentrations on RAW 264.7 cell viability and LPS-induced inflammation

To assess the cytotoxicity and anti-inflammatory potential of KLK29EVs and KEF14EVs, RAW264.7 cells were treated with a range of EV concentrations: extremely low (0.1-1 μg/mL), low (1-10 μg/mL), medium (10-50 μg/mL), and high (50-500 μg/mL). Cell viability was then evaluated (Figure 2). In both the KLK29EV (Figure 2A) and KEF14EV (Figure 2B) groups, cell viability showed a slight decline in the 0.1-10 μg/mL concentration range, but the differences were not statistically significant ($P > 0.05$), indicating minimal cytotoxicity at these levels. However, at 25 μg/mL of KLK29EVs, a significant reduction in cell activity was observed ($P < 0.05$), suggesting that concentrations of ≥ 25 μg/mL have a cytotoxic effect on RAW 264.7 cells. Furthermore, the toxicity increased in a dose-dependent manner at higher concentrations. At a concentration of 25 μg/mL, KEF14EVs caused a significantly greater reduction in cell viability compared to KLK29EVs ($P < 0.001$), indicating stronger cytotoxicity at this concentration. To refine the analysis, five concentrations within a predefined range were selected for a more detailed assessment. Specifically, KLK29EVs were tested at 0.1, 1, 5, 10, and 20 μg/mL, while KEF14EVs were assessed at 0.1, 0.5, 1, 5, and 10 μg/mL (Figure 2A and 2B). The results showed that KLK29EVs maintained cell viability between 96.54% and 98.39%, while KEF14EVs maintained viability between 97.34% and 99.06%. No significant cytotoxicity was observed across these concentrations for either EV type, indicating their relatively safety for cells. A slight, non-significant decline in viability was observed, consistent with the effect of OB-EV on RAW264.7

cell viability studied by Zhang et al.[27]. This slight reduction may be attributed to the maternal origin of the EVs and does not reflect true cytotoxicity.

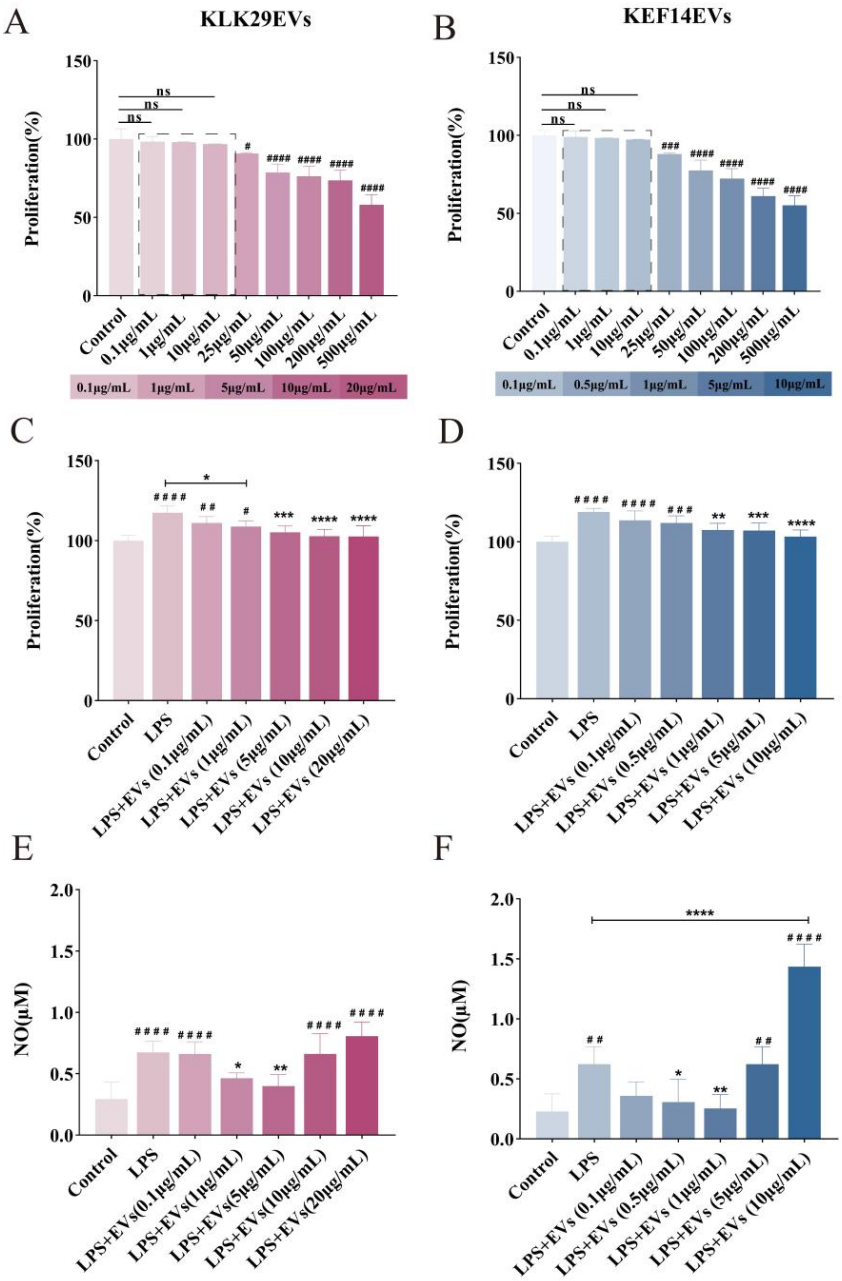


Fig. 2. Effects of different EV concentrations on RAW264.7 cell viability and the LPS-induced inflammatory response. The effects of varying EV concentrations (0.1, 1, 10, 25, 50, 100, 200, 500 μg/mL) on RAW264.7 cell viability (A, B), and the upper right figure shows the effects of the further preferred five EV concentrations on RAW264.7 cell viability (a,b). The effects of different concentrations (0.1, 1, 5, 10, 20 μg/mL) of KLK29EVs and (0.1, 0.5, 1, 5, 10 μg/mL) of KEF14EVs on the cell proliferation rate (C, D) and NO content (E, F) of LPS-induced RAW264.7 cells. (ns, no significance. #, *$P < 0.05$. ##, **$P < 0.01$. ###, ***$P < 0.001$. ####, ****$P < 0.0001$. # vs. control, * vs. LPS, calculated using Tukey's HSD test).

To investigate the anti-inflammatory potential of EVs, LPS-induced inflammatory RAW264.7 models were treated with the same concentration ranges of KLK29EVs and KEF14EVs. LPS stimulation significantly enhanced cell proliferation, confirming successful macrophage activation. Treatment with EVs at varying concentrations effectively attenuated this proliferation in a dose-dependent manner, suggesting a concentration-dependent inhibitory effect on inflammation (Figure 2C and 2D). This further confirms the

ability of EVs to regulate inflammation. As shown in Figure 2C, treatment with 1 μg/mL KLK29EVs significantly reduced cell proliferation compared with the LPS treatment ($P < 0.05$), although levels remained significantly higher than with the control treatment ($P < 0.05$). Higher KLK29EV concentrations (5, 10, and 20 μg/mL) resulted in an extremely significant reduction in proliferation ($P < 0.001$, $P < 0.0001$), with no statistical difference compared with the control group ($P > 0.05$). Similarly, KEF14EVs demonstrated a concentration-dependent inhibitory effect (Figure 2D). At concentrations of 1, 5, and 10 μg/mL, cell proliferation was significantly reduced compared with the LPS treatment ($P < 0.05$, $P < 0.001$, and $P < 0.0001$), with no significant difference from the control treatment ($P > 0.05$). These findings indicate that KLK29EVs (5-20 μg/mL) and KEF14EVs (1-10 μg/mL) exhibited no obvious toxicity to RAW264.7 cells while effectively reducing LPS-induced cell proliferation.

Because NO is a key pro-inflammatory mediator released by LPS-stimulated macrophages, its levels serve as a useful indicator of inflammatory status and offers an important basis for studying the inflammatory regulatory mechanism[28]. As shown in Figure 2E, NO levels decreased with increasing KLK29EV concentrations up to 5 μg/mL, followed by a rise at 10 and 20 μg/mL. Treatment with 1 and 5 μg/mL KLK29EVs significantly reduced NO content compared with the LPS treatment ($P < 0.05$, $P < 0.01$) and showed no significant variation compared with the control group ($P > 0.05$), highlighting their anti-inflammatory potential, with 5 μg/mL KLK29EVs being the most effective in inhibiting inflammation. KEF14EVs exhibited a similar trend (Figure 2F). NO levels in an inflammatory environment were consistent with those observed for KLK29EVs. The levels gradually decreased at 0.1, 0.5, and 1 μg/mL, and increased again at 5 and 10 μg/mL (Figure 2F). Compared with the LPS treatment, treatment with 0.5 and 1 μg/mL KEF14EVs significantly reduced the NO content ($P < 0.05$, $P < 0.01$), with no difference from the control treatment ($P > 0.05$). The 1 μg/mL KEF14EVs dose yielded the strongest anti-inflammatory effect. Interestingly, KLK29EVs provide sustained, broad-range modulation, whereas KEF14EVs deliver rapid, low-dose potency. These differences may stem from variations in the origin and mechanisms of EVs.

In summary, 5 μg/mL KLK29EVs and 1 μg/mL KEF14EVs demonstrated optimal anti-inflammatory effects while maintaining non-toxicity in RAW264.7 cells. Both reduced LPS-induced cell proliferation and NO production, making them promising candidates for further investigation in anti-inflammatory mechanism and potential therapeutic applications.

3.3.2 Regulatory effects of optimal EV concentrations on the inflammatory response of LPS-induced RAW264.7 cells

Following LPS stimulation, RAW264.7 macrophages underwent M1 polarization, characterized by significantly elevated expression of CD86, a classic M1 marker[29]. As shown in Figure 3B, CD86 expression in the LPS group (17.15%) was significantly higher than that in the control group (3.77%) ($P < 0.0001$), confirming the successful construction of an inflammation model and promotion of M1 macrophage polarization.

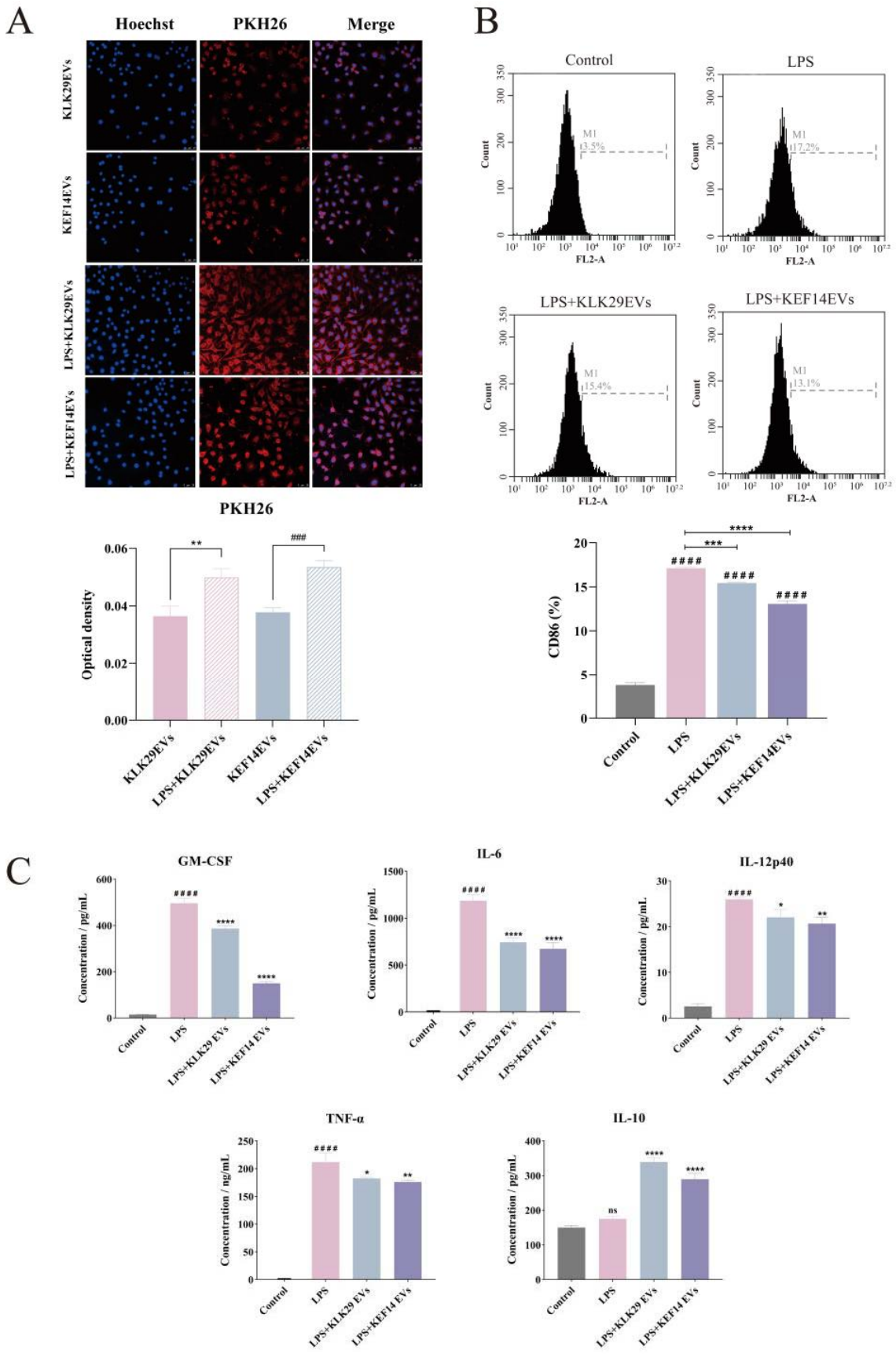


Fig. 3. EVs inhibit the inflammatory response triggered by LPS-induced RAW264.7 cells. EVs visualized through confocal microscopy and their degree of internalization by macrophages during inflammatory stimulation. Blue represents Hoechst-labeled nuclei, and red denotes PKH26-labeled EVs (A). The proportion of CD86 in different groups was determined through flow cytometry (B). The influence of different treatment groups on the concentration of inflammatory factors (C). (ns, there was no significance. #, *P < 0.05. ##, **P < 0.01. ###, ***P < 0.001. ####, ****P < 0.0001. # vs. control, * vs. LPS, calculated using Tukey's HSD test).

To assess EV uptake by macrophages and whether inflammation alters this process, fluorescently labeled KLK29EVs and KEF14EVs were incubated with both untreated and LPS-stimulated RAW264.7 cells. As shown in Figure 3A, both EV types exhibited strong perinuclear fluorescence signals in unstimulated cells, indicating efficient internalization. The fluorescence intensity of KEF14EVs appeared slightly higher than that of KLK29EVs, though the difference was not statistically significant ($P > 0.05$). Of note, inflammation triggered by LPS significantly enhances the cellular uptake of EVs. As shown in the PKH26 fluorescence bar chart, the fluorescence intensity of EVs derived from LPS-stimulated cells was significantly higher than that of unstimulated EVs (KLK29EVs: $P < 0.01$, KEF14EVs: $P < 0.001$). Although the fluorescence intensity of LPS-induced KEF14EVs was slightly higher than that of LPS-induced KLK29EVs, the difference was not statistically significant ($P > 0.05$).

Flow cytometry analysis was conducted to evaluate the effects of different EV treatments on the polarization status of RAW264.7 cells. As shown in Figure 3B, both the KLK29EV (15.43%, $P < 0.001$) and KEF14EV (13.01%, $P < 0.0001$) groups significantly reduced CD86 expression compared with the LPS group, indicating a strong inhibitory effect on M1 macrophage polarization. KEF14EVs exhibited a slightly greater inhibition than KLK29EVs. These findings suggest that both KLK29EVs and KEF14EVs exert anti-inflammatory effects, though further evaluation using additional indexes is warranted to comprehensively understand their mechanisms.

To further investigate whether EVs could suppress LPS-induced inflammation, a multiplex liquid-phase suspension chip assay was employed. As shown in Figure 3C, the LPS group significantly increased the levels of pro-inflammatory cytokines (GM-CSF, IL-6, IL-12p40, and TNF-α), whereas IL-10 did not significantly change compared with the control ($P > 0.05$). Following EVs treatment, both KLK29EV and KEF14EV groups exhibited a significant reduction in GM-CSF and IL-6 levels ($P < 0.0001$). IL-12p40 and TNF-α were significantly reduced in the KLK29EV group ($P < 0.05$), and even more so in the KEF14EV group ($P < 0.01$). Meanwhile, both EV treatments significantly upregulated IL-10 levels ($P < 0.0001$), with KLK29EVs showing a slightly stronger effect in enhancing the ability of resistance factors. The results indicate that KLK29EVs and KEF14EVs effectively modulate macrophage inflammatory responses by reducing pro-inflammatory cytokine secretion and enhancing anti-inflammatory cytokine production. This dual regulatory effect suggests that EVs exert their anti-inflammatory functions by modulating key inflammatory signaling pathways. Considering the central role of the NF-κB signaling pathway in inflammation, we next examined whether KLK29EVs and KEF14EVs regulate this NF-κB pathway, thereby deeply revealing their mechanism of action in inflammation regulation.

3.3.3 Inhibition of NF-κB signaling by EVs

To probe the mechanism, we specifically examined NF-κB signaling. As shown in Figure 4, LPS induction markedly increased the IκBα phosphorylation level ($P < 0.0001$) and p65 expression ($P < 0.0001$), indicating activation of the NF-κB signaling pathway. Upon treatment with either KLK29EVs or KEF14EVs, the ratio of p-IκBα/IκBα significantly decreased ($P < 0.0001$), and p65 expression was also significantly

reduced (KLK29EVs: $P < 0.0001$, KEF14EVs: $P < 0.001$). These findings suggest that both EV types suppress the activation of the NF-κB signaling pathway.

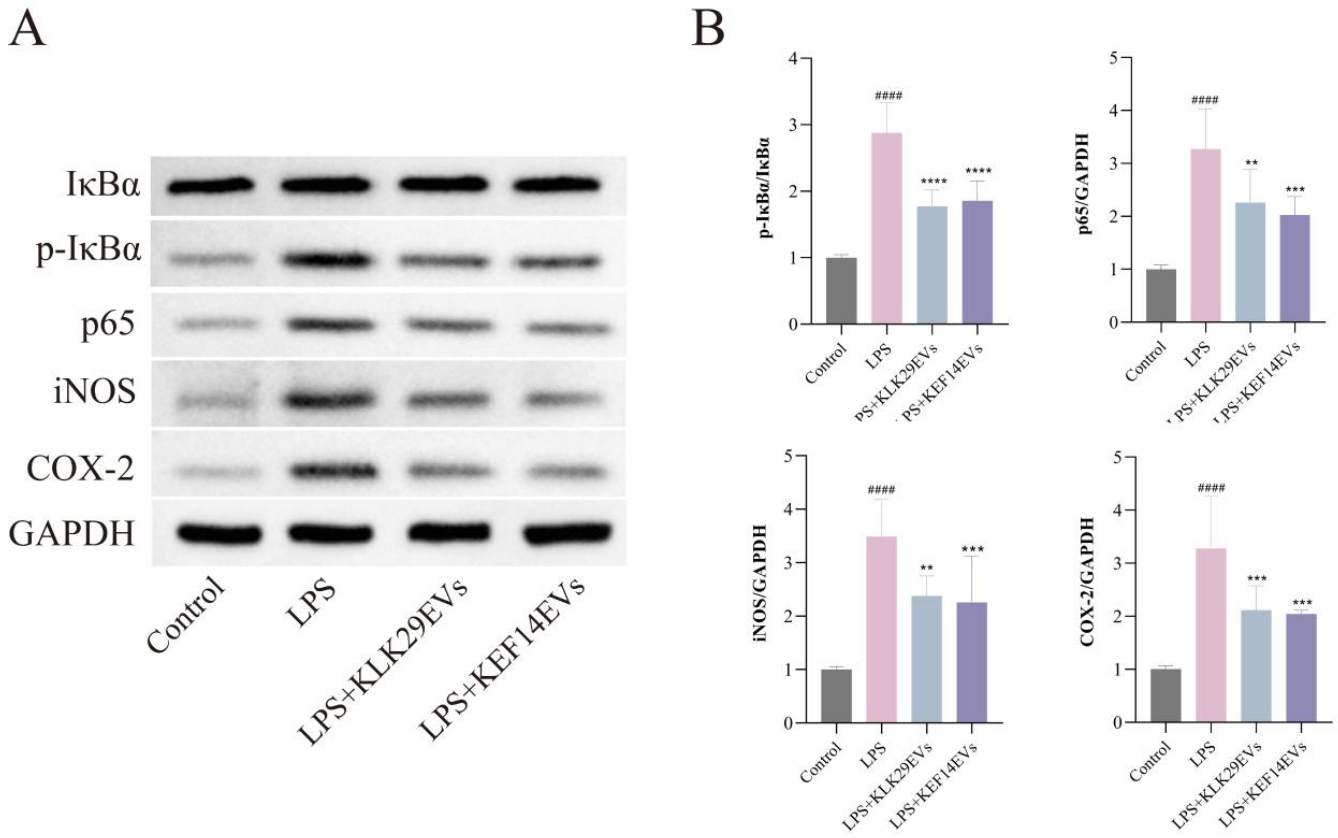


Fig. 4. Western blotting of the expression of NF-κB signaling pathway-related proteins in different treatment groups. Representative western blot images of p65, p-IκBα, IκBα, iNOS, and COX-2 in different treatment groups (A). The relative expression levels of p65, iNOS, and COX-2 proteins and the relative quantification of IκBα phosphorylation levels in different treatment groups (B). The data were normalized based on the gray values of western blotting images, and the results were expressed as mean ± SD (n = 6). (#, *$P < 0.05$. ##, **$P < 0.01$. ###, ***$P < 0.001$. ####, ****$P < 0.0001$. # vs. control, * vs. LPS, calculated using Tukey's HSD test)

The expression of iNOS and COX-2, downstream targets of the NF-κB signaling pathway, was significantly upregulated following LPS induction ($P < 0.0001$), reflecting pathway activation and downstream transcriptional activity. Treatment with KLK29EVs or KEF14EVs significantly reduced iNOS levels (KLK29EVs: $P < 0.01$, KEF14EVs: $P < 0.001$) and markedly decreased COX-2 levels ($P < 0.0001$). These changes align with the observed reductions in NO production and inflammatory cytokines, further supporting the conclusion that EVs mitigate inflammation by inhibiting the NF-κB signaling pathway. Based on the aforementioned results, the action mechanism diagrams of KLK29EVs and KEF14EVs regulating inflammatory cells are presented in Figure 5.

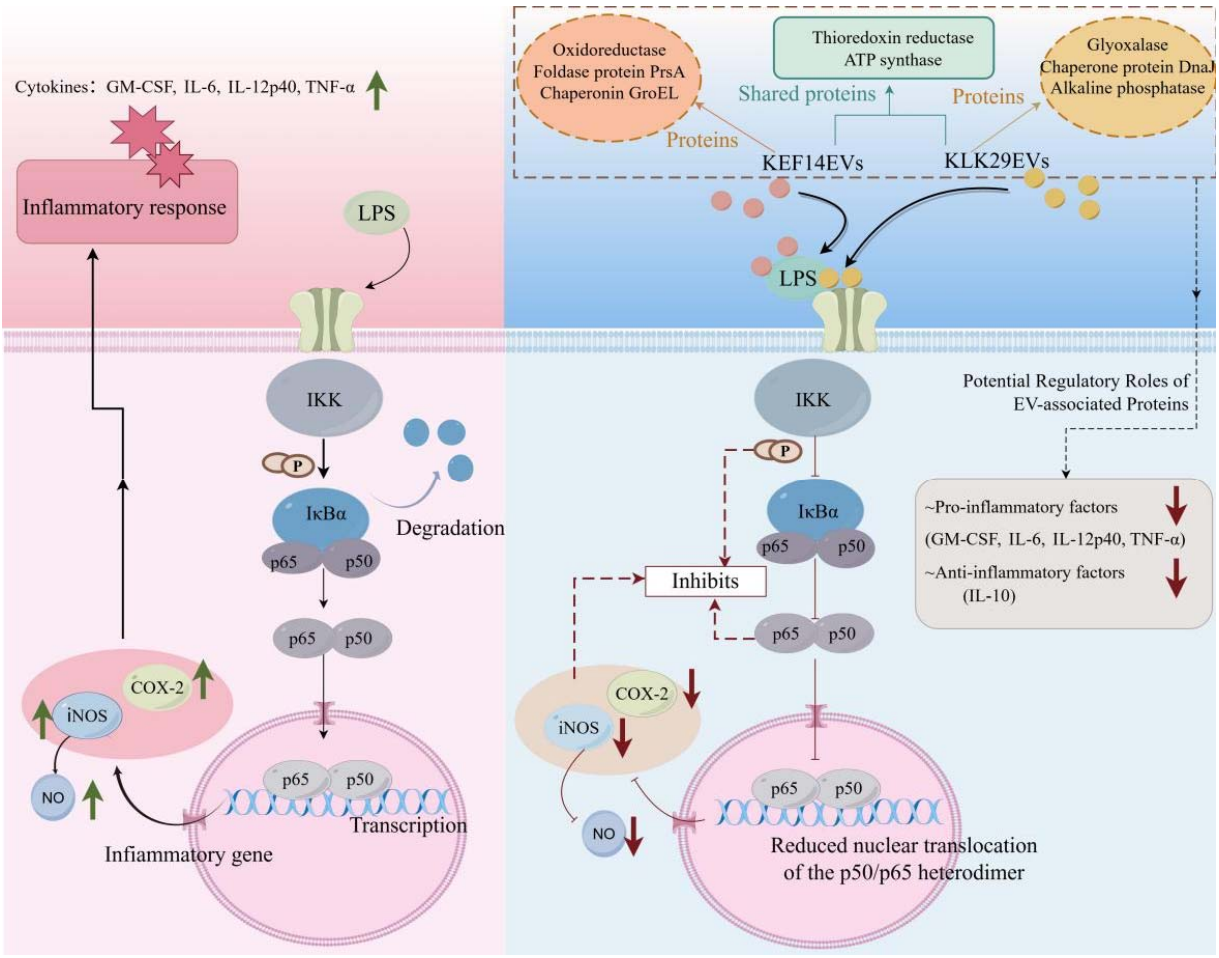


Fig. 5. In the LPS-induced inflammation model, KLK29EVs and KEF14EVs regulate the NF-κB pathway. The green arrow indicates LPS-mediated NFKB pathway activation, and the red arrow indicates the regulatory effect of KLK29EVs and KEF14EVs on this inflammatory pathway. The red dotted line indicates the potential anti-inflammatory regulation of EV-related proteins.

4. Discussion

Microbe-derived EVs are widespread and have been explored for diverse applications[19, 27, 28]. Recently, food-derived EVs have attracted attention because of their safety and probiotic potential[22, 29, 30]. In this study, we profiled kefir-derived LAB EVs at the proteomic level and showed that they alleviate LPS-induced inflammation in macrophages. Our findings suggest that kefir LAB EVs act mainly by suppressing NF-κB activation and rebalancing pro- and anti-inflammatory cytokines.

Bioinformatics analysis of proteomics data showed KLK29EVs and KEF14EVs had similar GO-MF annotations. Both were enriched in nucleotide binding, ion binding, small molecule binding, and carbohydrate binding. These functions point to roles in energy regulation, signaling, transport, glucose metabolism, and stress response—important for microbial adaptation and host interaction[22, 31-34].GO-BP analysis showed that KLK29EV proteins were more enriched in sugar metabolism and derivative synthesis than KEF14EVs, including the carbohydrate metabolic process, carbohydrate derivative metabolic process, nucleotide-sugar metabolic process, and monocarboxylic acid metabolic process (Table S2). These pathways are critical for acquiring carbohydrate precursors and facilitating transmembrane transport, suggesting a more crucial role for KLK29EVs in promoting EPS synthesis, consistent with previous results[9]. Furthermore, KLK29EVs were

enriched with oxidoreductases involved in stress response processes such as response to stress (GO:0006950), oxidative stress (GO:0006979), and response to stimulus (GO:0050896). KEF14EVs, meanwhile, were enriched with glyoxalase enzymes linked to the response to a toxic substance (GO:0009636). These findings imply that both EV types—particularly KLK29EVs—may contribute to host immune modulation and exert anti-inflammatory effects through stress-response pathways[35-38].

Meanwhile, KLK29EV and KEF14EV proteins are involved in protein folding (GO:0006457), including foldase protein PrsA and chaperonin in GroEL in KLK29EVs, and chaperone protein DnaJ in KEF14EVs, as well as in maintaining cell redox homeostasis (GO:0045454), such as through thioredoxin reductase. These proteins are closely related to cellular homeostasis[39, 40]. They may contribute to stabilizing the internal environment of host cells under stress by repairing damaged proteins and regulating the cellular redox state[40, 41]. Thus, KLK29EVs and KEF14EVs may help maintain host health. KEGG results show both share inflammation-related pathways(Table S3). Quorum sensing (QS) regulates bacterial behaviors like biofilm and EPS production[42-44] and can also affect host inflammatory mediators[45-47]. Oxidative phosphorylation, while essential for cellular energy production, can lead to excessive reactive oxygen species generation, triggering oxidative stress and subsequent inflammatory responses[48, 49]. Both KLK29EVs and KEF14EVs were found to contain ATP synthase-related proteins involved in this pathway, including the ATP synthase α, β, δ, and c subunits, and ATP synthase γ chain. Similar observations have been reported in proteomic studies of EVs from other bacteria[50-52]. These enzymes may help modulate the efficiency of oxidative phosphorylation and maintain redox homeostasis of cells, suggesting that ATP synthase could indirectly influence immune regulation by reducing oxidative stress, contributing to potential anti-inflammatory effects[48, 53, 54]. The ABC transporter pathway is also implicated in inflammation regulation. ABC transporter proteins have been shown to attenuate inflammatory diseases, such as atherosclerosis, by promoting reverse cholesterol transport and reducing lipid accumulation in macrophages[55-57]. In addition, the two-component system (TCS) identified in KLK29EV proteins represents a principal mechanism by which bacteria sense and respond to external stimuli. TCS can regulate diverse physiological processes, including stress responses and redox regulation[58]. Studies have confirmed that TCS influences probiotic activity and modulates inflammatory responses through the secretion of metabolites[59]. In KEF14EVs, alkaline phosphatases involved in metabolic pathways, such as glycerol phospholipid metabolism and phosphatidic acid biosynthesis, have been demonstrated-whether endogenously or exogenously supplemented-to significantly alleviate various inflammatory conditions[60]. Collectively, the proteomic results suggest that KLK29EV and KEF14EV proteins have promising potential in regulating inflammatory responses.

Regarding the viability of RAW264.7 cells treated with different EVs concentrations, a slight reduction in cell viability was observed following exposure to LAB-derived EVs, which is inconsistent with the results of some studies indicating that EVs from certain bacterial strains enhance cell viability[61]. Importantly, this does not imply that EVs exert a detrimental effect in our study. This observed variability may be attributed to the source-specific nature of EVs, as different EV components may elicit distinct adaptive cellular responses,

regulating cell behavior to maintain homeostasis or provide protection to cells[62, 63]. This specificity underscores the complexity and functional diversity of EVs in cellular response mechanisms. We also assessed NO production in LPS-induced inflammatory models treated with varying EV concentrations. KLK29EVs exhibited anti-inflammatory activity at extremely low concentrations (0.1 μg/mL), with peak inhibitory effects at 5 μg/mL. However, at higher concentrations (10 and 20 μg/mL), this effect gradually diminished, and NO production began to increase. KEF14EVs exhibited rapid suppression of NO at extremely low concentrations (0.1-0.5 μg/mL), reaching maximal inhibition at 1 μg/mL. Subsequently, in treatments above 5 μg/mL, KEF14EVs began to promote NO production, with a sharp increase in NO content observed at 10 μg/mL. These findings indicate that KLK29EVs and KEF14EVs exert concentration-dependent effects on inflammatory regulation. KLK29EVs showed a gradual and sustained anti-inflammatory effect across a broad concentration range, consistent with the role of *L. kefiri* in chronic inflammation[64, 65] and long-term immune regulation[13, 66]. In contrast, KEF14EVs acted rapidly and strongly at low doses, reflecting the capacity of *E. faecium* to swiftly adapt to environmental changes[67]. Some *E. faecium* strains suppress NF-κB and JNK(AP-1) activation in host cells[67]. Others modulate inflammation through non-MyD88 TLR2 signaling, leading to reduced inflammatory factor expression. Both mechanisms contribute to their anti-inflammatory effects[68]. As secretory products of *E. faecium*, EVs retain the parent strain's immunomodulatory functions but may enhance inflammatory regulation due to their efficient delivery mechanism[16]. Therefore, they may exert more rapid effects on host immune modulation than the bacteria themselves. At higher EVs concentrations, NO levels rose in both groups, particularly with KEF14EVs, but without cytotoxicity. This effect may result from iNOS overactivation or oxidative stress-related signaling, which drive excess NO release[69]. The precise mechanisms underlying this phenomenon warrant further exploration.

Visualization of EV internalization by macrophages under inflammatory conditions revealed that LPS induction significantly improved EV uptake. LPS exposure may trigger self-defense mechanisms that enhance EV uptake. On the one hand, macrophages increase phagocytic capacity[70]; on the other, membrane permeability rises, facilitating entry[71]. In addition, LPS may alter EV communication[72] or surface proteins[73], further improving uptake efficiency.

KLK29EVs and KEF14EVs can modulate inflammatory responses by inhibiting pro-inflammatory factors and promoting anti-inflammatory ones. Numerous studies have confirmed that EVs derived from *Lactobacillus* play crucial roles in regulating various inflammatory environments. For example, *Lactobacillus plantarum* Q7-EVs significantly reduced levels of pro-inflammatory cytokines in a DSS-induced model[74]; *Lactobacillus kefirgranum* PRCC 1301-EVs inhibited pro-inflammatory cytokine expression in Caco-2 cells[75]; and *Lactobacillus reuteri* BBC3-EVs alleviated LPS-induced intestinal injury and inflammation by regulating cytokine gene expression[76]. These studies further support the anti-inflammatory potential of KLK29EVs. Although research on EVs from *Enterococcus* species is limited, existing studies have suggested that EVs from *E. faecium* also contribute positively to inflammation regulation. For instance, Luo et al.[16] demonstrated that *E. faecium*-EVs pretreatment effectively alleviated ethanol-induced gastric inflammation

and promoted gastrointestinal recovery. Similarly, Sun et al. confirmed the anti-inflammatory effects of *E. faecium*-EVs in a mouse[18]. These findings reinforce the potential of *E. faecium*-EVs (KEF14EVs) in modulating inflammation.

The NF-κB signaling pathway is one of the most well-established mechanisms for regulating inflammatory responses[77]. Upon inflammatory stimulation, the NF-κB signaling pathway is activated through a cascade: IκB kinase is activated, leading to the phosphorylation and degradation of the IκB protein, which releases the NF-κB dimer to translocate into the nucleus and initiate the transcription of pro-inflammatory genes[78]. This study found that treatment with KLK29EVs and KEF14EVs inhibited IκBα phosphorylation, thereby retaining NF-κB dimers in the cytoplasm. Additionally, reduced p65 expression directly inhibited NF-κB transcriptional activity. By downregulating key downstream targets of the NF-κB signaling pathway, including iNOS and COX-2, both EVs effectively attenuated inflammatory signaling.

Bacterial EVs have previously been shown to modulate NF-κB activity in immune cells, such as Lactobacillus plantarum EVs suppressing NF-κB–dependent cytokine production.

Bacterial EVs have previously been reported to modulate NF-κB activity—for example, EVs from *L. plantarum* Q7[74]、*L. rhamnosus* (ATCC 7469)[79] and *L. gasseri* GFC-1220[80]. Our findings with kefir-derived LAB EVs are consistent with these observations: lower p-IκBα/IκBα ratios, reduced p65, and decreased iNOS/COX-2, indicating attenuation of NF-κB signaling. While other pathways may also contribute, these data provide mechanistic insight into the anti-inflammatory effects of KLK29EVs and KEF14EVs and support the potential of fermented food–derived bacterial EVs for therapeutic development.

5. Conclusion

In this study, we systematically profiled the proteomic composition of kefir-derived LAB EVs and validated their regulatory effects in an LPS-induced macrophage inflammation model. Both KLK29EVs and KEF14EVs were enriched in proteins associated with carbohydrate metabolism, substrate transport, and quorum sensing, which may contribute to nutrient supply, signal transduction, environmental adaptation, and host-microbe interactions. In the inflammation-regulation experiments, the two EV types were effectively internalized by macrophages at non-cytotoxic doses, resulting in reduced NO release, suppressed M1 polarization (CD86), downregulated pro-inflammatory cytokines, and upregulated anti-inflammatory cytokines, collectively constituting a comprehensive anti-inflammatory effect. In parallel, NF-κB pathway–related markers and downstream effectors were decreased. These findings indicate that KLK29EVs and KEF14EVs can effectively alleviate LPS-induced inflammatory phenotypes and support their potential as bioactive components in fermented foods.

Nevertheless, this study has certain limitations: it focused exclusively on LAB-derived EVs, without considering those released by other key members of the kefir microbiota (e.g., yeasts and acetic acid bacteria). The functional similarities, differences, and potential complementary effects of EVs from different microbial sources therefore remain to be clarified. The interactions among microbial EVs and their roles in

community homeostasis are not yet well understood. Moreover, Proteomic findings indicate only a potential association between EV protein cargos and immune regulation.

Future research should integrate multi-omics approaches (e.g., proteomics, transcriptomics, lipidomics and glycomics) to elucidate the compositional diversity, functional properties, and cooperative networks of kefir-derived EVs. On the basis of standardized EV isolation and quantification, combined in vivo and in vitro models are required to dissect the roles of key EV cargo-receptor signaling axes in NF-κB inhibition and cytokine regulation. Such efforts may contribute to the optimization of functional food formulations and provide a translational path from food-derived bioactive substances to therapeutic applications for inflammation-related diseases. Additionally, evaluating synergistic interactions among multi-source EVs in co-culture and their impact on bioactive metabolites (e.g., EPS) will be essential to elucidate the mechanisms underpinning kefir microbial ecosystem stability.

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.

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