The Prevention Effect of *Bacillus subtilis* on *Escherichia coli*–Induced Mastitis in Mice by Suppressing the NF-κB and MAPK Signaling Pathways

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Abstract

Mastitis, common inflammation of the mammary gland, caused by various factors, is a challenge for the dairy industry. *Escherichia coli* (*E. coli*), a Gram-negative opportunistic pathogen, is one of the major pathogens causing clinical mastitis which is characterized by reduced milk production and recognizable clinical symptoms. *Bacillus subtilis* (*B. subtilis*) has been reported to have the ability to limit the colonization of pathogens and has immune-stimulatory effects on epithelial cells. The purpose of this study was to explore the preventive role of *B. subtilis* H28 on *E. coli*–induced mastitis in mice. The mastitis model was established by nipple duct injection of *E. coli* into mice, while *B. subtilis* H28 was utilized 2 h before *E. coli* injection. Furthermore, pathological changes in the mammary gland were evaluated by hematoxylin–eosin (H&E) staining. Enzyme-linked immunosorbent assay (ELISA) was used to detect the levels of proinflammatory cytokines (TNF-α, IL-1β, and IL-6). We also observed changes in Toll-like receptor 4 (TLR4), nuclear transcription factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) expression by using western blotting. The results revealed that *B. subtilis* H28 pretreatment reduced neutrophil infiltration in the mammary gland tissues, significantly decreased the secretion of TNF-α, IL-1β, and IL-6, and downregulated the activation of TLR4 and the phosphorylation of p65 NF-κB, IκB, p38, and ERK. In conclusion, our results indicated that *B. subtilis* H28 can ameliorate *E. coli*–induced mastitis and suggest a new method for the prevention of mastitis.

Keywords *Bacillus subtilis* H28 · *E. coli* · Mastitis · TLR4 · NF-κB · MAPK

Introduction

Mastitis, a severe inflammation of the parenchyma of mammary glands generally caused by pathogens, has a huge influence on all lactating mammals and the dairy industry [1]. In cows, clinical mastitis, which is characterized by red, swollen, hot, painful discoloration at the site of the infected mammary gland, an increased number of somatic cells in the milk and reduced milk production, is commonly caused by

E. coli [2]. *E. coli* is an opportunistic pathogen that is one of the normal host microbiota, but it can invade the mammary gland to cause inflammation under some circumstances [3, 4]. Previous studies have shown that lipopolysaccharide (LPS), a component part of the cell wall of *E. coli*, has the ability to induce acute mastitis by activating the NF-κB signaling pathway after recognition by TLR4 [5, 6]. Although traditional antibiotics contribute to the treatment of cow mastitis, problems such as drug residues and resistance still threaten the dairy industry.

Bacillus subtilis (*B. subtilis*) has the ability to survive extreme environmental conditions such as high temperature and acidity conditions [7]. Furthermore, *Bacillus* strains also have probiotic potential by interacting with the internal milieu of the host by generating sundry antimicrobial peptides and small active molecules [8]. Recently, *B. subtilis* has been reported to decrease pathogen colonization by interfering with the quorum sensing [9], producing various antibacterial and antifungal compounds [10] and, affecting

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the immune stimulation of epithelial cells [11]. The findings from in vivo studies claim that *B. subtilis* exhibits an anti-inflammatory effect by mediating the secretion of TNF-α, IL-10, and IFN-γ in mouse models [12, 13]. Interestingly, *B. subtilis*, as one of the normal host microbiota, also exists in the mammary gland of cows [14], which provides strong support for the use of *B. subtilis* for the prevention of mastitis in dairy cows. However, the effects of *B. subtilis* H28 on *E. coli*–induced mastitis and the underlying immune mechanism are poorly understood.

In this study, we used a mouse model of mastitis to investigate the preventive effect of *B. subtilis* H28 on *E. coli*–induced mastitis by injecting *B. subtilis* H28 at different concentrations 2 h before *E. coli* injection. The results demonstrated that *B. subtilis* H28 ameliorated the pathological damage of mammary tissues, reduced the secretion of proinflammatory cytokines, and inhibited the activation of TLR4-mediated NF-κB and MAPK signaling pathways. Therefore, potential probiotics *B. subtilis* H28 that target breast pathogens may be an effective strategy to protect against mastitis.

Materials and Method

Animals

A total of 90 BALB/c mice (60 female and 30 male) aged 6–8 weeks were purchased from the Liaoning Changsheng Biotechnology Co. Ltd. The animal experiments were approved by the Animal Ethics Committee of Jilin University (pzpx20201023027). Females and males were mixed at a ratio of 2 to 1 in microisolator cages where water and food were provided freely until pregnancy.

Materials

ELISA kits of TNF-α, IL-6, and IL-1β were purchased from Biolegend, CA, USA. All the monoclonal antibodies, including β-actin, p65, p38, and the phosphorylation of p65 and p38, were purchased from Cell Signaling Technology, Beverly, MA, USA. Luria–Bertani (LB) medium, *Bacillus* medium, and MacConkey agar were purchased from Qingdao Haibo Biotechnology Co., Ltd, Qingdao, China.

Bacteria and the Culture Conditions

B. subtilis H28 was isolated from healthy cow's milk samples and identified by 16S rDNA sequencing and PCR. *E. coli* CVCC 1418 was purchased from China Veterinary Culture Collection Center (CVCC). In our study, *E. coli* was inoculated into a fresh LB medium at a ratio of 1:100 for approximately 6 h after *E. coli* was cultured overnight in LB medium at 37 °C and 120 r/min, and then the OD_{600} was approximately 0.5 (the concentration was approximately 10^8 CFU/mL). *B. subtilis* H28 was inoculated into *Bacillus* medium by the same inoculation method, and then, the OD_{600} was approximately 0.6 (the concentration was approximately 10^9 CFU/mL) after 6 h of growth.

Mastitis Model and Experiment Design

The model of mouse mastitis was established according to the previous studies [15, 16]. In brief, pregnant BALB/c mice were administered mammary gland injections at the fourth pair of nipples 5–7 days after delivery. The mice were randomly divided into 6 groups: a control group, *E. coli* group (10^7 CFU/100 μL), *B. subtilis* H28 (10^7, 10^8, and 10^9 CFU/100 μL) + *E. coli* and *B. subtilis* H28 group (10^9 CFU/100 μL). The *B. subtilis* H28 or PBS (control and *E. coli* group) was injected into each side of the nipple 2 h prior to *E. coli* (10^7 CFU/100 μL) treatment, and then 100 μL *E. coli* was administered in the same way. Then, 24 h after the last injection, the mice were euthanized, and mammary gland tissue was collected and stored at −20 °C until analysis.

Histological Evaluation

Mammary gland tissues were fixed in 4% formalin for at least 48 h and sliced by paraffin embedding. Then the slices were stained by hematoxylin and eosin after xylene dewaxing and rehydrating with graded alcohols. Finally, the pathological changes of the mammary gland were evaluated under a light microscope (×100).

Cytokine Assay

The proinflammatory cytokines were measured by using an ELISA assay kit according to the manufacturer's instructions. Mammary gland tissue (0.05 g) was weighed and ground into homogenate by adding 500 μL PBS. Then the supernatant was collected after centrifugation at 12,000 r/min for 10 min at 4 °C.

MPO Activity Determination

To homogenize the mammary gland tissues, HEPES was added, and then the precipitate was collected. Furthermore, the precipitate was ground and centrifuged after adding cetyltrimethylammonium chloride (CTAC), and the supernatant was collected for the examination of MPO, which was detected by calculating the absorbance (OD) at 450 nm.
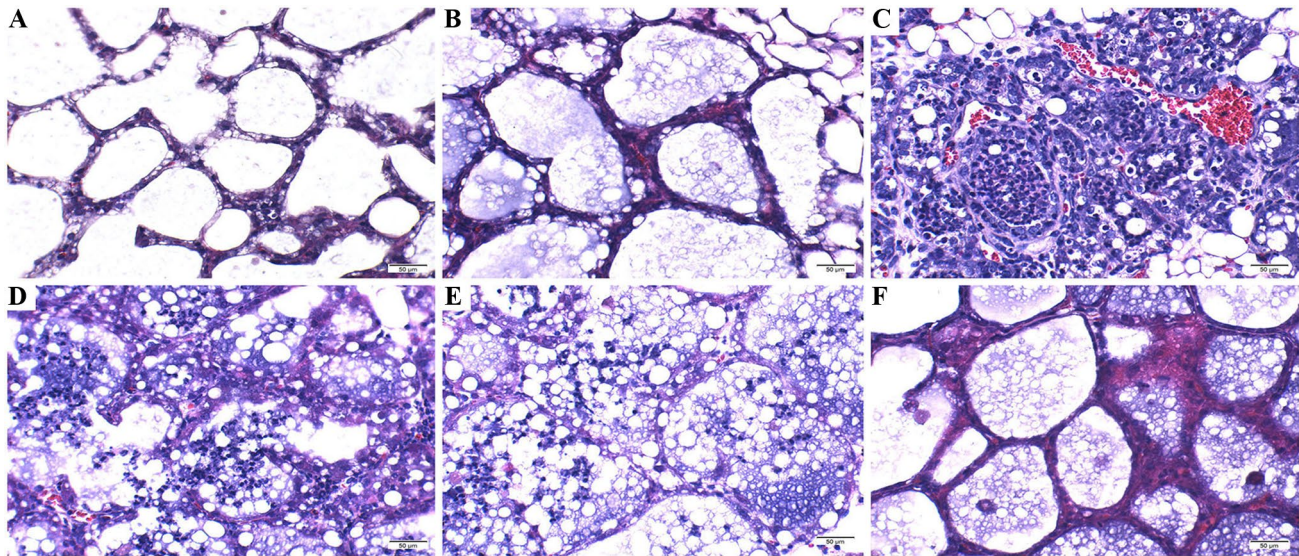
Fig. 1 Effects of *B. subtilis* H28 on mammary gland tissues histopathological changes. Histopathologic sections of mammary tissues (H&E, ×100). **A** The control group, **B** *B. subtilis* H28 group, **C** *E. coli* group, **D** *B. subtilis* H28 (10^7 CFU/mL) + *E. coli*, **E** *B. sub-tilis* H28 (10^8 CFU/mL) + *E. coli*, and **F** *B. subtilis* H28 (10^9 CFU/mL) + *E. coli* groups. The values presented are the means ± SEM of three independent experiments, * $P < 0.05$

Western Blot Analysis

To extract the total proteins of the mammary tissues, tissue protein extract was used. Then, the supernatant was collected, and the protein concentration was detected with a BCA protein assay kit. SDS-PAGE (10%) was used to separate the protein samples; subsequently, the protein samples were transferred to PVDF membranes and blocked in 5% skim milk for 2 h at room temperature. The PVDF membranes were incubated with specific primary antibodies against phospho-p38 (1:1000), phospho-ERK (1:1000), p38 (1:1000), ERK (1:1000), β-actin (1:1000), phospho-IκB (1:1000), IκB (1:1000), NF-κB p65 (1:1000), and phospho-NF-κB p65 (1:1000) at 4 °C overnight. Then, the membranes were washed 3 times with tris-buffered saline-Tween (TBST) for 10 min each time and incubated with goat anti-rabbit secondary antibodies for 2 h at room temperature. The membranes were detected by the ECL Plus Western blotting Detection System.

Statistical Analysis

These data were analyzed applying one-way ANOVA and Tukey's multiple comparisons test by using GraphPad Prism 5 (Manufacturer, La Jolla, CA, USA). All data are expressed as mean ± SEM.

Results

Effects of *B. subtilis* H28 on Mammary Gland Tissues Histopathological Changes in *E. coli*–Induced Mastitis

As shown in Fig. 1, the *E. coli* group had mammary gland structure destruction and inflammatory cell infiltration, while *B. subtilis* H28 pretreatment recovered the structure of the mammary gland and decreased the inflammatory infiltration. Notably, there was no significant pathological change in the *B. subtilis* H28-treated (without *E. coli*) group compared with the control group.

Effects of *B. subtilis* H28 on MPO Activity

MPO is a biomarker of neutrophil infiltration and can indirectly reflect the degree of inflammation [17]. As shown in Fig. 2, the results revealed that MPO activity in the *E. coli* group was significantly increased relative to that in the control group. In contrast, MPO activity was reduced in the *B. subtilis* H28-treated group compared with the *E. coli* group. The MPO activity was not increased markedly in the group treated with *B. subtilis* H28 alone.
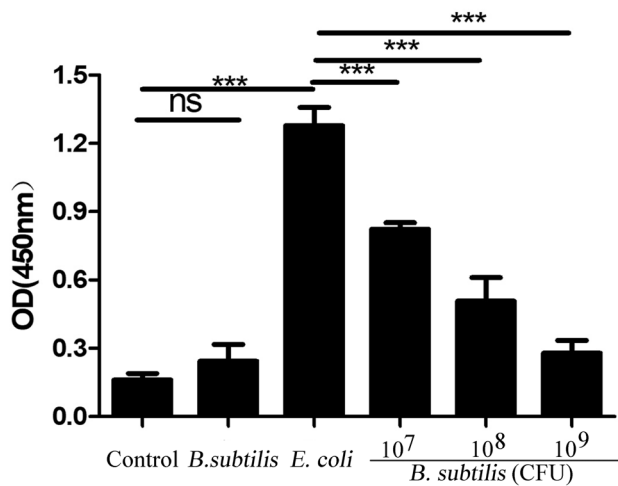
Fig. 2 Effect of *B. subtilis* H28 on MPO activity in *E. coli*–induced mastitis. MPO activity was determined at 24 h after *E. coli* treatment. The values presented are the means ± SEM of three independent experiments, * *P* < 0.05

Effects of *B. subtilis* H28 on Proinflammatory Cytokine Levels in *E. coli*-Induced Mastitis

To explore the anti-inflammatory effects of *B. subtilis* H28 on *E. coli*–induced mastitis, TNF-α, IL-1β, and IL-6 levels were detected by ELISA assays. As shown in Fig. 3, the expression of TNF-α, IL-1β, and IL-6 increased significantly compared with that in the control group after *E. coli* stimulation. However, *B. subtilis* H28 dramatically reduced the increases in their levels in a dose-dependent manner. No significant inflammatory factor release was observed in the *B. subtilis* H28-treated group compared with the control group.

Effects of *B. subtilis* H28 on the Activity of the NF-κB and MAPK Signaling Pathways

To elucidate the mechanisms of *B. subtilis* H28 on mastitis, we examined the effect of *B. subtilis* H28 on the activity

of the NF-κB and MAPK signaling pathways. The western blot results demonstrated that TLR4, phosphorylation of NF-κB p65, phosphorylation of IκB, phosphorylation of p38, and phosphorylation of ERK were upregulated in the *E. coli* group, but all of them were significantly decreased after *B. subtilis* H28 treatment compared with the *E. coli* group. However, there were no significant changes in these signaling pathways in the *B. subtilis* H28 group compared with the control group (Figs. 4 and 5).

Discussion

Mastitis is characterized by the destruction of the structure of the breast acinus and a large amount of neutrophil infiltration, accompanied by the secretion of inflammatory factors. Recently, an increasing number of studies have shown that probiotics have the potential to treat a variety of diseases, such as inflammatory bowel disease, obesity, and diabetes [14, 18, 19]. Unlike other probiotics such as lactobacilli and *Lacticaseibacillus rhamnosus*, which have clear probiotic effects and extensive research [20, 21]. However, the use of *Bacillus* has been controversial because certain strains of *Bacillus*, such as *Bacillus cereus*, can cause diarrhea by producing some toxins [22]. However, although some *Bacillus* may be harmful, *B. subtilis* has long been considered a safe and effective probiotic that is widely used in industry [8]. Recently, *B. subtilis* has been reported to reduce pathogen colonization by interfering with quorum sensing [9], to produce various antibacterial and antifungal compounds [10] and to affect immune stimulation of epithelial cells, which provides *B. subtilis* the possibility to protect against inflammation [23]. Hence, we have used a mouse model of mastitis to investigate the protective role of *B. subtilis* H28 against local inflammation of the mammary gland.

MPO is a functional marker and activation marker of neutrophils, and its level and activity changes represent the function and activity status of the PMN [17]. PMN can kill microorganisms in phagocytic cells and can also be secreted
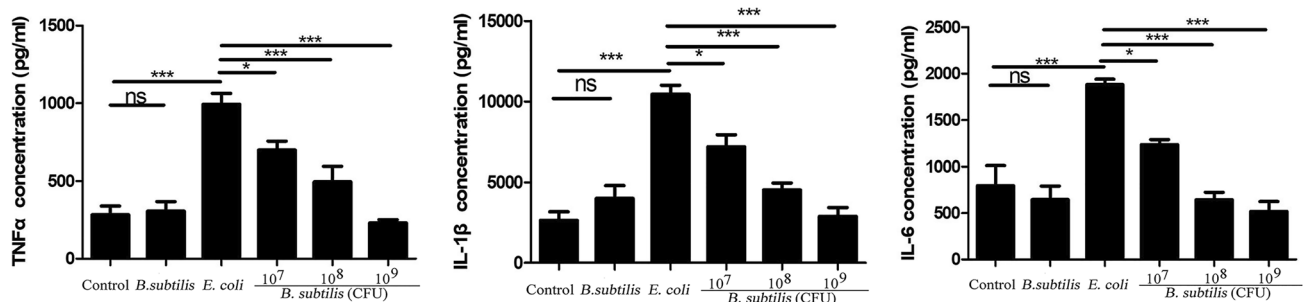

Fig. 3 Effect of *B. subtilis* H28 administration on pro-inflammatory cytokine production in mammary glands. The levels of pro-inflammatory cytokines in mammary gland tissues were detected at 24 h after *E. coli* treatment by using ELISA kits. The values presented are the means ± SEM of three independent experiments, * *P* < 0.05
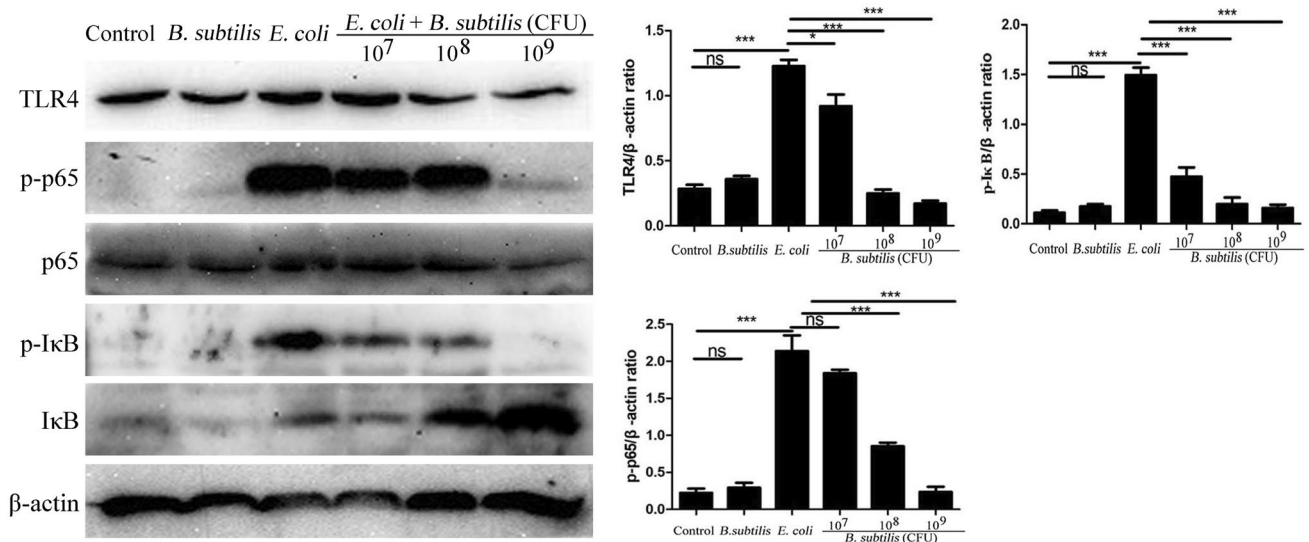
Fig. 4 Effects of *B. subtilis* H28 on the activity of the TLR4-mediated NF-κB signaling pathway. Effects of *B. subtilis* H28 on the expression of the TLR4-mediated NF-κB pathways were induced by *E. coli*.

All protein samples were analyzed by western blot with specific antibodies, and β-actin was used as a control. The values presented are the means ± SEM of three independent experiments. * $P < 0.05$

extracellularly to target multiple cells, but high concentrations of MPO can cause oxidative stress and tissue damage [24]. As shown in our study, MPO activity in the *E. coli* group was significantly increased relative to that in the control group, but MPO activity was reduced after *B. subtilis* H28 administration, while *B. subtilis* H28 administration without *E. coli* group did not show obvious amplification

compared with that in the control group. Cytokines are reactive indicator of inflammation, including proinflammatory and anti-inflammatory cytokines. TNF-α, IL-1β, and IL-6 are the main proinflammatory cytokines present in mastitic inflammation [25]. In our study, the results showed that *B. subtilis* H28 markedly reduced the production of TNF-α, IL-1β, and IL-6 in a dose-dependent manner, which
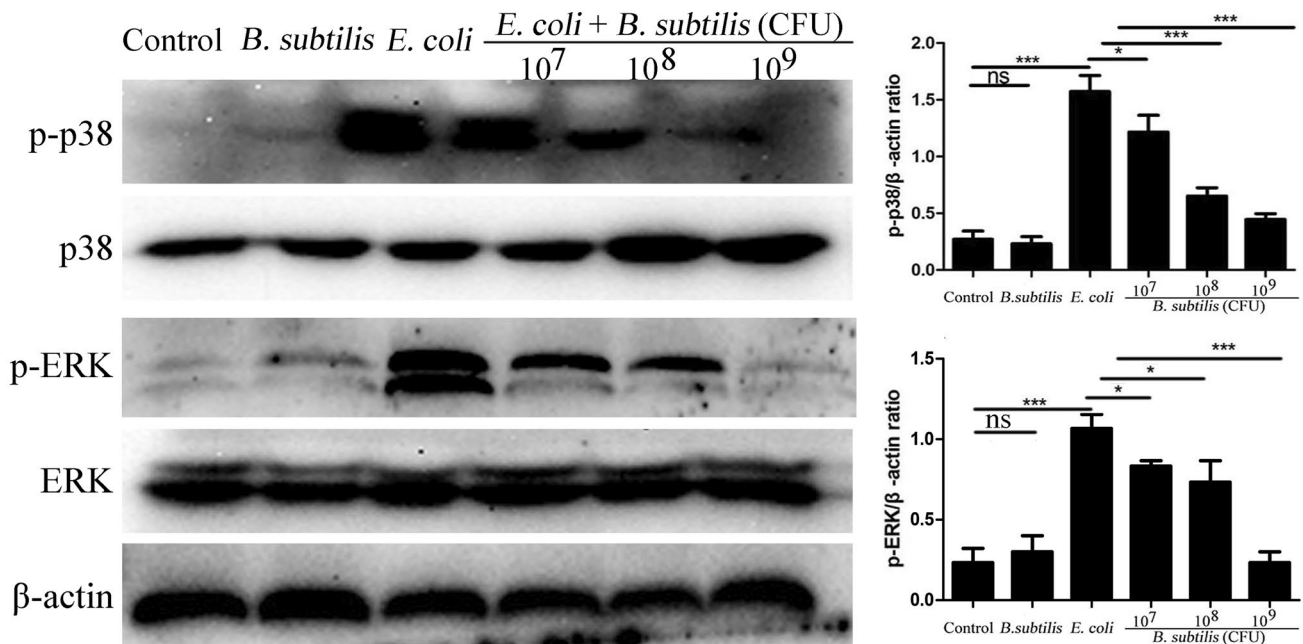

Fig. 5 Effects of *B. subtilis* H28 on the activity of the MAPK signaling pathways. Effects of *B. subtilis* H28 on the expression of the MAPK pathways were induced by *E. coli*. All protein samples were

analyzed by western blot with specific antibodies, and β-actin was used as a control. The values presented are the means ± SEM of three independent experiments, * $P < 0.05$

Fig. 6 The proposed mechanism of *B. subtilis* H28 on *E. coli*–induced mastitis in mice
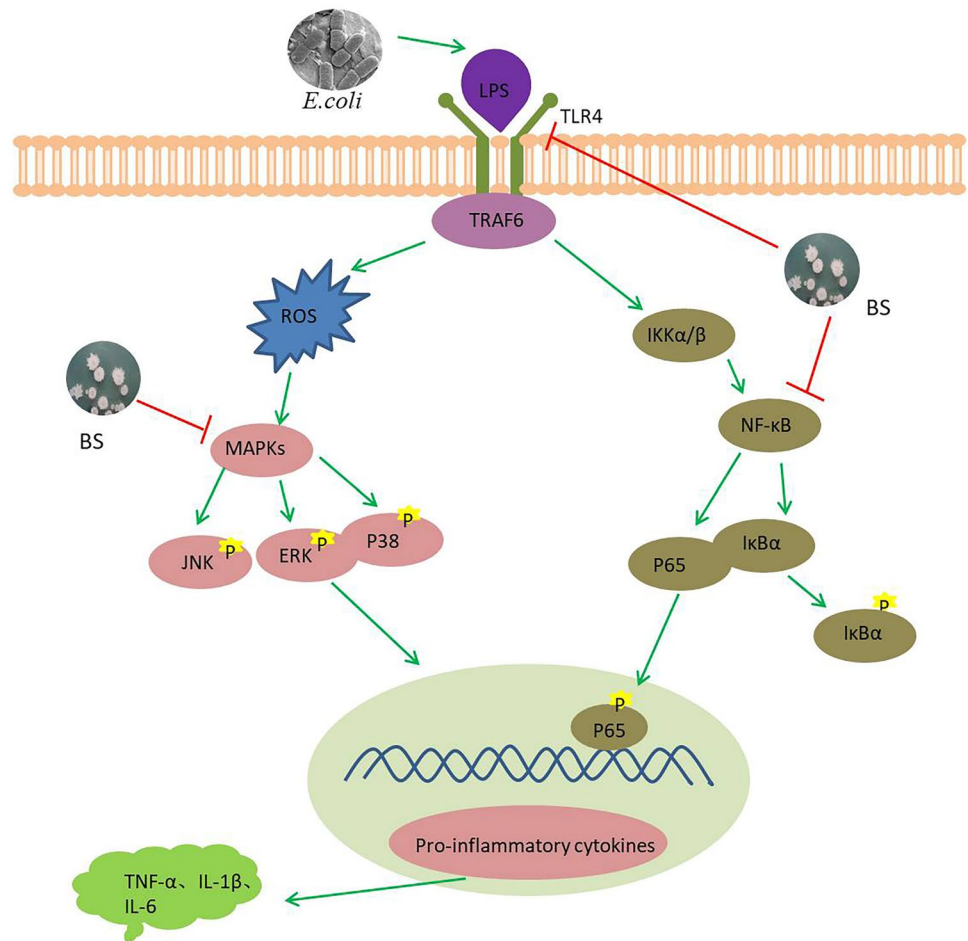
illustrated that the degree of *E. coli*–induced inflammation was ameliorated by *B. subtilis* H28.

To further clarify the mechanism by which *B. subtilis* H28 improves inflammation, we detected the NF-κB and MAPK signaling pathways. It has been reported that the Gram-negative bacteria, such as *E. coli*, are recognized by TLR4 [26], a pattern recognition receptor (PRR) on the cell surface. The downstream signaling pathways NF-κB and activator protein 1 (AP-1) are then activated in a myeloid differentiation primary response 88 (MyD88)-dependent or MyD88-non-dependent manner [27], ultimately promoting cytokine secretion. NF-κB, a fast-acting nuclear transcription factor involved in multiple proteins which mainly include RelA (p65), RelB, c-Rel, p50, and p52 in mammals [28], plays an important role in the inflammatory and immune response. Generally, IκB kinase (IKK) is activated to degrade IκB, which is normally bound to NF-κB in the cytoplasm of unstimulated cells. Then, the free NF-κB enters the nucleus and binds to genes with NF-κB binding sites to initiate transcriptional processes. In fact, TNF-α and pro-IL-1β are secreted during the initial transcriptional process following activation of NF-κB, while IL-6 is released during secondary transcription which is

related to inhibition of IκB [27]. In addition, the MAPK signaling pathways also have important regulatory effects on inflammation via the phosphorylation of p38 and ERK [29]. Our results showed that *B. subtilis* H28 downregulated the activation of TLR4 and the phosphorylation of p65 NF-κB, IκB, ERK, and p38, which suggests that the inhibitory effect of *B. subtilis* H28 on the secretion of pro-inflammatory cytokines may be due to the suppression of the NF-κB and MAPK pathways.

In summary, our study indicated that the preventive effect of *B. subtilis* H28 during *E. coli*–induced mastitis may be due to its ability to inhibit the TLR4-mediated NF-κB and MAPK signaling pathways (Fig. 6). Therefore, our study provides a support for the potential probiotic effect of *B. subtilis* H28, and *B. subtilis* H28 may be a promising candidate for the prevention of mastitis.

Availability of Data and Material All data and materials are available from the corresponding authors on reasonable request.

Declarations

Ethical Approval The animal experiments were approved by Jilin University Animal Ethics Committee, and the present study was performed according to the care and use of Laboratory Animals published manual by the US National Institutes of Health.

Consent for Publication All authors consent with the publication of this article.

Conflict of Interest The authors declare no competing interests.

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