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The Prevention Effect of *Lactobacillus plantarum* 17–5 on *Escherichia coli*-Induced Mastitis in Mice

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Abstract

Mastitis is the most economically important disease affecting the dairy industry worldwide. *Lactobacillus plantarum*, an important probiotic with a wide range of applications, has potential anti-inflammatory properties and has become a currently strong candidate for mastitis therapies. In the current study, we evaluated the prevention effect of *Lactobacillus plantarum* 17–5 on *Escherichia coli*-induced mastitis in mice. The results showed that pretreatment with *L. plantarum* 17–5 maintained the integrity of tight junctions; improved inflammatory injury; decreased MPO activity and the mRNA expression levels of *IL1β*, *IL6*, and *TNFα*; and inhibited the NF-κB and MAPK signaling pathways in mice mammary tissue. The results indicated that *Lactobacillus plantarum* 17–5 had excellent anti-inflammatory activities and could be developed into microecological preparation for clinical use to prevent mastitis.

Keywords *Lactobacillus plantarum* · *Escherichia coli* · Mastitis · Tight junction · Inflammation · NF-κB · MAPK

Introduction

Dairy cow mastitis is an inflammatory disease worldwide and causes severe economic losses in the dairy industry due to decreased milk production, higher veterinary care costs, and increased culling of dairy cows [1, 2]. *Escherichia coli* is one of the main pathogens in dairy cow mastitis and is widely present in bovine feces, humid soil, and composts [3]. Recent studies revealed that *E. coli* often causes acute inflammatory responses and might contribute to extensive mammary tissue damage [4]. There are currently no medications or other prophylactic methods effective against this disease, and common treatments are antibiotic treatment [5]. However, misuse of antibiotics inevitably leads to multi-antibiotic resistance and antibiotic residue, which causes threats to human and animal health globally [6]. Therefore, there is an urgent need to find effective and safe alternative antimicrobial agents for conventional antibiotics.

Lactobacillus plantarum is one of the most widely used probiotics with great beneficial effects on human and animal health [7]. Existing studies have shown that *L. plantarum* can produce lactic acid and various metabolites during colonization, which can effectively abrogate pathogenic bacteria growth and modulate immune functions [8]. In addition, some metabolites of *L. plantarum* may have anti-inflammatory properties in addition to their antimicrobial effects; this feature provides its therapeutic potential for various inflammatory diseases [9]. Fernsndez et al. found that oral administration of *L. salivarius* PS2 positively affected the prevention of infectious mastitis in late pregnancy [10]. Frola et al. have stated that intramammary infusion of *L. plantarum* CRL 1716 was an effective way of treating dairy cow mastitis [11]. Previous studies performed by our research team showed that *L. plantarum* 17–5 could attenuate *E. coli*-induced inflammatory responses in bovine mammary epithelial cells [12]. However, the effect of intramammary infusion of *L. plantarum* 17–5 on mice mastitis and its mechanism of action remains unclear. Here, our study establishes the murine model of mastitis using *E. coli*. The aim is to determine whether *L. plantarum* 17–5 has prevention effects on mastitis in vivo and provide a basis for developing and utilizing microecological agents.

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Materials and Methods

Bacteria and the Culture Conditions

The *Lactobacillus plantarum* 17–5 strain (ATCC 8014, provided by American Type Culture Collection, Manassas, VA, USA) was cultivated statically in de Man, Rogosa, and Sharpe (MRS) broth (Aobox, Beijing, China) at 37 ℃ under microaerobic conditions. *Escherichia coli* O111:K58 (CVCC1450, provided by China Constitute of Veterinary Drug Centre, Beijing, China) was grown overnight in Luria Broth (LB) medium (Aobox, Beijing, China) at 37 ℃ with shaking. The number of colony-forming units (CFUs) was counted after three generations.

Animals and Experiment Design

SPF-grade male and female Kunming mice (8 weeks old) were purchased from Liaoning Changsheng Biotechnology Corporation (Benxi, China). Females and males were placed in the same microisolator cage at a ratio of 2:1 until the females were pregnant, and water and food were provided ad libitum. Animal assays were approved by the Animal Ethics Committee of Hebei Agricultural University (protocol number 2020044). The mouse mastitis model was established by referring to previous studies [13, 14]. Briefly, after ether anesthesia, the tip of the L4 and R4 abdominal mammary glands was carefully snipped, and bacteria or PBS was injected into the mammary ducts 7 days after delivery. The lactating mice were randomly divided into six groups ($n=8$): the control group (PBS), the *E. coli* group (10^7 CFU/100 μL), *L. plantarum* (10^5, 10^6, and 10^7 CFU/100 μL) + *E. coli* and the *L. plantarum* group (10^7 CFU/100 μL). The *L. plantarum* or PBS was injected into each side of the nipple for 3 h prior to adding *E. coli* and then injected with *E. coli* using the same method. At 24 h after the last injection, mice were sacrificed, and the mammary gland tissues were collected and stored at − 80 ℃ until further analysis.

Histopathological Evaluation

The mammary tissues of the mice were observed for general condition and scored using a clinical scoring system ranging from 1 to 5, with higher scores indicating greater tissue damage. Specifically, 1 represents no damage, 2 represents slight redness, 3 represents slight redness and minor bleeding, 4 represents moderate redness and bleeding, and 5 represents severe redness and bleeding. Subsequently, tissue samples were fixed in 4% paraformaldehyde solution, dehydrated with gradient ethanol, and then embedded in paraffin.

The paraffin-embedded tissue sections were cut into 5 μm thickness, stained with hematoxylin and eosin (HE), and examined under an optical microscope. The same histological score (1 to 5) previously described was used for evaluating the degrees of tissue damage (necrosis and neutrophil and macrophage infiltration). The higher the score, the more serious the injury.

Immunofluorescence Staining

Paraffin sections were dewaxed with water, antigen repaired with sodium citrate, and blocked with 5% BSA (Solarbio, Beijing, China). Then, slides were incubated with primary antibody against claudin-3 (1:500; Bioss, Beijing, China) overnight at 4 ℃, then in FITC-labeled secondary antibody (1:200; Solarbio, Beijing, China) for 1 h at room temperature. After counterstaining with DAPI (Solarbio, Beijing, China), the fluorescence was observed under a fluorescence microscope.

MPO Activity Determination

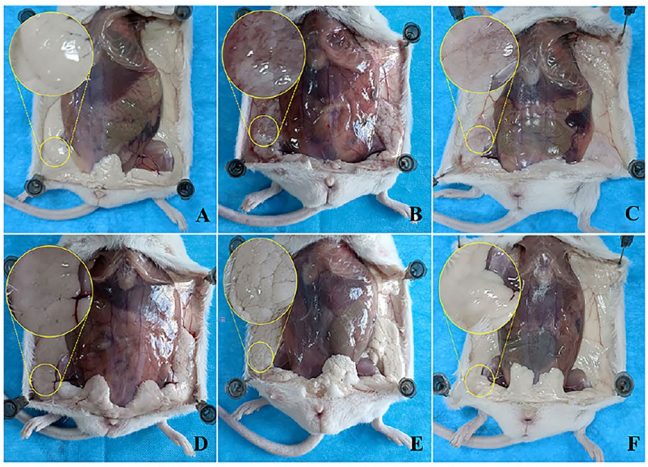
The mammary tissues were homogenized, and the homogenates were centrifuged at 2500 rpm for 10 min at 4 ℃ to obtain supernatants. The activity of MPO in mammary tissue homogenates was assayed using MPO Detection Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol.

qRT–PCR Analysis

Total RNA was extracted from mammary tissue using the Ultrapure RNA extraction kit (CWBio, Beijing, China). The concentration and purity of RNA samples were measured by NanoDrop-2000 (Thermo Scientific, DE, USA), and the integrity was detected by agarose gel electrophoresis. Then, RNA was reversely transcribed into cDNA using a reverse transcription kit (US Everbright Inc, CA, USA), and quantitative real-time PCR was performed according to the following procedures: 300 s at 95 ℃ followed by 45 cycles of 5 s at 95 ℃, 30 s at 57 ℃ and 15 s at 72 ℃. The efficiency of the amplification was evaluated by establishing the standard

Table 1 The primer sequences used for qRT-PCR

Gene	Primer sequence (5′-3′)
IL1β	TGAAATGCCACCTTTTGACAG
	CCACAGCCACAATGAGTGATAC
IL6	TGCCTTCTTGGGACTGAT
	CTGGCTTTGTCTTTCTTGTT
TNFα	GCCTCCCTCTCATCAGTTCTA
	GGCAGCCTTGTCCCTTG
GAPDH	AGGTCGGTGTGAACGGATTTG
	GGGGTCGTTGATGGCAACA
β-actin	TGCTGTCCCTGTA TGCCTCT
	GGTCTTTACGGA TGTCAACG



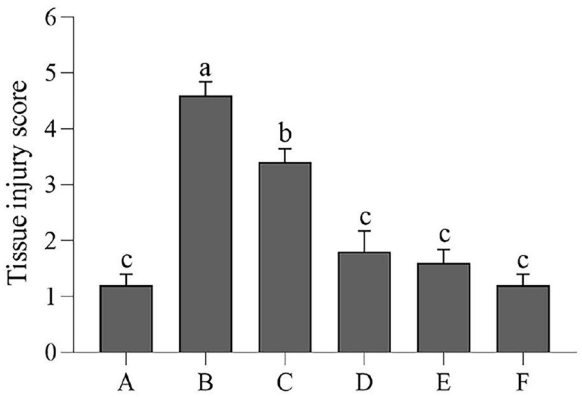


Fig. 1 Effect of *L. plantarum* 17–5 on the histopathological impairment in the mice mammary tissue. **A** The control group. **B** The *E. coli* group. **C**–**E** 10^5, 10^6, and 10^7 CFU/100 μL *L. plantarum* + *E. coli* group. **F** The *L. plantarum* group. The injury score from each group ranged from 1 to 5 with higher scores indicating greater tissue damage. Data shown as means ± SEM ($n = 5$) and different letters indicate significance at $P < 0.05$. The same as the following figures

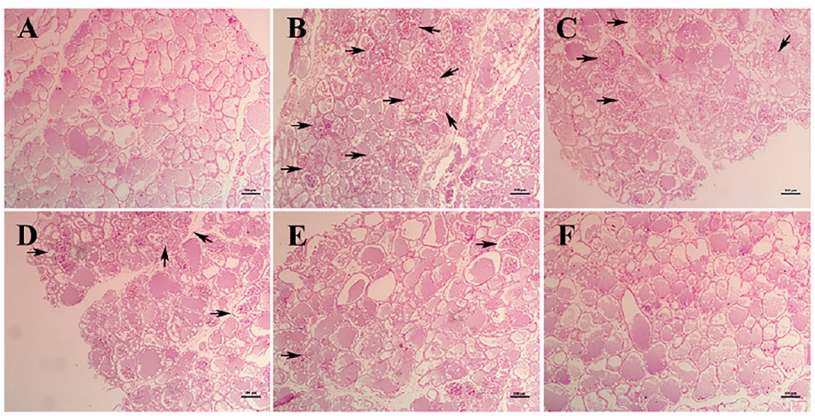
curve. Gene relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to the expression of *GAPDH* and *β-actin*. Primer sequences were listed in Table 1.

Western Blot Analysis

Total protein from mammary tissue was extracted using RIPA lysis buffer (Solarbio, Beijing, China), and its concentration was quantified by BCA protein assay kit (Solarbio, Beijing, China). A total of 30 μg of protein from each sample were separated by 10% SDS-PAGE gels, electrotransferred onto nitrocellulose membranes (Beyotime, Shanghai, China) and then blocked with 5% skim milk. The membranes were incubated with primary antibodies against claudin-3 (1:1000), occludin (1:1000), NF-κB p65 (1:1000), NF-κB phospho-p65 (1:1000), phospho-IκBα (1:500), and β-actin (1:1000) from Bioss Biotech Limited Company (Beijing, China) and antibodies against p38 (1:1000), phospho-p38 (1:1000), ERK (1:1000), phospho-ERK (1:2000), JNK (1:1000), phospho-JNK (1:1000), and IκBα (1:1000) from Cell Signaling Technology (MA, USA). After incubation with a secondary antibody (1:2000; Zhongshan Golden Bridge, Beijing, China), the NBT/BCIP color development kit (Solarbio, Beijing, China) was used to visualize the stainings, and ImageJ software (ImageJ Software Inc., MD, USA) was used for densitometric analyses of western blot bands.

Statistical Analysis

All data in this study was shown as means ± standard error of the mean (SEM). Comparisons between multiple independent groups were performed by one-way ANOVA and Tukey's or Dunnett's T3 tests. *P* values < 0.05 were considered significantly different.



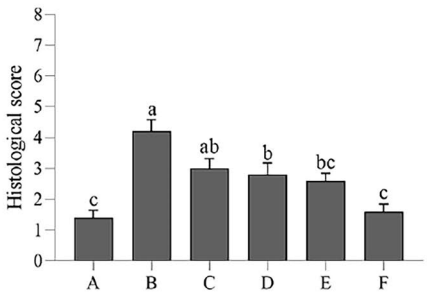


Fig. 2 Effect of *L. plantarum* 17–5 on the histopathological changes in the mice mammary tissue (H&E 100×). **A** The control group. **B** The *E. coli* group. **C**–**E** 10^5, 10^6, and 10^7 CFU/100 μL *L. plantarum* + *E. coli* group. **F** The *L. plantarum* group. Scale bars: 100 μm. The histological score from each group ranged from 1 to 5 with higher scores indicating greater tissue damage. Data were the mean ± SEM ($n = 5$)

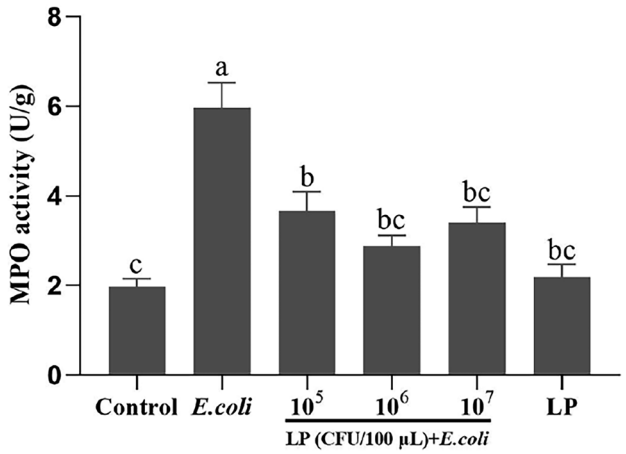


Fig. 3 MPO activity in mammary tissue from the control group, the *E. coli* group, and pretreatment with 10^5, 10^6, and 10^7 CFU/100 μL *L. plantarum* (LP) and 10^7 CFU/100 μL *L. plantarum* group. Data were expressed as means ± SEM ($n = 5$)

Results

Effect of *L. plantarum* 17–5 on Histopathological Changes in Mice Mammary Tissue

No visible redness, swelling, or bleeding were seen in the mammary tissues in the control group, high dose of *L. plantarum* pretreatment group, and the *L. plantarum* group, and the tissue injury scores were significantly lower ($P < 0.05$) in three doses of *L. plantarum* pretreatment groups compared with the *E. coli* group (Fig. 1). Obvious inflammatory changes were observed in the mammary tissues of the *E. coli* group with infiltration of neutrophils and macrophages in the mammary acini, ducts, and connective tissue. However, these histopathological changes were ameliorated in the *L. plantarum* pretreatment group, with a significant reduction ($P < 0.05$) in histological scores among medium and high doses of *L. plantarum* pretreatment groups (Fig. 2).

Effect of *L. plantarum* 17–5 on the MPO Activity in the Mammary Glands

As shown in Fig. 3, the MPO activity in the *E. coli* group increased significantly ($P < 0.05$) compared with the control group. Pretreatment with different doses of *L. plantarum* 17–5 significantly ($P < 0.05$) reduced these increases.

Effect of *L. plantarum* 17–5 on Tight Junction Proteins in the Mammary Glands

Immunofluorescence staining for the claudin-3 was performed in mammary gland sections (Fig. 4A). In control and *L. Plantarum* groups, claudin-3 was localized to the cell membrane at cell–cell contacts and showed a complete and continuous structure. In the *E. coli* group, the claudin-3 positive signals were intermittent and markedly weaker than the above groups showing that the tight junctions were disrupted. Pretreatment with *L. plantarum* alleviated the *E. coli*-induced damage in tight junction proteins.

Fig. 4 Effects of *L. plantarum* 17–5 on the structure and protein expression in the tight junction proteins. **A** Representative images of the FITC albumin staining in each group. Green shows the claudin-3 signal and blue shows the DAPI signal. (**a**) The control group. (**b**) The *E. coli* group. (**c**-**e**) 10^5, 10^6, and 10^7 CFU/100 μL *L. plantarum* + *E. coli* group. (**f**) The *L. plantarum* group. Scale bars: 100 μm. **B** Representative western blots showed expression of claudin-3 and occludin in each group. Data were expressed as means ± SEM from three independent experiments

To further evaluate the effect of *L. plantarum* 17–5 on tight junction protein level, we examined the levels of claudin-3 and occludin by western blot (Fig. 4B). As expected, the protein levels of claudin-3 and occludin in the *E. coli* group were significantly ($P < 0.05$) lower than those in the control group. However, the reduction of claudin-3 and occludin levels was alleviated in the *L. plantarum* pretreatment group.

Effect of *L. plantarum* 17–5 on the mRNA Expression of Inflammatory Cytokines in the Mammary Glands

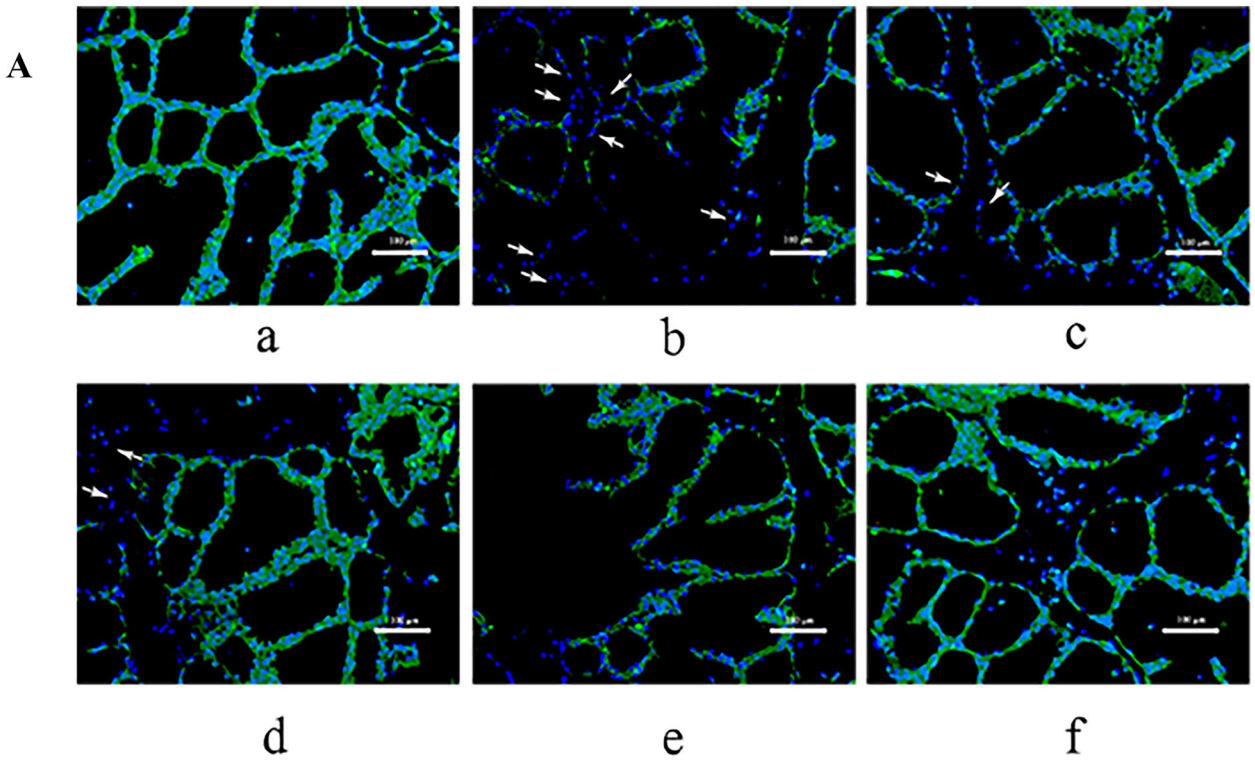
The results in Fig. 5 showed that the expression levels of *IL1β*, *IL6*, and *TNFα* in the *E. coli* group were significantly ($P < 0.05$) enhanced. However, these *E. coli*-induced expression alterations were partially inhibited ($P < 0.05$) by pretreatment with *L. plantarum* 17–5.

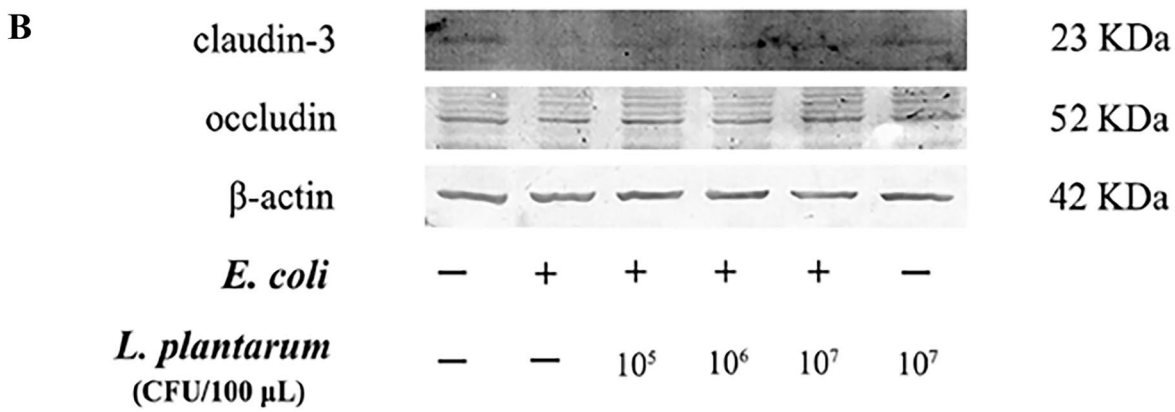
Protein Expression of the NF-κB and MAPK Signaling Pathways in the Mammary Glands

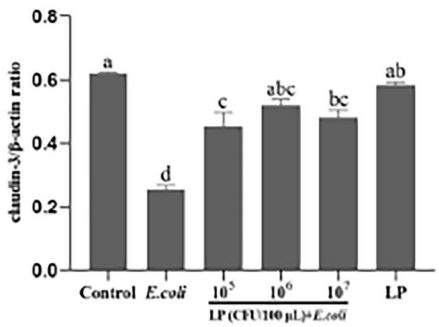
The western blot analysis of NF-κB and MAPK signaling pathway protein expression is shown in Figs. 6 and 7. The results showed that compared with the control group, the phosphorylation levels of p65, IκBα, p38, ERK, and JNK increased significantly ($P < 0.05$) after *E. coli* stimulation. However, the *L. Plantarum* pretreatment group suppressed these increases to varying degrees.

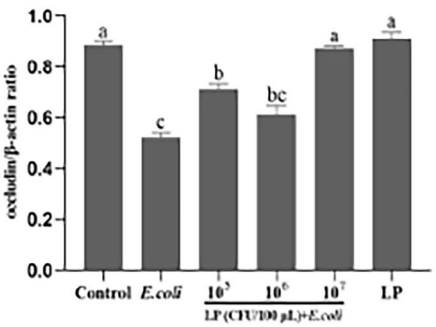
Discussion

E. coli is the most common environmental pathogen causing dairy cow mastitis in dairy herds [3]. Coliform mastitis is often characterized by a severe local and systemic inflammatory response, which causes huge economic


A
a
b
c
d
e
f


B
claudin-3
occludin
β-actin
23 KDa
52 KDa
42 KDa
E. coli
L. plantarum
(CFU/100 μL)
10^5
10^6
10^7


claudin-3/β-actin ratio
Control
E.coli
LP
LP (CFU/100 μL)+E.coli


occludin/β-actin ratio
Control
E.coli
LP
LP (CFU/100 μL)+E.coli

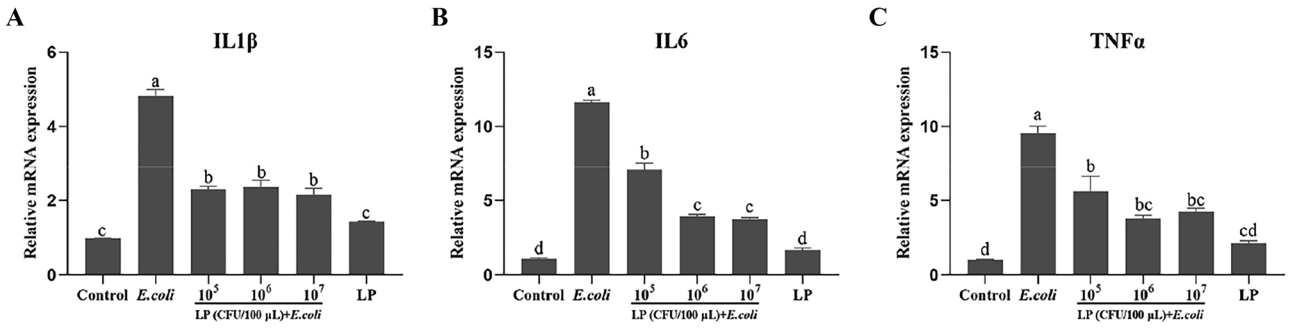


Fig. 5 The mRNA expression levels of *IL1β* (**A**), *IL6* (**B**), and *TNFα* (**C**) in the mammary tissue from each group. Data were expressed as means ± SEM (n = 3)

losses for dairy farmers due to reduced milk production and premature culling [15]. *Lactobacillus plantarum* has been continuously studied as a potential novel anti-inflammatory agent. The current studies show that *L. plantarum* can produce organic acids and bacteriocins, inhibit the growth of different pathogens, and exert an anti-inflammatory effect during the proliferation process [16]. At present, the management of dairy cow mastitis is predominantly accomplished through intramammary infusion [17, 18]. Although some scholars have expressed concerns about the intramammary infusion of active probiotics [19], more and more studies have shown that intramammary injection of *Lactococcus* not induces inflammation but enhances the expression of immune proteins in the mammary glands of healthy cows [20, 21]. Thus, this study explores the preventive effect of intramammary infusion of *L. plantarum* 17–5 on mice mastitis and sets the *L. plantarum* group to verify the safety of this method.

Mastitis is characterized by the destruction of the acinar structure and neutrophil infiltration in mammary tissue, accompanied by the secretion of pro-inflammatory factors [22, 23]. We next performed the histological evaluation of mice mammary glands to evaluate the effect of *L. plantarum* 17–5 on histological changes in mice mammary tissue. The results showed that the mammary gland tissue in the *E. coli* group had obvious redness, swelling and bleeding, and massive infiltration of inflammatory cells in the mammary tissue. However, these characteristics were significantly attenuated in the *L. plantarum* pretreatment group. This indicated that *L. plantarum* 17–5 might protect against inflammation and was consistent with the report by Chen et al. that *Lactobacillus plantarum* can alleviate the inflammatory response of LPS-induced murine mastitis [7]. Notably, there were no obvious pathological changes in the mammary gland tissue in the *L. plantarum* 17–5 group, indicating that *Lactobacillus plantarum* 17–5 does not cause an inflammatory response in mice mammary tissue; this is coincident with previously reported results [20].

The blood–milk barrier is an important physical barrier in organisms, which maintains normal lactation function and is an important barrier against pathogen invasion [24, 25]. The integrity of the blood–milk barrier primarily depends on mammary epithelial tight junctions (TJs) [26]. There are studies indicating that inflammation can disrupt the integrity of TJs and increase its permeability [27, 28]. To investigate the effect of *Lactobacillus plantarum* 17–5 on TJs in mice mammary tissue, we focused on changes in the transmembrane protein family claudin-3 and occludin closely related to TJs. Immunofluorescence staining showed that the claudin-3 signal in the *E. coli* group was significantly weakened, and the tight junction structure

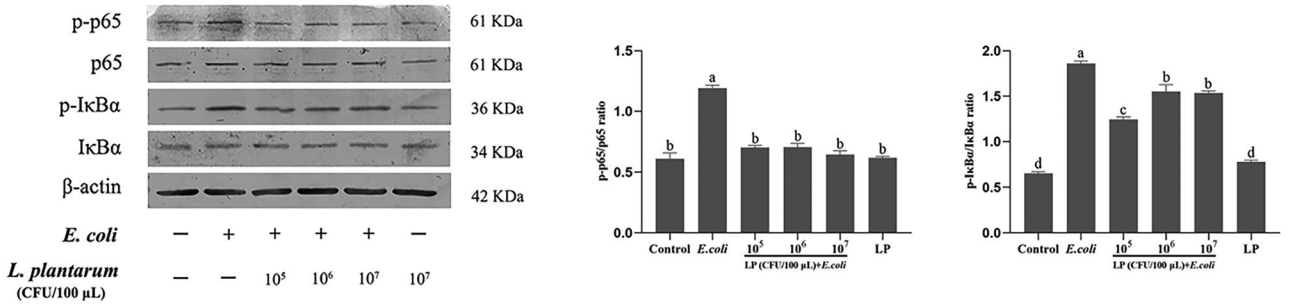


Fig. 6 Effects of *L. plantarum* 17–5 on NF-κB signaling pathway in the mammary tissue. Data were expressed as means ± SEM from three independent experiments

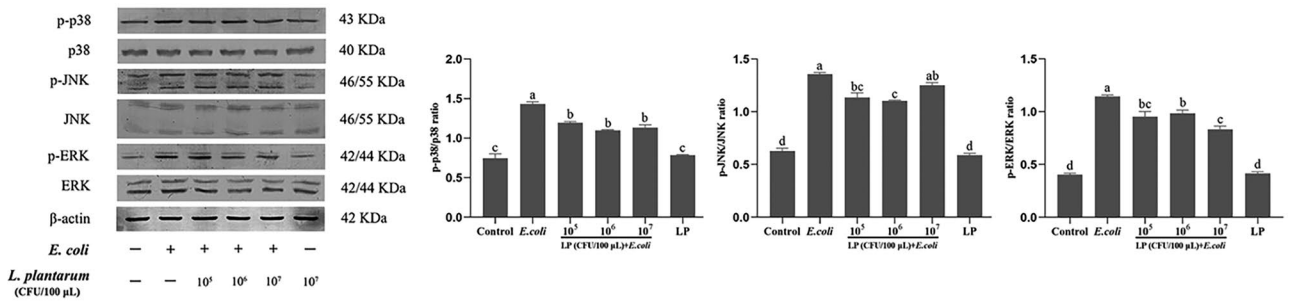


Fig. 7 Effects of *L. plantarum* 17–5 on MAPK signaling pathway in the mammary tissue. Data were expressed as means ± SEM from three independent experiments

was disrupted. In contrast, the claudin-3 signal in the *L. plantarum* 17–5 pretreatment group appeared stronger, and the tight junction structure was improved to some extent. Subsequently, we further detected the protein levels of claudin-3 and occludin in mice mammary tissue by western blot. As expected, claudin-3 and occludin levels were lower in the *E. coli* group and higher in the *L. plantarum* 17–5 pretreatment group, this suggests that the loss of aforementioned proteins led to the decrease in claudin-3 and occludin levels seen in the *E. coli* group. Similar findings were yielded by Zheng et al. [29].

MPO plays an important role in the process of inflammatory cells resisting microbial infection and is an important indicator for assessing neutrophil infiltration and damage in tissues [30]. In the present study, MPO activity was significantly higher in the *E. coli* group, indicating that inflammatory cells clustered around the injection site; this also validates the histopathological changes in mammary gland sections. Pretreatment with *L. plantarum* 17–5 could decrease the elevation of MPO activity, further ameliorating the aggregation of inflammatory cells and inflammatory injury in mice mammary tissue; this corresponds to previous reports [31]. Moreover, some pro-inflammatory cytokines such as IL1β, IL6, and TNFα are involved in the induction, amplification, and regulation of other inflammatory factors and play an important role in the development of inflammation and pathological processes [32–34]. Previous studies have shown that *L. plantarum* can reduce the secretion of IL1β, IL6, and TNFα in the mammary tissue [7]. Our results also indicated that *L. plantarum* 17–5 could inhibit the expression of the above cytokines and alleviate the inflammatory process in *E. coli*-induced mastitis.

To further clarify the mechanism of *L. plantarum* anti-inflammatory, we next detected the NF-κB and MAPK signaling pathways. NF-κB is a transcription factor with various biological activities involved in cell differentiation, inflammation, and immunomodulation [35, 36]. NF-κB normally exists in the cytoplasm in the inactive state; when stimulated by upstream signals, IκBα is rapidly degraded, and NF-κB is released into the nucleus to regulate downstream genes. Simultaneously, this effect is accompanied by increases in NF-κB and IκB phosphorylation [37]. In addition, the MAPK signaling pathways, which include p38 MAPK, ERK1/2, and JNK, are regulated by diverse transduction cascades [38]. It regulates inflammatory genes via phosphorylation of ERK, JNK, and p38 [39, 40]. In this study, we demonstrated that *E. coli* activated the NF-κB and MAPK signaling pathways in mice mammary tissue. However, pretreatment with *L. plantarum* 17–5 inhibited the phosphorylation levels of key proteins in these pathways. We speculate that the anti-inflammatory effect of *L. plantarum* 17–5 may involve inhibiting the NF-κB and MAPK signaling pathways.

Conclusion

In summary, our study indicated that pretreatment with *L. plantarum* 17–5 could alleviate inflammatory damage to the mammary tissue, decrease the expression of pro-inflammatory genes, and inhibit the activation of the NF-κB and MAPK signaling pathways in mice mammary tissue. Therefore, we believe that *L. plantarum* 17–5 has protective effects against *E. coli*-induced mastitis in mice and may be useful as a potential therapeutic agent for mastitis. Finally, a more comprehensive model evaluation should be conducted in vivo to advance their clinical applications further.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12602-023-10047-9.

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Author Contribution Ke Li designed the study. Ming Yang and Li Jia prepared materials. Ming Yang, Yinghao Wu, Lining Yuan, and Lianmin Li performed all experiments. Ke Li, Ming Yang, and Li Jia analyzed the data. Mengyue Tian, Jinliang Du, and Yuzhong Ma supervised and validated the project. Ke Li drafted the manuscript, and Yuzhong Ma revised it. All authors read and approved the final manuscript.

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Data Availability The original, full-length western blots were listed in the supplementary information (Additional file 1). Data generated during the presented study are available from the corresponding author (YZM) upon reasonable request.

Declarations

Ethics Approval and Consent to Participate All the animal experiments were approved by the guidelines of the Animal Care and Use Committee of Hebei Agricultural University (protocol number 2020044).

Competing Interests The authors declare no competing interests.

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