Commensal *Bacillus* From Cow Milk Inhibits *Staphylococcus Aureus* Biofilm Formation and Mastitis in Mice

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Abstract

Background: Mastitis, one of the most serious diseases in dairy industry, could cause tremendous economic losses worldwide and is commonly trigged by pathogen invasion. *Staphylococcus aureus* (*S. aureus*) -induced mastitis has been reported to play an important role in mastitis etiology characterized by high morbidity, recurrence, and increased antibiotic resistance, which may attribute to the formation of biofilm formation, a form of bacterial aggregation for better growth and resistance to adverse conditions. Probiotics *Bacillus* has been reported to disrupt bacteria quorum-sensing (QS) system, a central regulator for biofilm formation. However, whether commensal *Bacillus* affects *S. aureus* biofilm formation and consequent colonization during mastitis is still unknown.

Results Here, we identified that the Bacillus is associated with reduced colonization of S. aureus in the mammary gland of cows. Interestingly, Bacillus did not affect S. aureus growth but inhibited the biofilm formation of S. aureus by interfering with S. aureus QS signaling. The most obvious anti-biofilm effect was found in Bacillus subtilis H28, so it was selected for further study. We found that bacillus subtilis H28 treatment alleviated S. aureus-induced mastitis in mice, as showed by limiting pro-inflammatory cytokines production, enhancing barrier integrity, and reducing S. aureus burden. Consistently, Bacillus subtilis with the capacity to interfere S. aureus QS ameliorated S. aureus-induced NF-κB activation in mice mammary epithelial cells（MMECs）.

Conclusions: Collectively, our results indicate that commensal Bacillus inhibits S. aureus colonization and alleviates S. aureus-induced mastitis by influencing biofilm formation, which suggests a potential strategy for the decolonization of S. aureus and acts as a basis for the prevention and treatment of S. aureus-related disease.

Introduction

Mastitis is one of the most serious diseases in dairy cows, especially in high-yielding cows(1). The high incidence of mastitis and the difficulty of preventing and treating it has been one of the foremost diseases plaguing the health of the world's dairy industry (2). There is no effective vaccine for its prevention. Antibiotics are still the only means of clinical prevention and treatment of *S. aureus* (3). However, the non-standard use of antibiotics makes the drug increasingly serious resistant to *S. aureus* for a long time (4). In addition to improper production management and drug use, the complex biological functions of *S. aureus* also increase its bacterial resistance. It is reported that *S. aureus* has a strong biofilm-forming ability that was found to be significantly related to the severity of the disease (5). Much of the colonization and virulence production of *S. aureus* depends on its population sensing system (QS) and biofilm formation, and the microbial biofilm is responsible for the persistence of *S. aureus*-related disease (6). Therefore, difficulty of mastitis control induced by *S. aureus* may be related to the long-term colonization of the mammary site by *S. aureus.*

Biofilm is a kind of growth corresponding to planktonic bacteria in which bacteria adsorb to the surface of inert or active entities during the growth process to adapt to their living environment (7). *S. aureus* has a QS system, which is a central regulator in *S. aureus* pathogenicity (8). It can regulate adhesion and production of numerous virulence and pathogenic factors as well as the biofilm formation and heterogeneous resistance of *S. aureus* (9). The bacterial QS system is an intercellular communication mechanism used to synthesize, secrete and detect small signaling molecules to sense bacterial population densities and regulate the expression of specific genes in response to environmental changes (10). The QS system allows bacteria to function as multicellular organisms because the concentration of extracellular self-inducers increases as the number of bacteria grows. After reaching a certain amount, this molecule diffuses back into the bacteria and regulates the transcription of different genes related to biofilm secretion and other (11). The QS system is important in order to bacterial biofilm formation. Therefore, this QS system is a paramount target for the treatment of microbial biofilm associated infections (12). Once formed, biofilms protect bacteria from the action of traditional antibacterial drugs and exhibit multi-drug resistance, leading to the ineffectiveness of long-term antibiotic therapy (13). The biofilm also protects the bacteria from the immune response of the body and enables them to survive in a harsh environment(14). Therefore, the biofilm of *S. aureus* is considered an essential pathogenic feature.

Previous studies have found that live probiotic microorganisms are present in healthy milk, which considered to be the presence of self- or commensal microbiota. As one of the normal host microbiotas, *Bacillus* also is available in the mammary gland of cows (15). Different *Bacillus* strains showed antibacterial, antioxidant, and immunomodulatory activities in their hosts (16). Recently, probiotics such as *bacillus* have been utilized to prevent infection, because it is a nonpathogenic Gram-positive bacterium that can availably maintain a beneficial microflora balance in the gastrointestinal tract of a mammalian host (17). *B. subtilis*–fermented fermentation products can promote the growth performance of immune-stressed broilers and regulate the composition of intestinal microflora (18). In addition, the exclusion of pathogens by inhibition of bacterial biofilms is another potential property of the proposed *Bacillus* strain (19). Evidence accumulated from animal and in vitro studies indicates that *B. subtilis* yields a variety of substances, such as surfaceins, iturins and fengycins, which may contribute to antibacterial, anti-inflammatory, and immunomodulatory applications (17, 18). Specifically, a recent report showed that the secreted substance from *B. subtilis* abolished colonization with *S. aureus* by suppressing the production of the Arg-quorum-sensing signaling system (17). However, whether *B. subtilis* can reduce the colonization of mammary glands by *S. aureus* has not been reported.

Therefore, we hypothesized that there is interference between the mammary symbiotic bacterium *Bacillus* and the pathogenic bacterium *S. aureus*. *Bacillus* can reduce the colonization of *S. aureus* and thus relieve *S. aureus*-induced mastitis. Here, we found that *Bacillus* eliminated the colonization of the dangerous pathogen *S. aureus* in the mammary gland of cows. Further studies revealed that *Bacillus* disrupts the formation of biofilm and thus reduces the colonization of *S. aureus* probably by affecting the QS system of *S. aureus*. Using mice mastitis model, we also demonstrated that *Bacillus* ameliorated *S. aureus*-induced mastitis, as showed by improving mammary injury, limiting inflammatory markers, and promoting blood-milk barrier integrity. Collectively, our findings that *bacillus* with the capacity to eliminate

mammary gland pathogen colonization through disrupting biofilm formation protects against *S. aureuse*-induced mastitis in mice, suggests that probiotics that interfere with the formation of pathogenic biofilms may serve as a potential and effective st rategy to protect mastitis S. *aureus*-related disease.

Materials And Methods

Materials

TNF-α and IL-1β ELISA kits were purchased from Biolegend (CA, USA). Tissue protein extract and BCA Protein Assay Kit were bought from Thermo (Thermo, MA, USA). Trypticase Soy Broth (TSB) and bacillus medium were purchased from Qingdao Haibo Biotechnology Co., Ltd (Qingdao, China). Crystal Violet Stain solution, 1% was acquired from Solarbio (Solarbio, Beijing, China). All the monoclonal antibodies, including β-actin, p65, IκB, the phosphorylation of p65, phosphorylation IκB, ZO-1, Occludin, and Claudin-3 were recruited from Cell Signaling Technology (Beverly, MA, USA). MPO kit was available from Nanjing Jiancheng Co., Ltd (Nanjing, China).

Animals

A total of 60 Balb/c mice (40 females and 20 males) aged 6-8 weeks were purchased from the Liaoning Changsheng Biotechnology Co. Ltd. The animal experiments were subject to approval by the Animal Ethics Committee of Jilin University (KT202103058). Females and males are mixed in miniature isolation cages in about a ratio of 2 to 1 after adapting to the environment with free food and water. The mastitis model was established complying with the experimental animal manual. This study is built on the Handbook on the Care and use of Experimental Animals published by the National Institutes of Health.

Bacteria and culture conditions

Bacillus were isolated from healthy and mastitis milk samples from dairy cows in Baicheng, Jilin Province), ChiFeng, Inner Mongolia, and Weifang, Shandong Province, China. A total of 145 strains of *Bacillus* bacteria were separated and purified by the specific culture of *bacillus* medium plate. *S. aureus* (ATCC 35556) was acquired from American Type Culture Collection. In the present study, *S. aureus* was inoculated for about 6 hours at the condition of 37 ℃ and 120 r/min, and the OD 600 was about 0.5 (concentration was approximately 10^8 CFU/mL). Meanwhile, *B. subtilis* bacteria were inoculated into the TSB broth medium by the same inoculation method, and then the OD 600 reached about 0.6 (concentration was approximately 10^8CFU/mL) after 4 hours of growth.

Preparation of cell-free supernatant (CFS) from Bacillus culture and treatments

To prepare *Bacillus* CFS, *Bacillus* strains were cultured at 37 ℃ under shaking at 200 rpm overnight until the cultures reached an OD 600 of 0.6 ± 0.05. The CFS of bacterial culture was collected by centrifugation at 6000 g for 10 min, and then filtered through a 0.22 μm sterilizing-grade filter (Millipore, SLGV033RB, USA) to remove bacteria. To evaluate the effect of *Bacillus* CFS on *S. aureus* genes expression, overnight culture of *S. aureus* strains was collected by a centrifuge, washed with PBS, re-suspended at 10^8 CFU/mL

in TSB/PBS (1:1 v/v, control) or TSB/*B. subtilis* CFS (1:1 v/v) and incubated in 6-well-plate at 37 ℃ for 3 h. Finally, bacteria were collected for RNA extraction and analysis of genes expression.

Antibacterial experiment.

To determine the antibacterial effect of *B. subtilis* CFS on *S. aureus*, the Bacillus supernatant was obtained using the method described previously (18). Briefly, the supernatant of *Bacillus* bacteria was added into 96-well plates. 10 μL of *S. aureus* suspension (5×10^8 CFU/mL) from a fresh overnight culture was inoculated into 200 μL TBS (control), *Bacillus* CFS, TSB/ *Bacillus* CFS (1:1 v/v) or TSB/ *Bacillus* CFS (0.5:1.5 v/v) and incubated at 37 ℃ or 24 h. The growth of *S. aureus* was determined by monitoring OD 600 of the cell culture.

Biofilm formation and viability assay

To evaluate the effect of *Bacillus* CFS on *S. aureus* biofilm formation, 10 μL of *S. aureus* (5×10^8 CFU/mL) was added to 200 μL of TSB (control) or TSB/ *Bacillus* CFS (1:1 v/v) in each well on a 96-well plate and incubated at 37 ℃ for indicated time points without shaking. Next, the wells were washed three times with sterile PBS after the medium was removed. Finally, the plates were air-dried for 45 min and the adherent cells and matrix were stained with 1% crystal violet solution. To quantify the biofilm production, crystal violet was extracted by incubation in a solution (95% ethanol) at room temperature for 15 min, and absorbance was measured at 570 nm in a microplate reader.

RNA extraction and Quantitative real-time PCR (QRT-PCR)

Total RNA of *S. aureus* was extracted with a Bacterial RNA Extraction Kit (B518655-0050, Sangon Biotech, Shanghai, China) following the manufacturer's instructions. RNA purity was verified using a NanoDrop spectrophotometer (ND-1000, Nanodrop, USA). RNA was reversely transcribed using the 5× Prime Script RT Master Mix (RR036A, Takara, Shiga, Japan) according to the manufacturer's instructions. QRT-PCR was carried out using TB Green Premix Ex Taq II (RR820A, Takara, Shiga, Japan). Fold changes in level of chosing genes expression were determined using the $2^{-\Delta\Delta Ct}$ method.

Animal treatment and mastitis model

Totally forty female Balb/c mice 5-7 days after delivery were randomly divided into four groups: control group, *S. aureus* group (1×10^8 CFU per 100 μL PBS), *B. subtilis H28* group (1×10^8 CFU per 100 μL PBS), *B. subtilis H28* (1×10^8 CFU per 100 μL PBS) + *S. aureus* (1×10^8 CFU per 100 μL PBS) group. For induction of mastitis, *S. aureus* (1×10^8 CFU per 50 μL PBS) was an injected into each mammary gland of the mice using 100 μL syringes with a 30 gauge blunt needle. In the *B. subtilis H28* group, the mammary gland of mice received an injection dose of intramammary with *B. subtilis H28* (1×10^8 CFU per 100 μL PBS), later 2 hours *S. aureus* was an injected into each mammary gland. The control group mice received an injection dose of intramammary with an equal volume of sterile PBS. Twenty-four hours

later, the mice were sacrificed and the mammary gland tissues were harvested and stored at −80°C for subsequent detection.

Histological analysis

24 hours after *S. aureus* infection, the mammary gland tissues of each group were collected and fixed in 4% paraformaldehyde for 48 h. The sample was inserted into paraffin and cut into 4 μm sections. After deparaffinization, the sections were stained with hematoxylin and eosin (H&E), and histological analysis was conducted under an optical microscope. The main histopathological indicators are through hyperemia (grade 0-3, ranging from normal to severe, including normal, mild, moderate, and severe) and neutrophil infiltration (grade 0-5, grade 0, from nothing to transmural) to evaluate.

MPO activity assay

MPO activity is a functional and activation marker of neutrophils. The mammary gland tissue was harvested and homogenized on ice with reaction buffer (weight/volume ratio 1:19). The detection method of MPO activity was carried out according to the manufacturer's instructions (Nanjing Jiancheng Institute of Bioengineering, Nanjing, China).

ELISA assay

The inflammatory cytokine was established using an ELISA kit, according to the manufacturer's instructions. 10% tissue homogenate was prepared and centrifuged at 4°C, 12000 r/min, for 10 min. The lipid layer was removed, and the middle supernatant was collected for detection. Use a microplate reader to test the read absorbance of the sample at 450 nm and 570 nm.

Mammary S. aureus load assay

To assess the *S. aureus* burden in the mammary gland, mammary tissues were aseptically obtained in mice and homogenized in 1 mL of PBS. A 10-fold dilution of the tissue homogenate was plated in mannitol high salt agar plates. Bacterial colonies were counted and calculated following plate incubation at 37 ℃ for 18 h. Results of bacterial burden were expressed on a log10 scale.

Western Blotting Analysis

The BCA protein detection kit was utilized to detect the protein concentration in mammary gland tissues. The protein samples were separated by 10% SDS-PAGE, then protein samples were transferred to the PVDF membrane and blocked in 5% skimmed milk at room temperature for 2 h. Use primary antibodies β-actin (1:1000), phospho-IκB (1:1000), IκB (1:1000), NF-κB p65 (1:1000) ZO-1 (1:500) Occludin(1:1000) and Claudin-3 (1:1000)and phospho-NF-κB p65 (1:1000) in Incubate overnight at 4°C. The membrane was washed three times with TBST for 10 minutes each time and incubated with goat anti-rabbit secondary antibody for 2 h at room temperature. Finally, use of the ECL Plus Western blotting detection system to detect the cell membrane.

Immunohistochemistry (IHC)

The paraffin-embedded glass slides were dewaxed in xylene and different concentrations of alcohol. The antigen is restored with 0.01 M citrate buffer. Incubate in 3% H_2O_2 and then in diluted goat serum. The sections were incubated with primary antibodies: Claudin 3 (1:500) at 4°C. After washing 3 times, the sections were incubated with HRP-labeled goats with anti-rabbit secondary antibody (1:500, ZS-Bio, Beijing, China) at 37°C for 15 min. This section was stained with DAB and observed under a microscope.

Statistical analysis

GraphPad Prism 5 (Manufacturer, La Jolla, CA, USA) was performed for statistical analysis. The data are presented as mean ± SEM. $p < 0.05$ indicated statistical significance by one-way ANOVA and Tukey's multiple comparisons test.

Results

Increased S. aureus **colonization contributes to cow mastitis and is associated with reduced commensal** Bacillus.

To investigate the correlation between *S. aureus* and *Bacillus* colonization in the mammary gland, we performed the culture-based analysis of milk samples with swabs taken from healthy non-mastitis cows and mastitis cows. The absolute abundance of total *S. aureus* and *Bacillus* on milk swabs was evaluated by manual colony counting for colony-forming units (CFUs) on a selective egg yolk mannitol salt agar and *Bacillus* culture medium respectively. As showed in Fig. 1A, we can clearly observe substantial differences in the quantity of *S. aureus* between health and mastitis cows that more colonization of *S. aureus* in mastitis cows. Meantime, we found that healthy cow had more *Bacillus* colonization than mastitis cows (Fig. 1B). Previous studies have found that laboratory isolates of *Bacillus* species can reduce the colonization of *S. aureus* (19). One explanation for the larger amount of *S. aureus* from mastitis milk compared to normal milk was that *Bacillus* from normal milk may affect colonization of *S. aureus*. Therefore, we next sought to further determine whether was a correlation between *Bacillus* and *S. aureus*. We started raising a striking correlation between the presence of *Bacillus* bacteria and the absence of *S. aureus.* We first assessed the effect of the presence or absence of *S. aureus* colonization on the number of *Bacillus*. The results showed in that the number of *Bacillus* decreased in the presence of *S. aureus* colonization in both health and mastitis cows (Fig. 1C). We then analyzed the effect of *Bacillus* on *S. aureus* colonization and found that lower load of *S. aureus* was detected in healthy and mastitis cows when *Bacillus* was colonized (Fig. 1D). These results are mentioned that there is a negative correlation between *S. aureus* and *Bacillus* in the milk.

Commensal Bacillus **inhibits** S. aureus **colonization through affecting the biofilm formation of** S. aureus.

It has been declared in the literature that *Bacillus* can inhibit the population sensing system of *S. aureus*, thus reducing the colonization of the intestinal tract by *S. aureus* (18). We therefore hypothesize that

Bacillus may exert a broad mechanism for comprehensively inhibit *S. aureus* colonization in the mammary gland. In the results of the flow disease survey, it was shown that the presence of *Bacillus* in the mammary gland could be a possibility determining factor for the absence of *S. aureus* in cows. We first analyzed whether there is a growth-inhibitory effect of the *Bacillus* isolates on *S. aureus*. To detect antimicrobial growth effect, we randomly isolated about 145 individual *Bacillus* isolates from the milk samples and then conducted an unbiased analysis of antimicrobial activity by determining the capacity of the sterile conditioned supernatant of each individual isolate to inhibit *S. aureus* growth. However, we found no difference in the antibacterial effect between *S. aureus*-negative and -positive *Bacillus* (Fig. 2A). Therefore, a growth-inhibitory effect not is able to explain the observed complete correlation between the presence of *Bacillus* and the absence of *S. aureus*, and rules out a bacteriocin-mediated phenomenon. The factors that are important in order to *S. aureus* mammary colonization are poorly understood. A previous study has implicated that quorum-sensing (QS) is a requirement for *S. aureus* to colonize the intestine, and discovering that secreted *Bacillus* function as QS blockers to achieve complete eradication of intestinal *S. aureus* (19). We then examined the anti-*S. aureus* biofilm capacity of *S. aureus*-negative and -positive *Bacillus* and found that *S. aureus*-negative *Bacillus* had higher anti-*S. aureus* biofilm capacity than *S. aureus*-positive *Bacillus* (Fig. 2B). We next determined the ability of biofilm formation between health and mastitis cow isolated *S. aureus* and showed that *S. aureus* from mastitis cow had higher biofilm formation capacity than *S. aureus* from non-mastitis. Collectively, these results suggest that *Bacillus* inhibits *S. aureus* colonization by affecting the biofilm formation of S. aureus.

Bacillus **inhibits** S. aureus **biofilm formation by regulating QS signaling.**

To detect anti-biofilm activity, we randomly isolated about 145 individual Bacillus isolates from each milk culture swab and then conducted an unbiased analysis of anti-biofilml activity by measuring the capacity of the sterile conditioned supernatant of each individual isolate to inhibit S. aureus biofilm (Fig. 2A). The showed that the anti-biofilm effect of different *Bacillus* species varied significantly, and we performed the strain with the best anti-biofilm effect for an intensive investigation which was identified by 16S RNA sequencing and was named as *Bacillus subtilis* H28 (*B. subtilis* H28) (Fig. 3B). As shown in Fig. 3C, B. *subtilis* H28 strain did not produce any inhibition loop on agar. Moreover, biofilm formation in static *S. aureus* culture was evaluated by crystal violet staining. Results showed much faint staining in the culture of *B. subtilis* H28 treated *S. aureus* (Fig. 3D), which was significantly different from the group with the addition of *S. aureus* alone indicating that *bacillus* has an inhibitory effect on biofilm production of *S. aureus*. Moreover, we counted the *S. aureus* in the biofilm and showed that *B. subtilis* H28 significantly reduced the number of *S. aureus* in the biofilm (Fig. 3F).

To confirm whether the *B. subtilis* H28 supernatant had antibacterial activity, 5×10^8 CFUs of *S. aureus* were grown for 48 h in 25%, 50%, and 100% supernatant of *B. subtilis* H28 in polystyrene plates for 2 days at 37°C. As shown in Fig. 4A, different concentrations of *B. subtilis* H28 supernatant had no effect on bacterial growth. To test for the effects of *B. subtilis* H28 on biofilm formation in vitro, S. aureus was treated as similarly and biofilm was stained with crystal violet and determined at OD 570 nm. We found that *B. subtilis* H28 reduced cell attachment and biomass in a concentration-dependent manner (Fig. 4A).

To test whether *B. subtilis* H28 has a disruptive effect on biofilms already formed in vitro, 5×10^8 *S. aureus* cells were incubated in polystyrene plates at 37°C for 2 days. Afterwards, 25%, 50%, and 100% *B. subtilis* H28 supernatants were added and incubation was continued for 24 h. We demonstrated that *B. subtilis* H28 inhibited the biofilm already formed in a dose-dependent manner (Fig. 4B). However, a non-inhibitory *bacillus* cannot destroy the biofilm formed by *S. aureus* (Fig. 4B).

Biofilm formation involves the expression and regulation of multiple genes (20). The QS system controls *S. aureus* biofilm formation and release of virulence factors (21). Researchers have indicated that the accessory gene regulator (Agr) system regulates the QS system of *S. aureus* (22). *S. aureus* secretes the polysaccharide intercellular adhesion (PIA) is a factor necessary for the bacterial aggregation phase of its biofilm formation, and PIA synthesis is mainly encoded by the ica manipulator(23, 24). The RNAIII activating peptide is considered as an auto-inducible peptide that phosphocreeatine its target molecules, activates the Agr system, and regulates biofilm formation (25, 26). We then analyzed the effect of *B. subtilis* H28 on the expression of genes involved in population sensing (ArgA and RNA III) and biofilm formation gene ica. We showed that *B. subtilis* H28 treatment significantly down-regulated the mRNA expression of all the above genes (Fig. 4C-E). Taken together, these results indicate that *Bacillus* inhibited the biofilm formation of *S. aureus* by regulating the QS system.

B. subtilis **H28 protects against** S. aureus **induced mastitis in mice**

We then investigated whether *B. subtilis* H28 could ameliorate *S. aureus* induced mastitis. The results showed that *S. aureus* infection resulted in observable pathological damage of the mammary gland, including edema, inflammatory cell infiltration, and disarrangement of the mammary gland structure (Fig. 5A-B). However, pre-treatment with *B. subtilis* H28 significantly alleviated these pathological damages of the mammary gland induced by *S. aureus* (Fig. 5A-B). The mammary gland enumeration of bacterial burdens revealed that *S. aureus* mice harbored higher bacterial burdens, while *B. subtilis* H28 treatment did substantially reduce bacterial burdens in the mammary gland (Fig. 5C). *S. aureus* treated mice had higher MPO activity than control mice, while it was reduced by *B. subtilis* H28, and administration of *B. subtilis* H28 alone did not affect the MPO activity when compared with the control group (Fig. 5D). In addition, *B. subtilis* H28 inhibited the production of TNF-α and IL-1β, the major pro-inflammatory cytokines in mastitis, induced by *S. aureus* (Fig. 5E-F).

B. subtilis **H28 improves the blood-milk integrity by increasing expressions of the tight junction proteins**

To assess the effect of *B. subtilis* H28 on *S. aureus*-induced damage of the epithelial barrier integrity, we examined the expression of ZO-1, Occludin, and Claudin-3 by western blotting. Following *S. aureus* infection, the mammary gland markedly reduced the expression of ZO-1, Occludin, and Claudin-3 when compared to the mammary gland without *S. aureus* stimulation (Fig. 6A-C). However, *B. subtilis* H28 treatment increased the levels of ZO-1, Occludin, and Claudin-3 compared to *S. aureus*-infected mice (Fig. 6A-C). Furthermore, we confirmed Claudin-3 level of immunochemistry and showed that *B. subtilis H28* increased the Claudin-3 expression (Fig. 6D). To determine whether *B. subtilis* H28 inhibits the QS system

and virulence, we measured the transcriptional level of AgrA, RNAIII, SarA through RTQ-PCR. We found that *B. subtilis* H28 could down-regulate the expression AgrA, RNAIII, and SarA as compared with that of the control group (Fig. 7A-C). Together, these results suggest that *B. subtilis* H28 inhibits *S. aureus* colonization and alleviates *S. aureus*-induced barrier damage by improving tight junctions.

B. subtilis H28 **inhibited** S. aureus**-induced inflammatory response in mouse mammary epithelial cells**

To verify the anti-inflammatory effect of *B. subtilis H28*, the mouse mammary epithelial cells were pretreated with *B. subtilis H28* then stimulated with *S. aureus*. The activation of the NF-κB signaling pathway is responsible for the release of pro-inflammatory cytokines, such as TNF-α and IL-1β. Chemokines are a class of specific small-molecule proteins that play critical roles in the recruitment and activation of leukocytes (27). CXCL3 controls the migration and adhesion of monocytes by interacting with a cell surface chemokine receptor called CXCR2, which affects the secretion of cytokines (28). We found that *S. aureus* increased TNF-α, IL-1 β, IL-6 and CXCL3 mRNA levels (Fig. 8A-D) but reversed by *B. subtilis H28* treatment in a dose-dependent manner (Fig. 8A-D). Moreover, we found that *B. subtilis H28* reduced the expression ica, RNAIII, and SarA compared to *S. aureus* treatment in a dose-dependent manner (Fig. 8E-F).

B. subtilis H28 **limites the activation of NF-κB and increases tight junction protein expression in MMECs**

In order to study the effect of *B. subtilis H28* on the integrity of the mammary gland epithelial cell induced by *S. aureus*, we found that *S. aureus* reduced the expression of Claudin-3, ZO-1, and Occludin. However, treatment with *B. subtilis H28* increased the levels of Claudin 3, ZO-1 and Occludin in a dose-dependent manner (Fig. 9A-C). This is compatible with the results of in vitro experiments. To test whether the anti-inflammatory properties of *B. subtilis H28* resulted from the regulation of the activation of the NF-κB, a predominant signaling pathway in *S. aureus* induced mastitis and associated with barrier injury, we assessed the levels of phosphorylation (p-) levels of p65 and IκB by western blotting and showed that the levels of p-p65 and p-IκB were significantly increased in the *S. aureus* group, while *B. subtilis H28* treatment markedly inhibited the activation of the NF-κB signaling pathway in a dose-dependent manner (Fig. 9A and E-F). Collectively, these results suggest that *B. subtilis H28* alleviates *S. aureus*-induced mastitis by improving tight junction protein expression and limiting NF-κB pathway activation.

Discussion

Mastitis constitutes one of the aggressive diseases that affecting the development of dairy farming. *S. aureus* is the most frequent and important pathogen causing mastitis in dairy cows (29). *Bacillus* as one of the normal host-microbiota exists in the mammary gland of cows (30). But the functional *Bacillus* and their role in *S. aureus*-induced mastitis pathogenesis remain poorly defined. The aim of our study was to elucidate the distinctive contribution of *Bacillus* in *S. aureus* mastitis. In studies, we found that there is interference between the mammary symbiotic bacterium *Bacillus* and the pathogenic bacterium *S. aureus*. *Bacillus* can down-regulate the gene expression of the QS system of *S. aureus* to inhibit the biofilm formation of *S. aureus* and thus reduce the amount of colonization of *S. aureus.* In treatment with

Bacillus in mice mastitis, the result showed that preparations of the *Bacillus* were as effective as commonly used antibiotics relieving *S. aureus*-induced mastitis for the treatment of intramammary infections and did not show adverse effects on mammary tissue.

Probiotic nutrition is frequently claimed to be improve health. Probiotic bacteria obtained with food are thought to decrease colonization by pathogens, and thus reduce susceptibility to infection (31-33). Some probiotic strains produce bacteriocin proteins, which can kill phylogenetically related pathogenic bacteria (31). It is particularly noteworthy human data indicate that probiotic *Bacillus* can comprehensively eradicate intestinal as well as nasal *S. aureus* colonization (18). Epidemiological studies have established that there is interference between the mammary symbiotic bacterium *Bacillus* and the pathogenic bacterium *S. aureus*. *Bacillus* can reduce the colonization of mammary glands by *S. aureus* in dairy cows. it was shown that the presence of *Bacillus* in the gland mammary could be a potential determining factor for the absence of *S. aureus* in cows.

Several studies have reported that in addition to the potential probiotic properties of *Bacillus* strains, the *Bacillus* exhibited strong anti-cholesterol, anti-biofilm, and antioxidant properties, making the strain with additional functional abilities (34). *B. subtilis* exerts an antimicrobial effect against a broad spectrum of pathogens through direct bactericidal activity or indirect enhancement of immune response, such as interrupting quorum-sensing regulatory system by production of fengycins (18), inhibiting *S. aureus* adhesion and biofilm formation by production of surfactant (35), and the enhancing anti-microbial function of macrophage (36). We study has confirmed a potent inhibitory capacity o*f B. subtilis H28* against both planktonic and biofilm *S. aureus* in vitro, which may prominently suppress the expression of genes associated with *S. aureus* adhesion, biofilm formation.

QS plays an essential role in biofilm formation, in the production of virulence factors and antimicrobial resistance (37). There is evidence that quorum sensing in *S. aureus* is important for the construction and dissolution of biofilm communities (38). The QS system controls *S. aureus* biofilm formation and release of virulence factors (21, 39). Researchers believe that the accessory gene regulator (agr) system regulates the QS in *S. aureus* (40). In fact, agr plays a pivotal role in regulating virulence factor expression (41), making it a potential therapeutic target (42). Biofilm formation is believed to require the adhesion of cells to a solid substrate, which creates multiple layers of cells. Intercellular adhesion requires PIA, which can be synthesized by-products of the intercellular adhesion (ica) locus(23, 24). The RNAIII activating peptide is thought to be a type of auto-inducing peptide (25, 26) that can phosphorylate its target molecule to activate the agr system, which increases the production of auto inducing peptides and AgrC to enhance the adhesion of the bacteria [36, 37]. Recent findings indicate that RNAIII is a regulatory mRNA molecule that not only regulates biofilm formation but also induces toxin production, such as enterotoxin, plasma-coagulase, hemolysin, and thermostable nuclease(26, 43). In addition, it is a fellow of the staphylococcal accessory regulator A (SarA) family of transcriptional regulators (44). Recent evidence indicates that SarA, a central regulatory element that controls the production of *S. aureus* virulence factors, is essential for the synthesis of polysaccharide intercellular adhesin (PIA) (45). We have shown that *B. subtilis* H28 could inhibit biofilm formation by *S. aureus* phenotypically. To determine whether *B. subtilis H28* inhibits

the QS system and virulence, we analyzed the effects of *B. subtilis H28* on the expression of *S. aureus* genes involved in quorum sensing (AgrA and RNAIII) and biofilm formation (Ica and SarA). We found that *B. subtilis* H28 could down-regulate the expression of genes agrA, RNAIII, ica, and sarA associated with the QS system. *B. subtilis* H28 effectively inhibited the QS system of *S. aureus* in a dose-dependent manner, resulting in inhibition of biofilm formation. *B. subtilis* H28 might be considered a QS inhibitor because of its ability to block the cell-to-cell signal transduction that is regulated by the QS system, and thus inhibiting expression of QS-related genes. The specific mechanism by which *B. subtilis* H28 inhibits the QS system requires further study and discussion, but the net outcome of QS inhibition is inhibition of biofilm formation. Therefore, we believe *B. subtilis* H28 is potential novel treatment against *S. aureus* biofilm-related infections.

Therefore, we further investigated in the next study whether *B. subtilis* H28 can have a protective effect against mastitis caused by *S. aureus.* Recently, the intramammary infusion of lactic acid bacteria has emerged as a potential new alternative to antibiotics for preventing and treating bovine mastitis (46). We took the same mammary gland infusion and an important finding in this study is that the intramammary infusion of *B. subtilis H28* can significantly reduce *S. aureus* colonization to alleviate *S. aureus*-induced mastitis.

Our study suggests valuable translational applications regarding alternative strategies to combat antibiotic-resistant *S. aureus*. *Bacillus*-containing probiotics could be used in order to simple and safe *S. aureus* decolonization strategies. In that regard, it is particularly noteworthy that our data indicate that probiotic *Bacillus* can eradicate mammary *S. aureus* colonization. Such a probiotic approach would have numerous advantages over the present standard topical strategy involving antibiotics. *Bacillus* provides a reasonable reference for the treatment of inflammatory diseases. Therefore, our study provided support for the probiotic effect of *Bacillus* and *Bacillus* may have the potential as a promising candidate for the treatment of mastitis.

At the same time, the study has a few limitations. The specific mechanisms responsible for the inhibition of the QS system by *B. subtilis* H28 need to be further investigated and discussed. In addition to this, it was observed *Bacillus* can reduce the colonization of mammary glands by *S. aureus* in cows and was confirmed in mice. However, all the experiments evaluating the effects of this defect were carried out in mice, which can be seen as a potential limitation. Further interventional studies in cows should be made to evaluate the proposed therapeutic strategy. Our future research will focus on the cows in vivo to identify the precise mechanism by which *B. subtilis H28* inhibits S. aureus biofilm formation.

Conclusions

Collectively, our results indicate that commensal Bacillus inhibits S. aureus colonization and alleviates *S. aureus*-induced mastitis by influencing biofilm formation, which suggests a potential strategy for the decolonization of S. aureus and acts as a basis for the prevention and treatment of S. aureus-related disease.

Declarations

Author contributions

Min Qiu performed the article writing and result evaluation. Caijin Zhao performed histologic analysis and article revision. Xiaoyu Hu performed the final revision of the article and provided expert opinion. Lianjun Feng, Siyuan Gao contributed to animal experiment. Naisheng Zhang and Yunhe Fu contributed to the study design.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Compliance with ethical standards

Ethical statement

The permit number (202103058) was assigned by the Institutional Animal Care and Use Committee (IACUC) of Jilin University for animal experiments approvals. The full proposal was reviewed by the IACUC ethics committee, which approved the animal care and use permit license. All experiments comply with the manual of the care and use of laboratory animals published by the US National Institutes of Health.

Data Availability

All data analyzed during this study are included within this article. Any other data are available from the corresponding author upon reasonable request.

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Figures

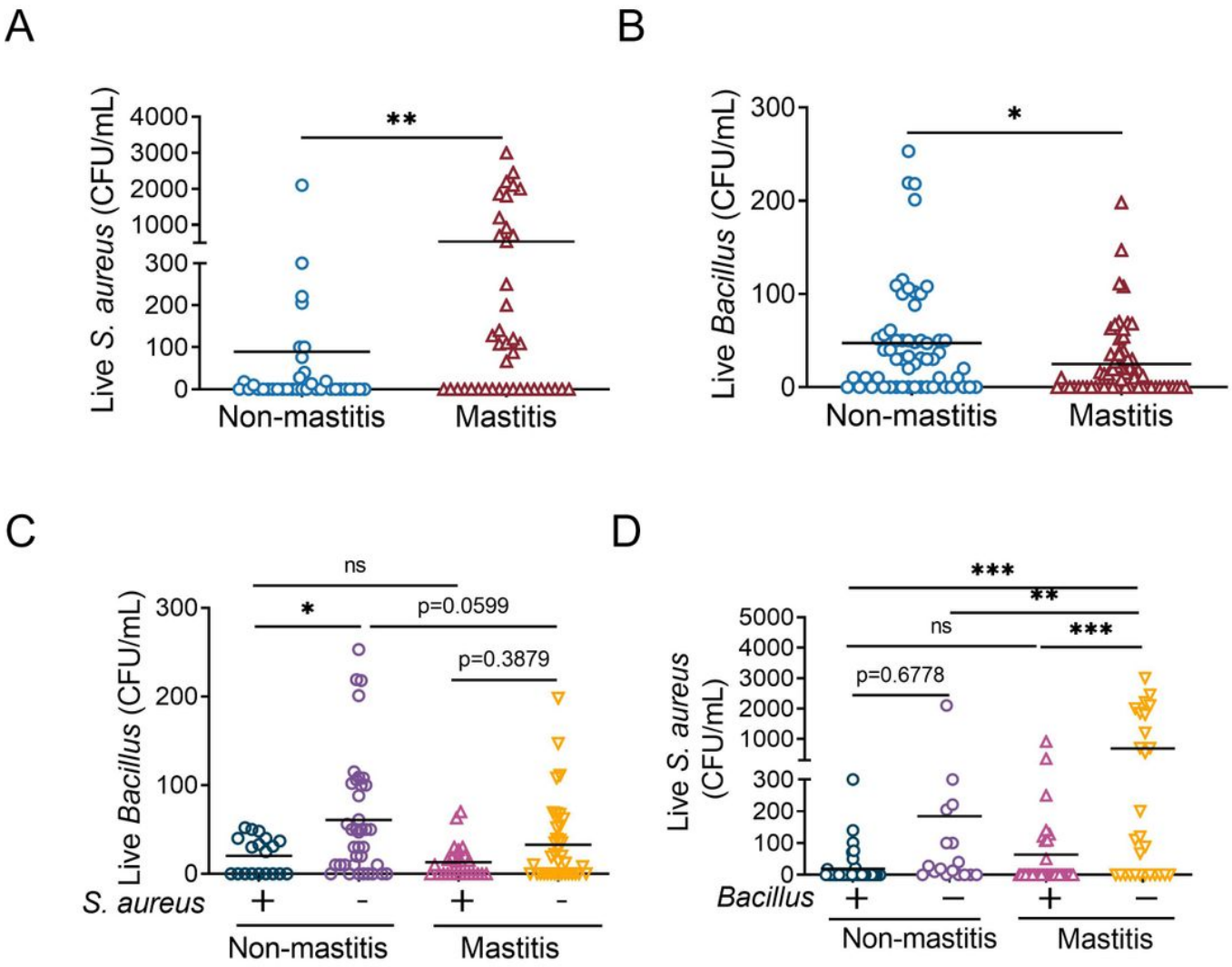

Figure 1

Mastitis cows have increased S. aureus and reduced Bacillus colonization in the milk. (A) Live S. aureus CFUs in non-mastitis cows and mastitis cows. (B) Live Bacillus CFUs in non-mastitis cows and mastitis cows. (C) Effect of the presence or absence of S. aureus on the live Bacillus CFUs in non-mastitis cows and mastitis cows. (D) Effect of the presence or absence of Bacillus on the live S. aureus CFUs in non-

mastitis cows and mastitis cows. One-way analysis of variance was used for statistical analysis. *p < 0.05 and *p < 0.01 and ***p□0.001 indicate significant differences from each group. ns, no significance.

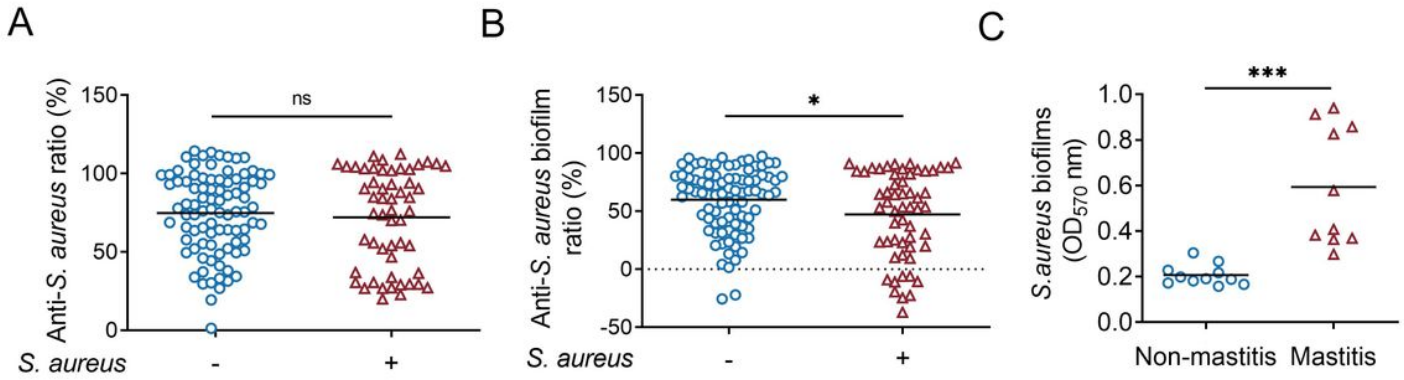

Figure 2

S. aureus from mastitis have higher biofilm formation capacity but inhibited by commensal Bacillus. (A) Effect of Bacillus on the growth of S. aureus. (B) Effect of Bacillus on the biofilm of S. aureus. (C) S. aureus biofilm forming ability in non-mastitis cows and mastitis cows. One-way analysis of variance was used for statistical analysis. The values presented are the mean ± SEM (n=4). *p < 0.05 and *p < 0.01 and ***p□0.001 indicate significant differences from each group. ns, no significance.

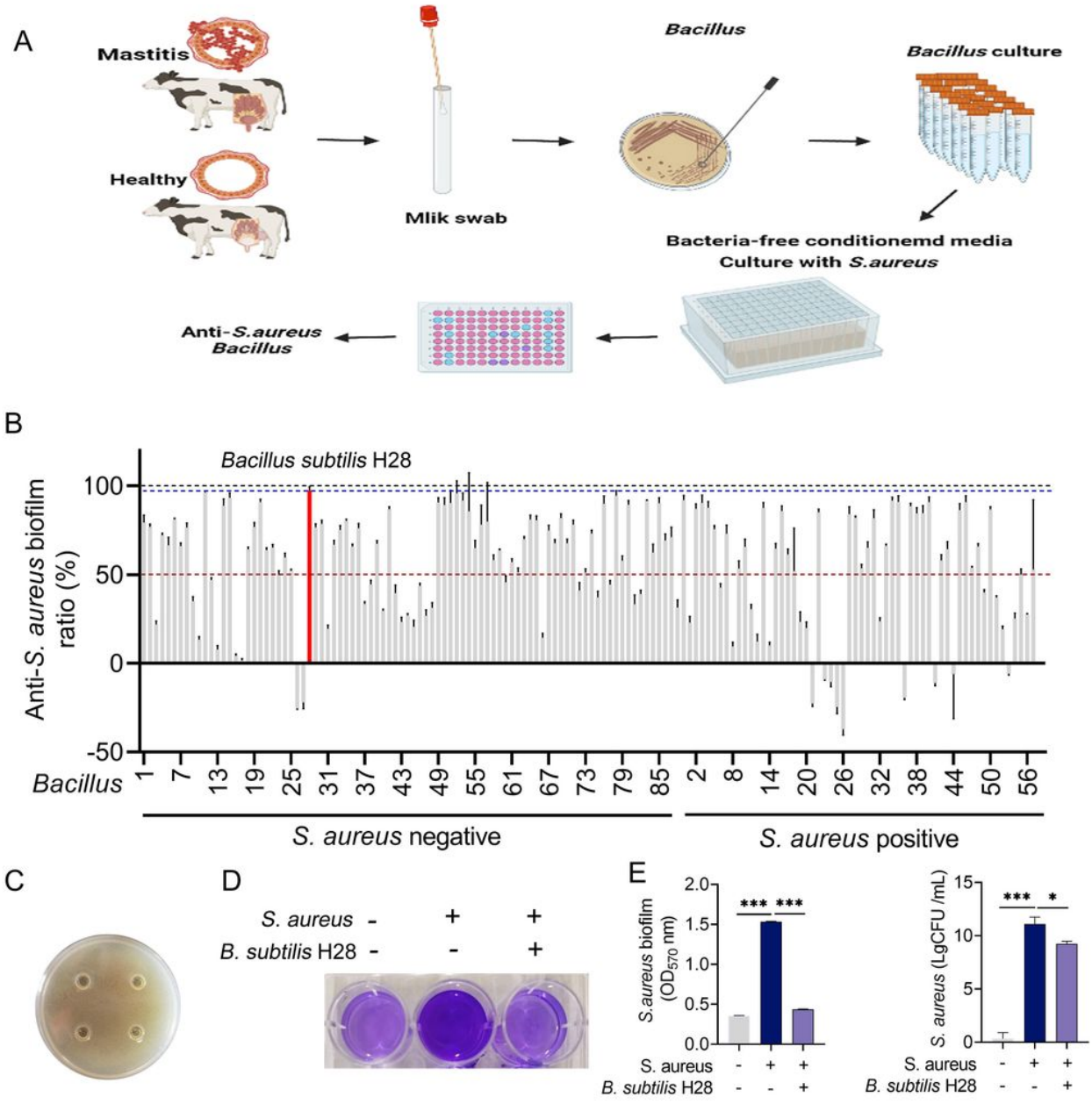

Figure 3

B. subtilis H28 isolated from healthy cow inhibited S. aureus biofilm. (A) Schematic of a high-throughput anti-biofilm screening of Bacillus against S. aureus. (B)Percentage of anti-biofilm effect of Bacillus without (≤50%) or with anti-biofilm activity against S. aureus (≥50%). The first 87 strains of Bacillus were isolated from healthy cows. The last 58 Bacillus strains were isolated from cows with S. aureus-mastitis. Red is the strain with the best biofilm effect against S. aureus, i.e., B. subtilis H28. (C) Antibacterial effect of B. subtilis H28 detected by inhibition circle. a and b add TBS, c and d add B. subtilis H28 culture filtrate. (D) Representative images of crystal violet staining for S. aureus biofilm. Quantitative analysis of biofilm formation by crystal violet-staining was dissolved and measured at 570 nm in a microplate reader. (E) Intra-biofilm S. aureus count. One-way analysis of variance was used for statistical analysis.

The values presented are the mean ± SEM (n=4). *p < 0.05 and *p < 0.01 and ***p□0.001 indicate significant differences from each group. ns, no significance.

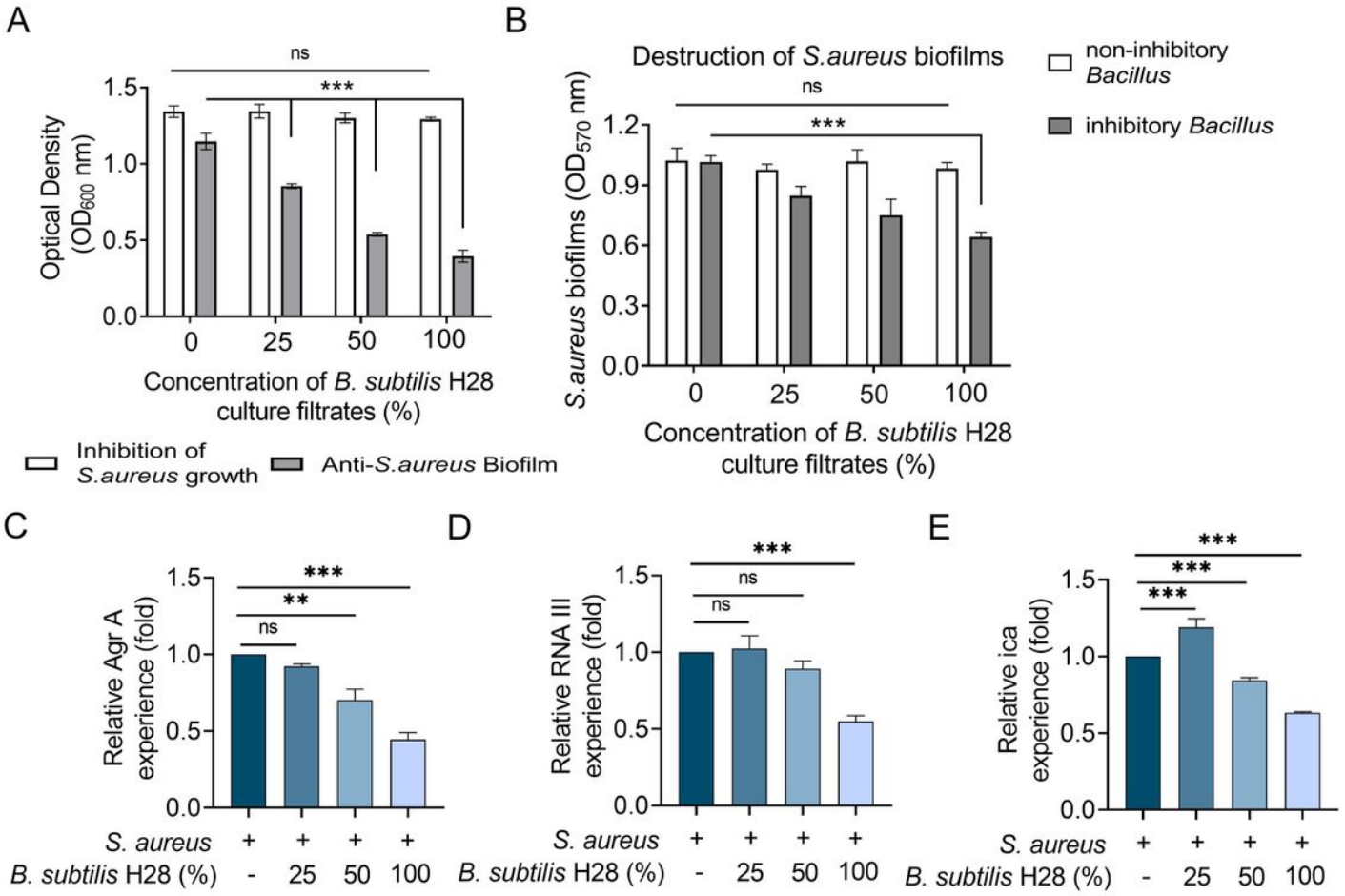

Figure 4

B. subtilis H28 limits S. aureus biofilm formation by inhibiting QS system (A) Effect of 25%, 50%, 100% B. subtilis H28 culture filtrates on the growth of S. -aureus and on S. aureus biofilm. (B) Destructive effect of 25 %, 50 % and 100 % B. subtilis H28 culture filtrate on the formed biofilm of S. aureus. Meanwhile, non-inhibiting Bacillus, which has no biofilm inhibitory effect on S. aureus, was used as a comparison. (C) Relative expression of the AgrA was identified using qPCR. (D) Relative expression of the RNA□were identified using qPCR. (E) Relative expression of the ica was identified using qPCR. One-way analysis of variance was used for statistical analysis. The values presented are the mean ± SEM (n=3). *p < 0.05 and *p < 0.01 and ***p□0.001 indicate significant differences from each group. ns, no significance.

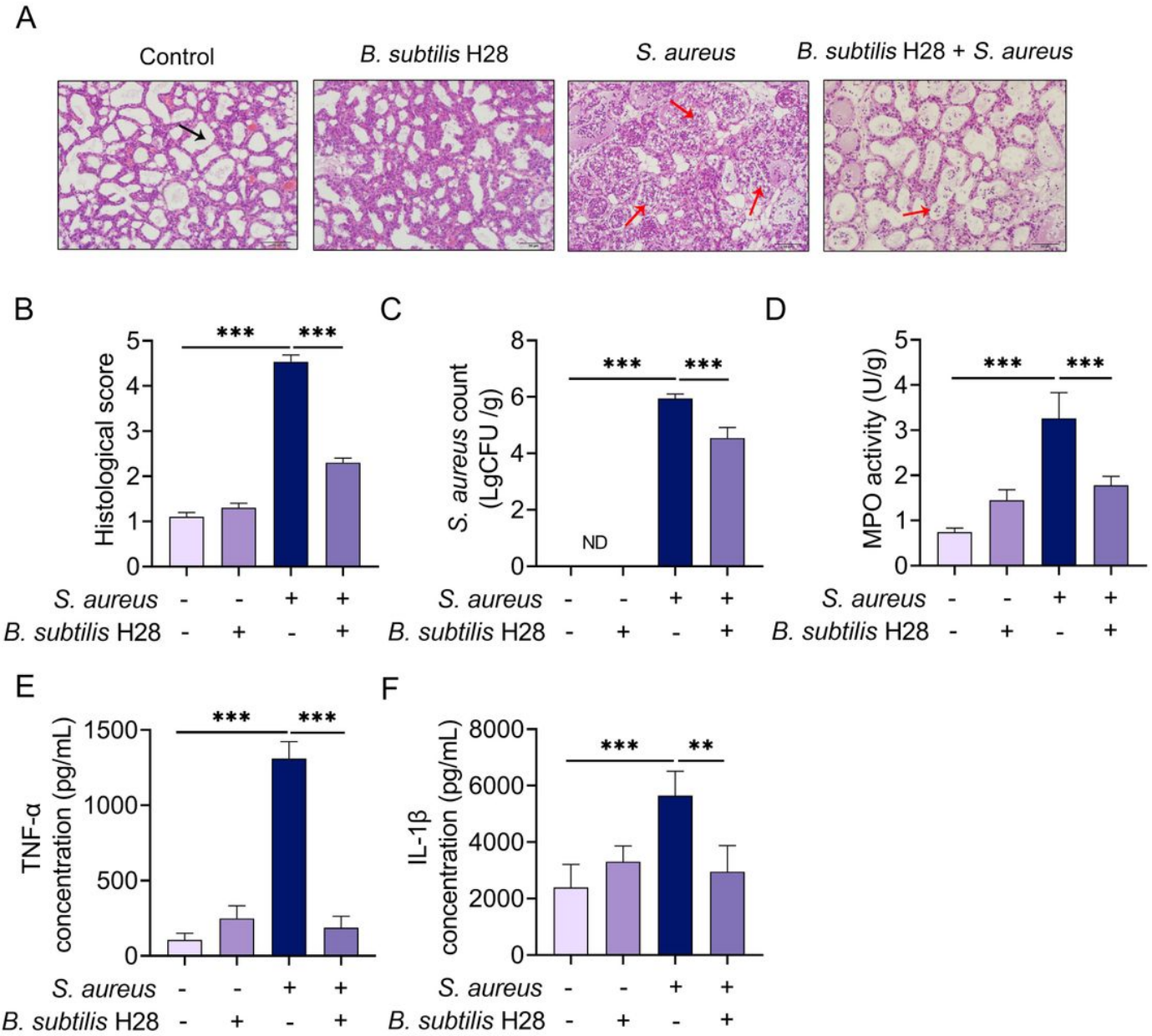

Figure 5

B. subtilis H28 alleviates S. aureus induced mastitis in mice. B. subtilis H28 (1 × 108 CFU) was administered to the mammary glands of mice 2 h before infection with S. aureus (1 × 108CFU). Twenty-four hours later, mammary gland tissues were collected and used for testing. (A) Pathological changes were detected with H&E (200×). The black arrow indicates normal alveolar cells. The red arrow indicates the inflammatory cells infiltration. (B) Histopathologic scoring. (C) Mammary tissue S. aureus count. (D) MPO activity. (E)TNF-α and (F) IL-1β in the mammary gland tissues. The values presented are the mean ± SEM (n=5-6). One-way analysis of variance was used for statistical analysis. *p < 0.05 and *p < 0.01 and ***p□0.001 indicate significant differences from each group. ns, no significance.

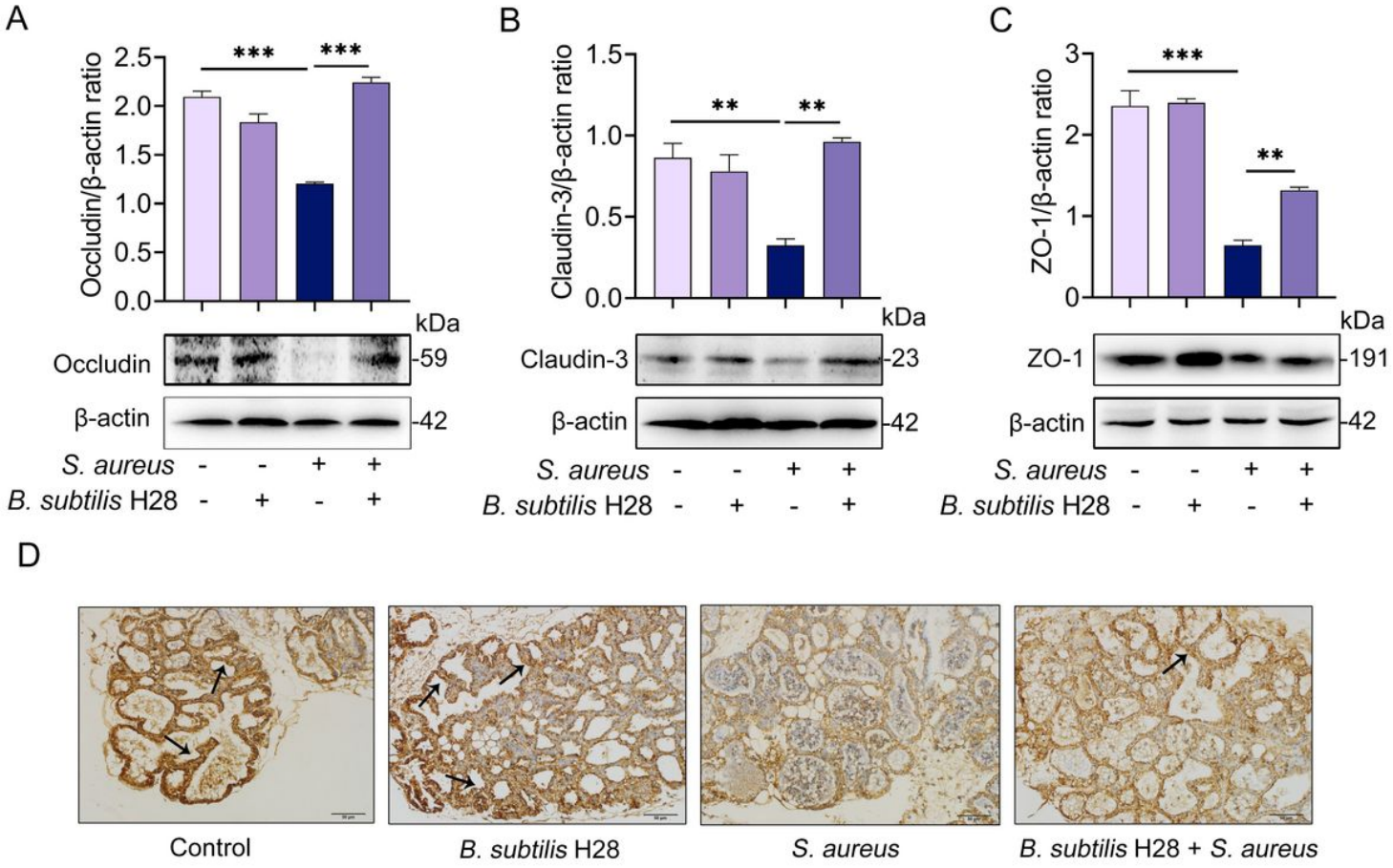

Figure 6

Effects of B. subtilis H28 on the distribution of tight junction proteins Expression of tight junction proteins by western blotting, including Occludin, Claudin-3, and ZO-1, was tested 24 h after infection in in mice mammary gland. The values presented are the mean ± SEM (n=3-4). One-way analysis of variance (A-C) was used for statistical analysis. *p < 0.05 and *p < 0.01 and ***p 0.001 indicate significant differences from each group. ns, no significance. (D) Representative images of mammary gland immunohistochemistry (IHC) sections stained with Claudin-3 antibody (scale bar, 50 μm). The black arrow indicates the positive staining.

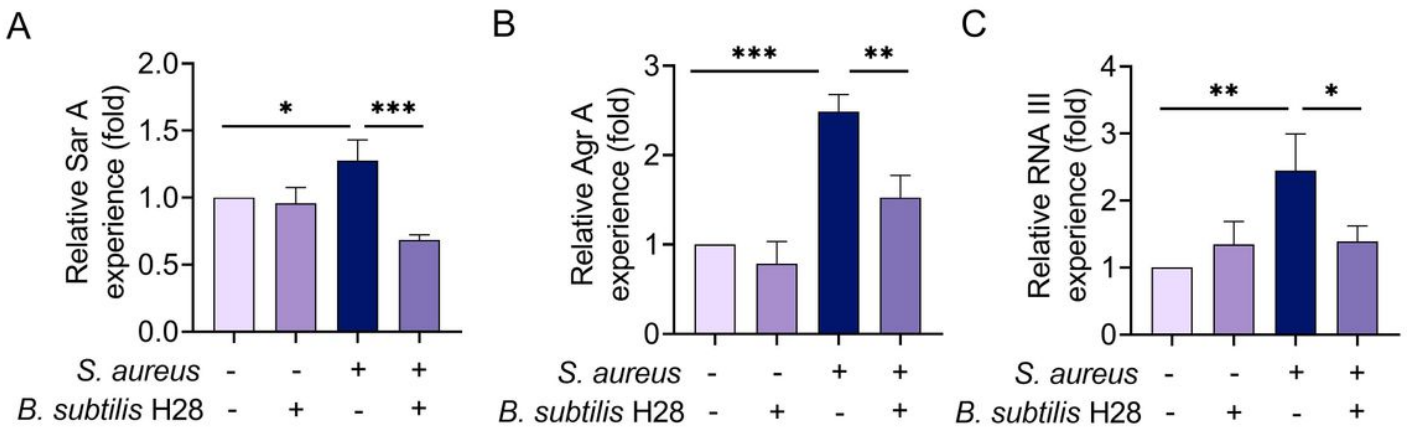

Figure 7

Effect of B. subtilis H28 expression of QS related genes in mice mammary gland (A) Relative expression of the SarA was identified using qPCR. (B) Relative expression of the RNA was identified using qPCR. (C) Relative expression of the Agr A was identified using qPCR. The values presented are the mean ± SEM(n=3). One-way analysis of variance was used for statistical analysis. *p < 0.05 and *p < 0.01 and ***p0.001 indicate significant differences from each group. ns, no significance.

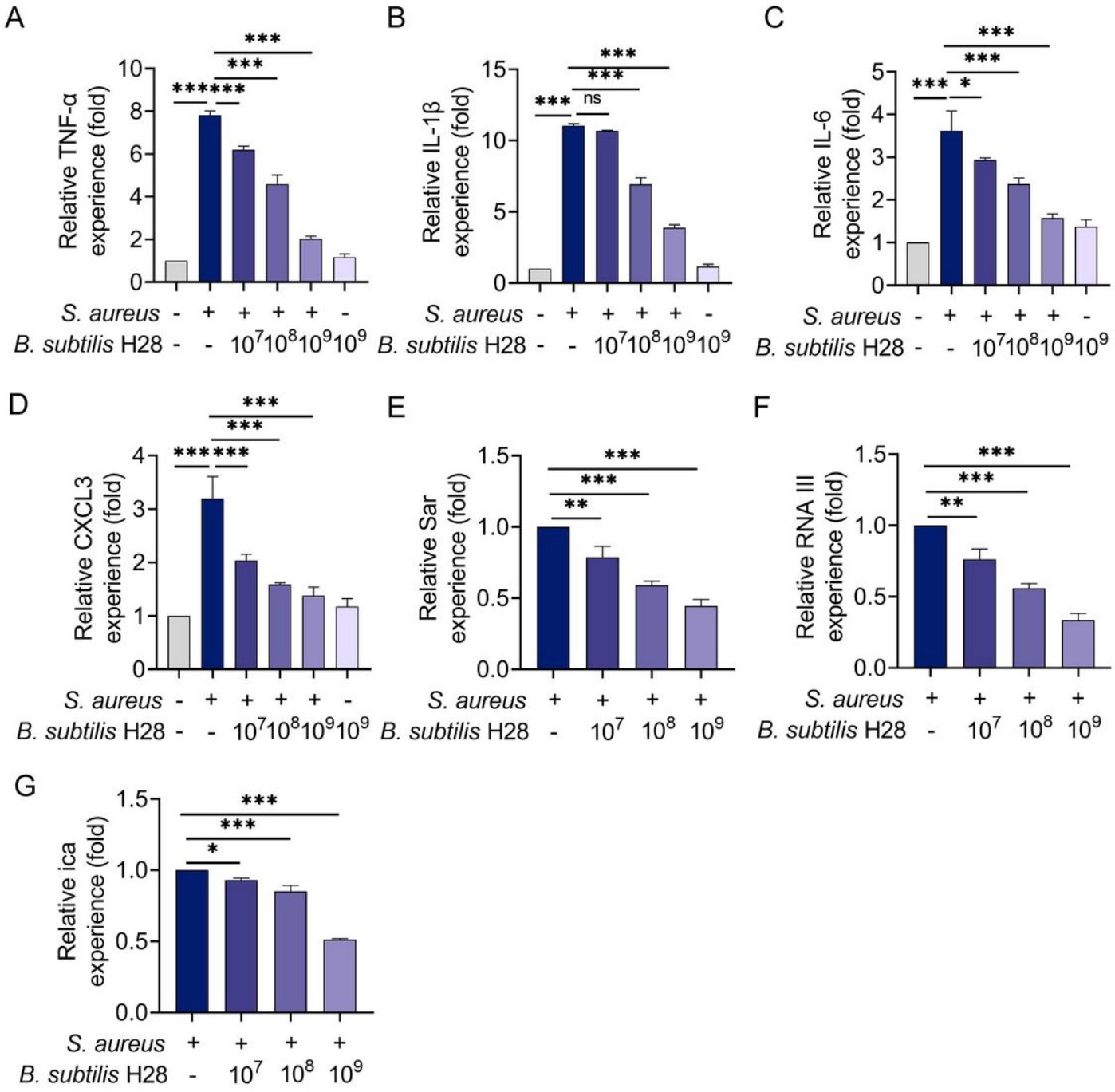

Figure 8

B. subtilis H28 inhibited S. aureus-induced inflammatory response and expression of QS genes in mice mammary epithelial cells. (A-C) The TNF-α, IL-1β and IL-6 mRNA levels in mice mammary epithelial cells were identified using qPCR. (D) The CXCL3 mRNA levels in mice mammary epithelial cells were identified using qPCR. (E) Relative expression of the Sar was identified using qPCR. (F) Relative expression of the RNA was identified using qPCR. (G) Relative expression of the ica was identified using qPCR. The values presented are the mean ± SEM (n=3). One-way analysis of variance was used for statistical analysis. *p < 0.05 and *p < 0.01 and ***p0.001 indicate significant differences from each group. ns, no significance.

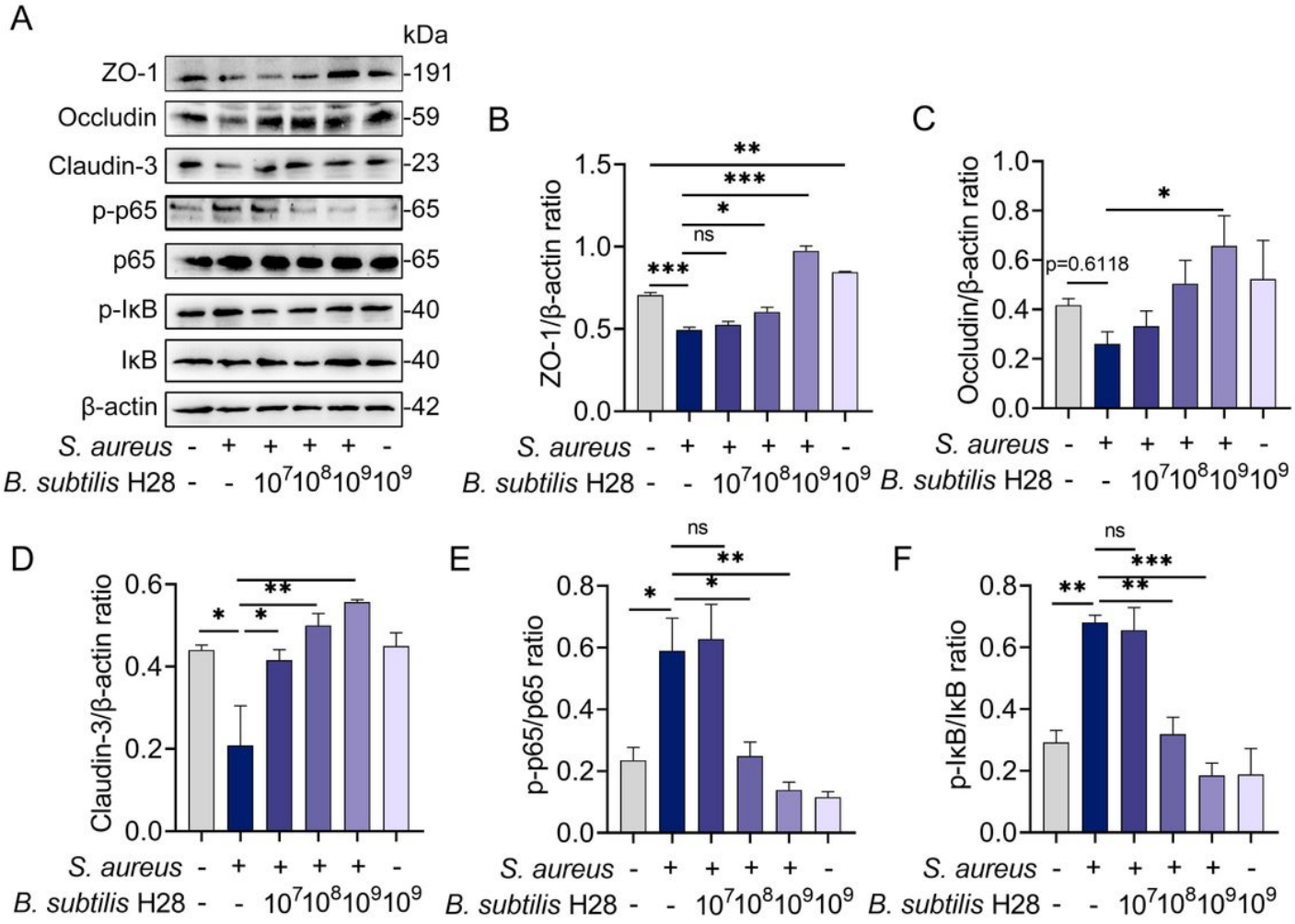

Figure 9

B. subtilis H28 increases the tight junction proteins and limits NF-κB activation in mice mammary epithelial cells. Effect of B. subtilis H28 on the expression of NF-κB pathways were induced by S. aureus. All protein samples were analyzed by Western blot with specific antibodies and β-actin was used as a control. (A) Protein strips. (B) ZO-1/actin ratio (C) Occludin/actin ratio. (D) Claudin-3/actin ratio. (E) p-p65/p65 ratio. (F) p- IκB/ IκB ratio. The values presented are the means ± SEM (n=3-4). One-way analysis of variance was used for statistical analysis. *p < 0.05 and *p < 0.01 and ***p 0.001. ns, no significance.