The correlation and mechanism between rumen microbiota disturbance and mastitis in dairy cows

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Research

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Abstract

Background: Mastitis, which is a highly prevalent disease in dairy cows and economically costly to the dairy industry worldwide, is commonly believed to be caused by local infection of the mammary gland. Recently, studies have suggested that the gut microbiota plays an important role in the development of cow mastitis, yet the mechanisms linking the gut microbiota to mastitis remain unclear. This study assessed the effects and mechanisms of rumen microbiota on mastitis based on the subacute rumen acidosis (SARA) model induced by feeding a high-concentration diet (HCD) to Holstein cows .

Results: Lipopolysaccharides (LPS)-derived from the rumen translocated into the blood and then accumulated in the mammary glands by damaging the integrity of the blood-milk barrier and induced an inflammatory response in the mammary glands of SARA cows. Furthermore, elevation of the abundance of *Stenotrophomonas* was one of the endogenous factors to induce mastitis during cows suffering SARA. Finally, we found that rumen microbiota imbalance induced by SARA resulted in increased severity to mastitis by inhibiting the clearance of pathogens from the mammary glands in SARA cows.

Conclusions: This study identifies rumen microbiota dysbiosis and LPS translocation from the rumen to the mammary gland as important factors to induce mastitis in cows. In addition, rumen microbiota imbalance increased mastitis susceptibility by inhibiting the ability of the mammary glands to clear out pathogens. Our studies provide a basis for novel therapeutic strategies that exploit the gut microbiota in cow mastitis.

Background

Milk production represents an important sector of agriculture. Ruminant species are the primary animals that produce milk agriculturally, in which cows account for approximately 84 % of total worldwide milk production[1]. Mastitis, a major symptom of inflammation of the mammary gland, is one of the most serious diseases of dairy cows, and its occurrence has a massive negative effect on animal well-being and farm economics due to treatment costs and a reduction in milk production[2, 3]. It is commonly believed that the major cause of mastitis is opportunistic pathogens, such as *Staphylococcus aureus*, *Streptococcus uberis*, and *Escherichia coli*, invade and colonize the mammary gland[4-6]. However, recent research has demonstrated that microbiological diagnoses of milk derived from cows that suffered from mastitis include 27.3 % culture-negative[7]. Thus, it is possible that factors other than these mastitis-associated pathogens are associated with the development of mastitis, such as gut microbiota[8].

The rumen, as one of the most important digestive organs in ruminants, plays an important role in many diseases of dairy cows. In order to maximize milk yield, cows are often fed a high-concentrate diet (HCD) to meet the nutritional demands of lactation. However, the long-term overfeeding of cows with an HCD often leads to a metabolic disorder termed subacute ruminal acidosis (SARA), which mainly characterized by a prolonged decline in ruminal pH, causing rumen microbial disbalance[9-11]. Epidemiological investigation indicated a prevalence of SARA ranging from 18-40 % in early and mid-

lactation dairy cows due to the addition of a high proportion of concentrate to the diet[12-14], and reports also demonstrated that most cases of mastitis take place in the early lactation phase[15]. It is suggested that the occurrence of cow mastitis is associated with the development of SARA. In addition, SARA is a typical case model of rumen microflora disorder in cows, and it is also indicated that changes in the rumen microbiota community may be associated with the development of cow mastitis. LPS, one of the main constituents of the cell walls of gram-negative bacteria, is an important factor that induces inflammation. Because an abundance of gram-negative bacteria is present in the rumen of cows, the rumen is considered the repository of LPS. During the lactation period, the mammary gland blood volume of the cow accounts for 8 % of the total body blood volume. It is indicated that LPS circulating in the blood may enter the mammary gland and induce the development of mastitis, especially during the lactation period.

The somatic cell count (SCC), consisting mostly neutrophils, is the gold standard for the clinical diagnosis of mastitis. Neutrophils translocation from blood to milk must occur through the blood-milk barrier, which is an important gatekeeper that hinders the movement of molecules between the blood and tissues[16]. Evidence has shown that gut microbiota play an important role in the integrity of the blood-brain barrier[17], blood-testis barrier[18], and gut barrier[19], and research has also suggested that injection of LPS by the mammary gland damaged the function and structure of the blood-milk barrier[20]], lowering the threshold for neutrophils to cross the barrier. When LPS enters the mammary gland and stimulates the neutrophils, a large number of pro-inflammatory cytokines are produced and induce the inflammatory response of the mammary gland. In view of the above theoretical basis, we put forward the theoretical hypotheses: when stress factors such as overfeeding of HCD result in rumen dysbacteriosis, a large amount of LPS was released and translocated into the bloodstream, causing subclinical endotoxemia. When the cow is subjected to lactation stress, circulating LPS enters the mammary gland, damages the blood-milk barrier, reduces the threshold for neutrophils migration across the blood-milk barrier, and induces a large number of neutrophils to enter mammary gland, which leads to mastitis. Thus, in the present study, cow SARA induced by HCD was used as the model of rumen dysbacteriosis to study the correlations and mechanisms between rumen microbiota and mastitis of lactating dairy cows.

Results

SARA induces the system inflammatory response in dairy cows

In order to exclude the effect of experimental period on milk composition, we detected the milk composition of another eight Hosltein cows with the same standards of the cows that used to establish SARA model (lactation days and weight similar and non-pregnant cows). The results showed that there were no significant changes in milk yield, milk fat, milk protein, lactose, urea nitrogen and SCC of healthy cows during the experiment period (Fig. 1). To evaluate the HCD-induced SARA model in cows, we measured the dry matter intake (DMI) and milk yield weekly. After 8 weeks of feeding an HCD, the DMI and milk production were significantly reduced when compared to the baseline from before HCD feeding

(see supplementary fig. 1A-B). In addition, the pH of the rumen fluid was obviously reduced, and a pH value < 5.8 was sustained for more than 3 h at different periods of time in the cows fed an HCD for 8 weeks (see supplementary Table S1), indicating that SARA was efficiently provoked[21]. In addition, levels of pH in feces were also significantly reduced, but there were no changes in the blood in SARA cows compared to the control cows (see supplementary Table S1). Abundance of evidence proved that a large number of LPS-derived rumen of SARA cows can be translocated into the bloodstream, and lead to systemic inflammatory response[22]. In the present study, the levels of LPS in rumen fluid, feces, and tail vein significantly all increased in cows happened SARA (Fig. 2A-C). To evaluate the host system inflammatory response, we tested the clinical health parameters, including rectal temperature, pulse rate, and respiratory rates, has not changed in SARA cows (see supplementary Table S2). However, the numbers of neutrophils in blood has markedly increased (see supplementary Table S3), and the levels of Ca and PHOS in plasma were significantly reduced in cows suffered from SARA (see supplementary Table S4). In addition, inflammatory cytokines, including TNF-α, IL-1β, IL-6, IL-17, as well as the levels of acute phase proteins serum amyloid-A (SAA) in plasma all significantly increased in SARA cows (Fig. 2D-H). Plasma levels of ALB, GLB, A/G, TP, and GLU were assessed as a indicator of the metabolism and inner organ status of animal[23]. The higher levels of ALB and the lower levels of GLU (see supplementary fig. S2C and S1G), but another has no changes in plasma were observed in SARA cows (see supplementary fig. S1D-F). The liver is an important metabolic and immune organ of ruminants, it plays an important role in removing the LPS in bloodstream[24]. On the contrary, over-production of LPS also can promotes the occurrence of inflammatory reactions and the aggregation of immune cell, and thus interferes with the metabolism of substances in the liver[25]. SARA cows showed a severe pathological damage, including inflammatory cells infiltration and liver cells injury ballooning (Fig. 2I-J), and up-regulated the level of AST (Fig. 2K). LPS entering the blood from the rumen needs to cross the rumen wall, thus, the rumen epithelial permeability was assessed in cows from control and SARA. Hispathological changes suggested that rumen epithelium of SARA was incomplete, and greater numbers of immune cells infiltrated into the rumen epithelium of SARA cows (Fig. 2M-N). Furthermore, the tight junction proteins of rumen barrier testing indicated that the proteins expression of Claudin-1, Claudin-3, Occludin, and ZO-1 all significantly reduced in SARA cows when compared to the control cows (Fig. 2O). Moreover, histopathologic changes of intestine tissues indicated that gut epithelium desquamation and severe cellular damage in SARA cows when compared to the control cows, and SARA cows showed significantly higher epithelial damage scores in intestines (Fig. 2P-Q). In addition, the expression of Claudin-1, Claudin-3, Occludin, and ZO-1 were all significantly reduced in SARA cows when compared to the control cows (Fig. 2R). These results suggested that cows SARA increase the permeability of the rumen epithelial, and induces rumen-derived LPS is released into the bloodstream, damaging the liver function and inducing systemic inflammation.

SARA induces the inflammatory response in the mammary gland by increasing blood-milk barrier permeability in dairy cows

Milk composition analysis showed that the contents of milk fat, milk protein, fat/protein ratio and dry matter were markedly reduced (Fig. 3A-C and Fig. 3E). The content of lactose in milk from SARA cows was unchanged (Fig. 3D) and urea nitrogen significantly increased when compared to the mil from control cows (Fig. 3F). Importantly, SCC and SAA, two important indicators of mastitis, also significantly increased after cows suffered from SARA (Fig. 3G-H). In addition, the levels of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, were obviously increased in the milk (see supplementary fig. S2A-C) and the mammary glands (Fig. 3I-K) from cows suffering from SARA. Histopathological analysis from SARA cows showed a large amount of inflammatory cell infiltration, thickening of the alveolar wall, and mammary gland destruction in the mammary gland and a higher inflammation score (Fig. 3L-M). Furthermore, we assessed whether cow mastitis induced by SARA was associated with LPS migration from the rumen to the mammary gland via the bloodstream. The levels of LPS in the lacteal veins, milk and mammary glands were all significantly increased in cows with SARA (Fig. 3N-P). LPS is a strong activator of innate immune responses and is recognized by TLR4, which then activates activated NF-κB to induce the transcription of TNF-α, IL-1β, and IL-6. The expression of TLR4, phosphorylation NF-κB p65, and phosphorylation IκBα were upregulated in the mammary glands of SARA compared with control cows (Fig. 3Q). The blood-milk barrier is a specific structure that plays an important role in preventing foreign matter from the blood or external environment entering the mammary gland[26]. To evaluate the permeability of the blood-milk barrier in control and SARA cows, we detected the tight junction proteins that make up the blood-milk barrier. The expression of Claudin-1, Claudin-3, Occludin, and ZO-1 was significantly reduced in SARA cows (Fig. 3R). In addition, blood-derived proteins in milk, including IgG and lactate dehydrogenase (LDH), serve as indicators of the integrity of the blood-milk barrier[27]. The production of IgG and LDH obviously increased both in the blood and milk of SARA cows when compared to the control cows (see supplementary fig. S2D-G). These results suggest that SARA of cows leads to rumen-derived LPS entering the mammary gland through blood circulation, damaging the blood-milk barrier, and inducting inflammation of the mammary gland in cows.

Dissimilarity of milk, rumen fluid, feces, and blood microbiota in cows from control and SARA

To explore the development of mastitis induced by SARA was associated with the changes with microbiota, we collected milk, rumen fluid, feces, and blood samples from the same 8 Holstein dairy cows on day 0 and 8 weeks after feeding an HDC to detect the community of microbiota using the V4 region of the bacterial 16S ribosomal RNA (rRNA) gene amplified PCR from all 64 samples. 16S rRNA sequencing resulted in 6,835,632 reads, with an average of 77677.64 ± 2614.504 reads (standard error of the mean) from each sample. The estimators of community richness (chao 1, and ace) and richness (shannon index and simpson) of milk, rumen fluid, and feces in SARA cows were significantly reduced compared to the control cows, but there were no significant effects on the richness of blood (see supplementary fig. S3). The Top 10 phyla and the top 30 genera in relative abundance were used to analyze the bacterial community structure of rumen fluid, milk, feces, and blood of cows. At the phylum level, Proteobacteria, Firmicutes, Bacteroidetes, and Tenericutes were the four dominant phyla in all samples by relative abundance (Fig. 4A and supplementary Table S5). T-test analysis showed that in the rumen fluid

microbiota, Proteobacteria was significantly increased in SARA cows, while Firmicutes, Tenericutes and other low abundance phyla, including Spirochaetes, Gracilibacteria, Euryarchaeota, Fibrobacteres, and Kiritimatiellaeota were significantly reduced in SARA when compared to the control cows (Fig. 4B). The changes of milk microbiota was similar to the changes of rumen microbiota, as shown in Proteobacteria was significantly increased, while other phyla, including Firmicutes, Bacteroidetes, Tenericutes, and Actinobacteria were significantly reduced in SARA cows when compared to the control cows (Fig. 4C). In feces microbiota, the relative abundance of Proteobacteria significantly increased, while the relative abundance of Melainabacteria was reduced in SARA cows when compared to the control cows (Fig. 4D). However, there are no detected changed significantly phyla in blood between control and SARA cows (see supplementary Table S6).

To test the microbiota elements at the genus level, the sequence showed that *Stenotrophomonas*, *Bacteroides*, *Exiguobacterium*, and *Sphingononas* were the most prominent genera in all simples (Fig. 5A and Table S7). Significant differences in the genus of milk, rumen fluid, feces, and blood between control and SARA cows were further assessed by t-test analysis. *Stenotrophomonas* and *Succinivibrio* were increased, while the other ten genes were reduced in rumen from SARA cows compared to those from control cows (Fig. 5B). The changes of milk microbiota was also similar to the changes of the rumen microbiota, as shown in the relative abundance of *Stenotrophomonas*, *Sphingomonas* and *Brevnudimonas* were obviously elevated, while other detected 14 genera all were reduced in milk from SARA cows compared with those from control cows (Fig. 5C). Microbiota in feces from cows indicated that except *Stenotrophomonas*, other genera, including *Succinivibrio* , *unidentified-Prevatellaceae*, *unidentified-Erysipelotrichaceae*, *Unidentified-Clostridiales*, *Roseburia*, *Oscillibacter*, *Anaeroplasma*, and *Marvinbryantia* were significantly increased, and other detected five genera were reduced in the SARA group (Fig. 5D). In addition, there are no significantly changes in microbiota in blood from SARA and control cows (see supplementary Table S8). These results showed that the changes of the microbiota of milk and rumen fluid samples after SARA have similarities. It is indicates that there may be a certain correlation between milk microbiota and rumen microbiota of dairy cows.

Similarity of bacterial community composition in the milk, rumen fluid, feces, and blood of control and SARA cows

To explore the correlation of microbiota structure in milk, rumen fluid, feces, and blood, we carried out a similarity analysis on the microflora of four different samples from control cows. Although the rumen fluid and feces are the most similar at the level of diversity of microbiota (Fig. 6A and supplementary fig. S4A), the milk and rumen fluid present the most similar microbiota abundance (Fig. 6B and supplementary fig. S4B). In addition, a principal coordinate analysis (PCoA) plot, based on the bray curtis distance matrices, indicated that the microbiota composition of milk was more similar to the microbiota composition of rumen fluid (Fig. 6C). Interestingly, nonmetric multidimensional scaling (NMDS) ordination performed on the Bray-Curtis dissimilarity showed that the bacterial community profiles of rumen fluid was closer to those of milk in SARA cows than in control cows (Fig. 6D). To confirm the results in the NMDS, the microbiota community dissimilarity was also evaluated by MRPP, which was

based on the Bray-Curtis distance matrices. Application of MRPP to milk and rumen fluid and milk and feces indicated that rumen fluid (HM-HR vs SM-SR, expected-delt = 0.621 vs 0.446), but not feces microbiota (HM-HF vs SM-SM, expected-delt = 0.649 vs 0.683), were more similar to milk microbiota in SARA cows when compared to the control cows (Fig. 6E-F). These results suggest that the major changes in the bacterial community profile of milk, rumen fluid, feces, and blood during SARA lead to more similar community profiles among milk and rumen fluid.

Stenotrophomonas from the rumen maybe a factor to induce inflammation in the mammary gland in cows suffering from SARA

To evaluate whether a unique bacterial was associated with the occurrence of mastitis by analyzing core genera shared in milk, rumen fluid, feces, and blood in both control and SARA cows using Venn diagrams. A total of 772 core genera in control cows and 824 core genera in SARA cows overlapped among the milk, fluid rumen, feces, and blood samples (Fig. 7A-B). This is indicated that changes in bacterial abundance is more important for the development of mastitis than unique differences in bacterial communities. Furthermore, core genera accounted for 13.87 % of all milk bacteria in control cows and 21.25 % in SARA cows, 18.87 % of all rumen fluid bacteria on control cows and 22.26 % on SARA cows, 25.76 % of all feces bacteria on control cows and 25.46 % on SARA cows, 22.37 % of all blood bacteria on control cows and 25.46 % on SARA cows (Fig. 7C). This suggested that some bacteria may migrate among the milk, rumen fluid, and blood, resulting in a consistent change in the proportion of the shared core genera in these samples after cow suffered SARA.

To identify the specific bacteria that may be translocated from rumen to mammary gland via blood and be associated with mammary gland inflammation, we performed a biomarker analysis using linear discriminant analysis (LDA=4.0) effect size (LEfSe) and a cladogram generated from LEfSe analysis on the microbiota community of milk, rumen fluid, feces, and blood. At geneus levels, only Only *Stenotrophomonas* and *Sphingomonadaceae* were enriched in milk from SARA cows (Fig. 8A-B). Additionally, the relative abundance of the *Stenotrophomonas* in rumen fluid and feces all significantly increased in SARA cows when compared to the control cows (Fig. 8C-F). However, there was no detection of *Stenotrophomonas* enrichment in the blood of SARA cows, and we did not isolated the *Stenotrophomonas* in the blood (data has no shown). This results suggested that the consistency of the changes in the milk microbiota and rumen microbiota caused by SARA may not be due to the migration of *Stenotrophomonas* through the blood. However a large amount of *Stenotrophomonas* derived from the rumen may be associated with the development of mastitis. To rule out the possibility that mastitis in SARA cows is caused by common pathogenic microorganisms in the environment, we tested the relative abundance of common mastitis pathogens, including *Staphylococcus*, *Streptococcus*, and *Escherichia coli*, in the milk of control and SARA cows. The results showed that the abundance of *Staphylococcus*, *Streptococcus*, and *Escherichia coli* was reduced in SARA cows we observed when compared to the control cows (see supplementary fig. S5A-D). These results indicated that the development of mastitis in SARA cows may be caused by endogenous pathogens, such as *Stenotrophomonas*, rather than by common pathogen infections.

Treatment of *Stenotrophomonas. maltophilia* (*S. maltophilia*) induced mastitis in mice

To detect the relationship of elevated *Stenotrophomonas* in the rumen of SARA cows and the occurrence of mastitis, we detected the effected of *S. maltophilia* (the only species of the *Stenotrophomonas*) on the mastitis in mice. The results showed that gavaged of *S. maltophilia* results in the damages of mammary gland tissues (Fig. 9A-B), and increased the production of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 in the mammary glands (Fig. 9C-E). In addition, to detect whether *S. maltophilia* on the mammary gland surface can induce mastitis, the feces from mice that gavaged *S. maltophilia* were collected and smeared on the surface of healthy mice. H&E staining and ELISA analysis showed that no inflammation was present in the mammary glands of the mice smeared with feces from the mice that gavaged *S. maltophilia* duration of experiment (Fig. 9A-E). These results suggested that gut-derived *S. maltophilia* is an important factors to induce the development of mastitis.

Increased severity to *S. aureus* mastitis in cows suffering from SARA

To evaluate the effect of SARA on the severity of bovine mastitis , we tested the milk composition in cows of control and SARA after infection with *S. aureus*. Milk composition analysis demonstrated that milk fat was reduced after *S. aureus* infusion, and the contents were recovered when the 24 h after *S. aureus* treatment. However, a significant difference in SARA-positive and SARA-negative was only found 4 h after the *S. aureus* infusion (Fig. 10A). Moreover, infusion of *S. aureus* results in increased milk protein content in both SARA-positive and SARA-negative cows. A difference was found 24 h after *S. aureus* treatment in SARA-positive and SARA-negative cows (Fig. 10B). Furthermore, the lactose and dry matter content was significantly reduced after *S. aureus* infection in both SARA-positive and SARA-negative cows. However, no difference was found in SARA-positive vs SARA-negative until 24 h after *S. aureus* infusion (Fig. 10C-D). Infusion of *S. aureus* elevated the levels of SCC until the end of the observation time. More SCC was also detected in SARA-positive than in SARA-negative animals 12 h after *S. aureus* infusion, although there was no significant difference (Fig. 10E). Further research was conducted to detect the load of *S. aureus* in milk of SARA-positive and SARA-negative after receiving the *S. aureus* challenge. As shown in Fig. 10F, the bacterial loads in milk 4 h after *S. aureus* infusion reached the maximum, and then the loads of *S. aureus* were gradually reduced due to the clearance effect of the mammary gland on *S. aureus*. However, there were significantly higher bacterial loads in the milk of SARA-positive cows than that of SARA-negative cows from 12 h until 24 h after *S. aureus* challenge. These results suggested that cow rumen microbiota disturbance induced by SARA may increase the severity to mastitis by reducing the clearance ability of the mammary gland to *S. aureus* infection.

Discussion

Mastitis, affects nearly all lactating mammals and is generally thought to be an inflammatory response of the mammary gland that is caused by local infection with pathogens in the environment. Over the last few years, however, some new important factors that caused mastitis have been revealed, such as gut microbiota[8]. However, the regulatory mechanism of gut microbiota on mastitis remains largely unknown.

In the present study, we assessed the effects and mechanisms of rumen microbiota on mastitis based on a SARA model induced by HCD feeding in cows using a 16S rRNA gene V4 region analysis on the IsonS^{TM}XL platform. In addition, the microbial changes may result from a direct or indirect effect of lifestyle, dietary habit, genetic, or other factors that vary between individuals. In order to eliminate adverse effect brought by inter-individual variations, a self-comparative analysis was proposed to reduce selection biases and achieve more reliable results[28]. Thus, in this study, the samples collected before treatment were used as a control group and compared with the following treatment procedures.

Dairy cows are often fed HCD to meet the nutritional demands of high milk production. However, feeding HCD for long periods of time in cows often leads to the development of SARA, in which the rumen pH falls below 5.6 and then results in a rumen microbiota imbalance[29]. Once cows suffer SARA, a large amount of bacterial endotoxin (LPS), a very important pro-inflammatory factor, is released in the rumen ant translocated into the bloodstream across the epithelium of the rumen[30]. During the lactation period, the mammary gland blood volume of cow accounts for 8 % of the total body blood volume. It is suggested that every 1 L of milk secretion, the udder must have 400 to 500 L of blood flow, that is, 280 mL of blood flow every second. The large blood flow of lactating cows' mammary glands suggests that LPS in the blood may circulate into the mammary gland, and then induce the inflammatory response of the mammary gland. Studies also indicated that long-term overfeeding of an HCD results in rumen-derived LPS across the blood circulation, induced inflammatory and immune responses and increased the synthesis of LAP by NF-κB signaling in the mammary gland of lactating cows[31]. In addition, overproduction of pro-inflammatory cytokines activated the apoptosis pathway and induced mammary cell apoptosis in lactating cows[32]. Increased amounts of LPS induced by SARA also affect the milk fat and milk protein by enhancing the methylation of genes relevant to fat synthesis and inhibiting the expression of methylation of genes associated with protein synthesis[33, 34]. We found that the level of LPS in rumen fluid, jugular vein, lacteal vein, milk, and mammary gland all increased in SARA cows, and the content of LPS was higher in mammary gland and milk than in the jugular vein and lacteal vein. These results suggest that LPS accumulates in the mammary gland through the bloodstream. Additionally, the SCC was significantly increased, and milk fat, protein, fat/protein, and dry matter were reduced in milk from SARA cows. In addition, the blood-milk barrier is a specific structure that plays an important role in preventing foreign matter, such as LPS, from the blood or external environment entering the mammary gland[26]. Studies provided that infusion of LPS disrupted the blood-milk barrier by regulating the expression of Claudins in alveolar epithelial tight junctions through activating the TLR4 signaling pathway[35]. In the present study, cows that received SARA had a reduced expression of tight junction proteins in the mammary gland. The results suggested that SARA of cows led to rumen-derived LPS entering the mammary gland through blood circulation, damaged the blood-milk barrier, and then induced inflammation of the mammary gland in cows.

Rumen microbiota was associated with the mammary health in cows. Previous studies demonstrated that the rumen bacterial community was difference in cows between the low and high SCC[36].In addition, Ma et al., through transplantation feces of mastitis cows to mice, induced inflammation of the mammary

gland, indicated that intestinal microbiota are one cause of mastitis[8]. Moreover, recent research shown that the development of mastitis may be closely associated with changes in milk microbiota in lactating cows[37]. Evidence has shown that cow mastitis-associated pathogens such as *Streptococcus uberis*, although present in small quantities, are part of the normal milk microbiota; thus, the development of mastitis may be a dysbiosis of milk microbiota rather than a simple primary infection[37, 38]. Others suggested that the microbiota community in milk is relevant to inflammation status and showed that the bacterial flora are more abundant but less diverse in milk of cows suffering from inflammation[39]. In the present study, we found that elevated the abundance of *Stenotrophomonas* in rumen was associated with mastitis during cows suffering SARA. In addition, although ruminants, milk, and feces harbored distinct microbiota, the community of bacterial flora in the rumen and milk of cows suffering from SARA are more similar to the rumen and milk of control cows, both were characterized by significantly increased abundance of *Stenotrophomonas*. This is suggested there must be some close association between the rumen microbiota and milk microbiota, and this association may be played an important role in the development of mastitis. Similarly, Mao et al., showed that the levels of *Stenotrophomonas* was increased in raw milk of SARA cows and may increase the risk of dairy cows suffering from mastitis[40]. An epidemiologic study of bovine protothecal mastitis indicated that the disease might be associated with persistent infection in the gut and that the source of infection is feces. It is suggested that microbial translocation occurs, with *P. zofii* potentially being disseminated from the gut to the mammary glands or the other organs[41]. A hematogenous route of bacterial infection cannot be discounted because SARA has have been reported to be associated with liver abscesses[12], which are one of the important causes of bacterial infection. In addition, recent research has demonstrated that uterine pathogens can be transferred from the gut to the uterus by bloodstream[42]. Whether dynamic changes of rumen microbiota and milk microbiota was associated with the translocate of pathogenic through blood was detected in the present study. LEfSe analysis showed that *Stenotrophomonas* was enriched in the milk of cows that received SARA, and *Stenotrophomonas* was identified as more predominant in clinical mastitis milk samples. Additionally, the proportions of *Stenotrophomonas* not only in milk but also in rumen fluid, and feces, are among the most high genera and significantly increased in all samples in SARA cows. However, there are no bacteria enriched and isolated in blood of SARA cows. Thus, the consistency of the changes of microbiota in rumen and milk caused by SARA may not be due to the migration of bacteria through the blood, and the correlative mechanism still needs further study.

Previous works and the present study found that a large amount of LPS derived from rumen was transferred to the bloodstream and then persisted in the host for a long period, with adverse health effects[22]. We hypothesized that this condition, when the mammary gland is challenged by pathogens, would lead to increased susceptibility of mastitis. Thus, we detected changes in SCC and milk composition in both SARA-positive and SARA-negative cows after challenge with *S. aureus.* Infusion with *S. aureus* induced a strong increase in SCC, and the number of SCC in SARA-positive cows was slightly higher than the SCC in SARA-negative cows. S. Aditya et al., showed no difference in the levels of SCC in control and SARA cows after mammary gland infusion of LPS[43]. In addition, the P.N. Gott., demonstrated

that SCC for cows fed the control diet was lower than for cows fed a high-starch diet, and the changes in SCC caused a similar change after infusion of LPS. They explosion that might present a certain endotoxin tolerance at the local of the mammary gland[44]. However, a recent study showed that after LPS infusion, cows suffering from SARA present a higher concentration of SAA, another important indicator of bovine mastitis[45, 46], in milk[47]. However, the difference in the experimental results should be further studied. Moreover, milk constituent analysis showed that challenged *S. aureus* led to a reduction in the content of milk fat, lactose and dry matter, and increase the content of milk protein after *S. aureus* infusion, where some changes in milk constituents were more pronounced in SARA-positive than SARA-negative cows. The result was similar to that reported by S. Aditya[43]. Bovine mastitis is often caused by infection with environmental pathogens. Once the pathogens are inside the teat, leucocytes are transferred to the mammary gland from the blood to recognize and phagocytose the pathogen[48]. Additionally, a large quantity of inflammatory cytokines, such as TNF-α, IL-1, and IL-8, are released and induce an the inflammatory response in the mammary glands[48, 49]. Previous studies showed that gut microbiota play an important role in the phagocytic activity of PMNs, and evidence showed that depletion of the gut microbiota impaired host defense and increased susceptibility by PMNs[50, 51]. In the present study, we found that the load of *S. aureus* was higher in the milk of SARA-positive cows than in the milk of SARA-negative cows from 12 h to 24 h after *S. aureus* infusion. This suggested that the increased mastitis susceptibility of SARA-positive animals might be associated with the lower clearance of neutrophils in milk, but the specific mechanism needs to be further studied.

Conclusion

In conclusion, these results demonstrated that the milk, rumen, feces, and blood harbored unique structures of bacterial communities. Rumen microbiota disturbance may be associated with the changes of milk micorbiota, and high abundance of *Stenotrophomonas* in rumen may be led to the development of mastitis. In addition, LPS originating from the rumen may be translocated to the mammary gland by the bloodstream and then induce inflammation of the mammary gland. On the other hand, the chronic, large-scale passage of LPS from the rumen to the blood circulation, compromises the health of the host and then damages the ability of the mammary gland to clear out pathogens, thus increasing susceptibility of mastitis. Our studies provide a basis for novel therapeutic strategies that exploit the rumen microbiota against mastitis in cows.

Materials And Methods

Animals and experimental protocol.

Eight late-lactating Holstein cows (average body weight, 547±51 kg, lactation days and weight similar and non-pregnant) were used in this experiment. For fifteen days before the start of the experiment, cows were offered free access to a diet containing a forage-to-concentrate ratio (F:C) of 40:60 to ensure adaptation to the diet. After 8 weeks, cows received a high-concentrate diet (HCD) comprising 30 %

forage and 70 % mixed concentrate. The rumen fluid, milk, feces, and blood were harvested before fed with HCD were used as a control group and compared with following treatment with HCD cows. Throughout the experiment, the cows were fed daily at 5:00 and 18:00, and they had free access to water during the experimental period. Detailed methods for the intramammary infusion of S. aureus are provided in the online supplementary file.

Mastitis induced by *S. maltophilia* in mice

The operation methods are provided in the online supplementary file.

Sample collection and analysis

Milk production and feed intake were recorded daily. The rumen fluid, milk, feces, and blood were collected at 0 and 8 weeks after introduction of the HCD. Details on the methods are provided in the online supplementary file.

LPS analysis

The levels of LPS from samples of rumen fluid, feces, plasma, milk, and mammary glands were detected by a chromogenic endpoint assay (Chinese Horseshoe Crab Reagent Manufactory Co., Ltd., Xiamen, China) with a minimum detection limit of 0.1 EU/mL (rumen fluid and feces) or 0.01 EU/mL (plasma, milk, and mammary gland) according to the manufacturer's instructions.

Pro-inflammatory cytokines, LDH, IgG, and SAA assay

Mammary gland tissues from control and SARA cows were weighed and homogenized with PBS (1:10, w/v) and centrifuged at 2000 g for 40 min at 4 °C. The lipids on the surface were removed, and the supernatants were collected. Milk was centrifugated at 1000 g for 20 min at 4 ℃, and the supernatants were collected. The levels of TNF-α, IL-1β, IL-6, IL-17, LDH, and IgG in plasma of different samples were tested by ELISA kits (Lanpai BIO, Shanghai, China) according to the manufacturer's instructions. SAA in the milk and plasma were detected by using a biochemical analyzer (Diano, China).

Western blot analysis

The total proteins from the rumen, mammary gland, and intestine tissues were extracted with tissue protein extracted reagent (Thermo Scientific, MA, USA), and the concentration of proteins was detected using a BCA protein assay kit (Thermo Scientific, MA, USA). The proteins were fractionated by 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (Bio Trace, Pall Co., USA). The membranes were blocked with 3 % BSA at room temperature at shaking for 2 h, and then incubated with anti-Clauind-1, anti-Claudin-3, Occludin, ZO-1, anti-phospho-IκBα (Cell Signaling Technology Inc., Beverly, MA), anti-IκBα, anti-phospho-NF-κB p65, anti-NF-κB p65, and anti-TLR4 at 4 °C overnight. Following TBS-T washing, all membranes were incubated with secondary antibody for 1 h. After washing with TBS-T. The targeted proteins were detected by Supersignal West Pico

Chemiluminescent Substrate (Thermo Scientific, USA), and the bands were imaged by a Protein Simple imager (ProteinSimple, Santa Clara CA, USA).

Histopathologic analysis

The tissues of the mammary gland, liver, rumen, and intestine of cows were histopathologic analyzed and pathological scored on an provided on online Supplementary file.

DNA extraction, 16S rRNA gene amplification, Ion S5^{TM} XL sequencing, and data analysis

Total genome DNA from rumen fluid, milk, feces, and blood was extracted by a CTAB/SDS method. The DNA concentration and purity of each sample were detected by 1 % agarose gels and the concentration of DNA was diluted to 1 ng/μL by sterile water. The 16S rRNA was amplified by specific primer (16S V4:515F-806R) with the barcode targeting the V4 region. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). PCR products were mixed with an equal volume of 1×loading buffer (containing SYB green) and subjected to electrophoresis on a 2 % agarose gel for detection. PCR products were mixed in equidensity ratios, and the products were purified with Gene JET^{TM} Gel Extraction Kit (Thermo Scientific) . Sequencing libraries were generated using Iso Plus Fragment Library Kit 48 rxns (Thermo Scientific) following manufacturer's instructions. The library quality was evaluated by a Qubit@ 2.0 Fluorometer (Thermo Scientific). Finally, the library was sequence on an Ion S5^{TM} XL platform and 400 bp/600 bp single-end reads were generated. In addition, pyrosequencing-derived data analysis was provided on online supplementary files.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 6.01 (GraphPad Software, Inc., San Diego, CA). All data are expressed as the means ± SEM. Differences between date means were determined using one-way ANOVA (Dunnett's t-test) and the two-tailed t-test. A $p < 0.05$ was considered to be statistically significant.

Declarations

Acknowledgments

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Availability of data and materials

Datasets supporting the conclusion of this article are available in the Supplementary files. Any other datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

.Author's contributions

Xiaoyu Hu contributed to article writing, literature search, results evaluation, establishment of SARA model in dairy cows. Jian Guo performed establishment of SARA model in dairy cows. Jian Guo and Peng Jiang performed inflammatory biomarkers detection. Ruiying Mu performed establishment of SARA model in dairy cows. Caiju Zhao and Yongguo Cao contributed to literature search and results evaluation. Naisheng Zhang and Yunhe Fu contributed to study design and the final revision of the article and results evaluation.

Ethics approval and consent to participate

The full proposal was reviewed by the Institutional Animal Care and Use Committee (IACUC) of Jilin University 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.

Consent for publication

No applicable

Conflicts of interest

None of the authors have a financial interest in any of the products, devices, or Materials mentioned in this manuscript. The authors declare that they have no conflicts of interest.

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Figures

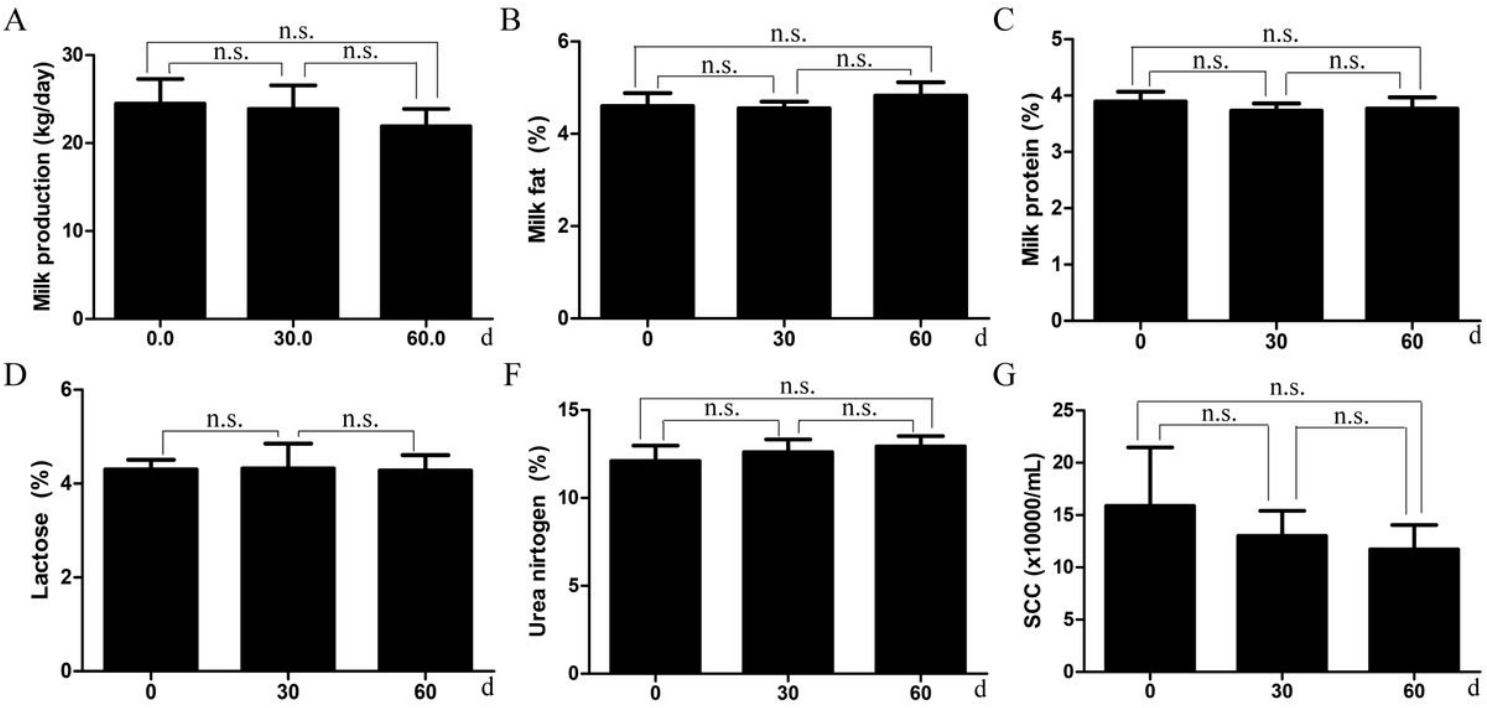

Figure 1

Effect of experimental period on the changes of milk composition. (A) Milk production, (B) milk fat, (C) milk protein, (D) lactose, (E) urea nitrogen, (F) SCC of milk from the healthy cows throughout the experimental period. n.s. indicates a no significant difference between the different groups.

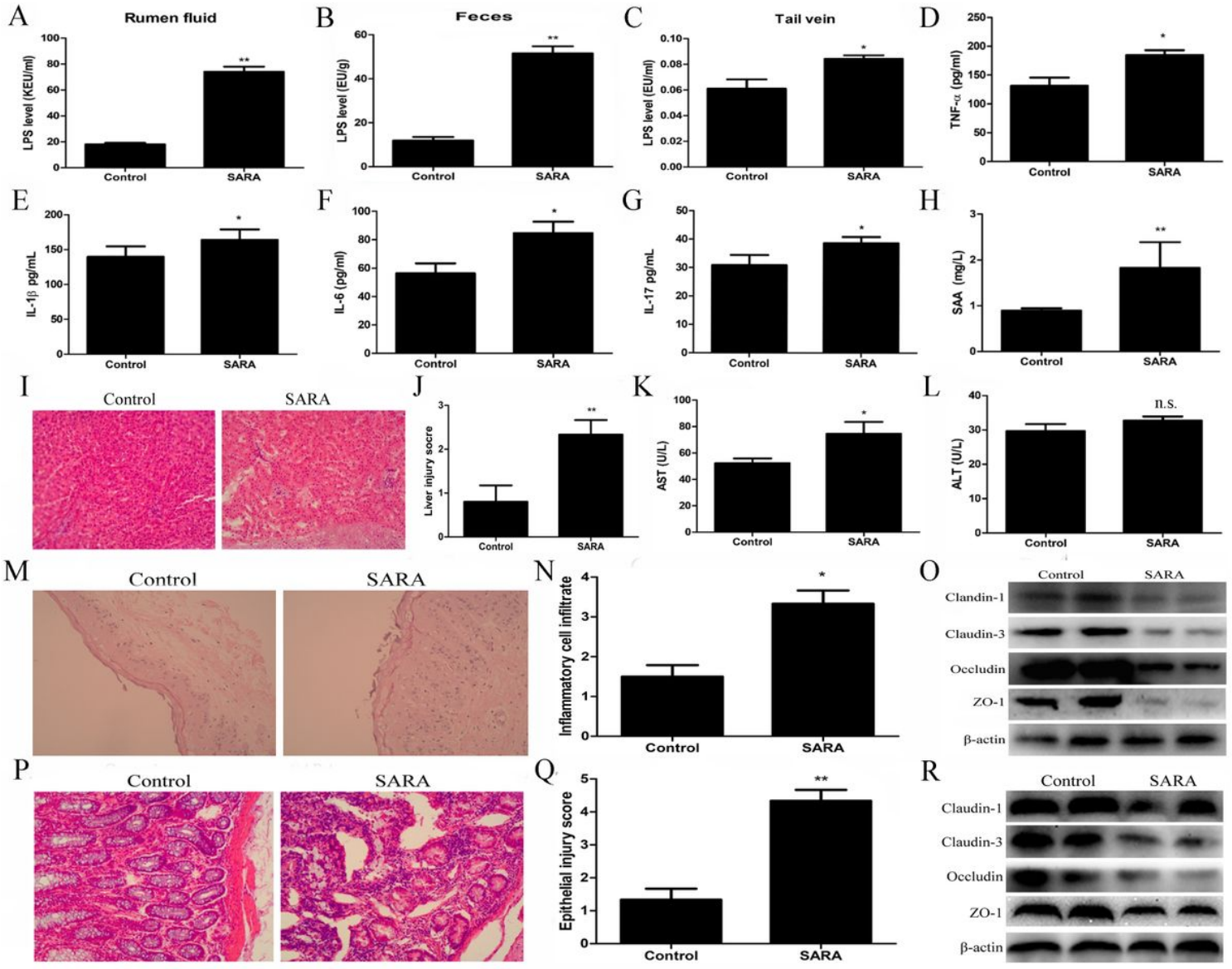

Figure 2

SARA induces the system inflammatory response in dairy cows. (A) LPS from the rumen fluid, (B) feces, and (C) tail vein of control and SARA cows. (D) TNF-α, (E) IL-1β, (F) IL-6 (G) IL1-17, and (H) SAA levels in the blood of control and SARA cows. (I) Histological analysis and (J) liver injury score of the liver between control and SARA cows. (K) AST, and (L) ALT concentration in blood of control and SARA cows. (M) Histological analysis and (N) inflammatory cell infiltration score of rumen between control and SARA cows. (O) The expression of tight junction proteins Claudin-1, Claudin-3, Occludin, and ZO-1 of rumen between control and SARA cows. (P) Histological analysis and (Q) epithelial injury score of intestines between control and SARA cows. (R) The expression of tight junction proteins Claudin-1, Claudin-3, Occludin, and ZO-1 of intestines between control and SARA cows. $p < 0.05$ indicates a significant difference between the different groups

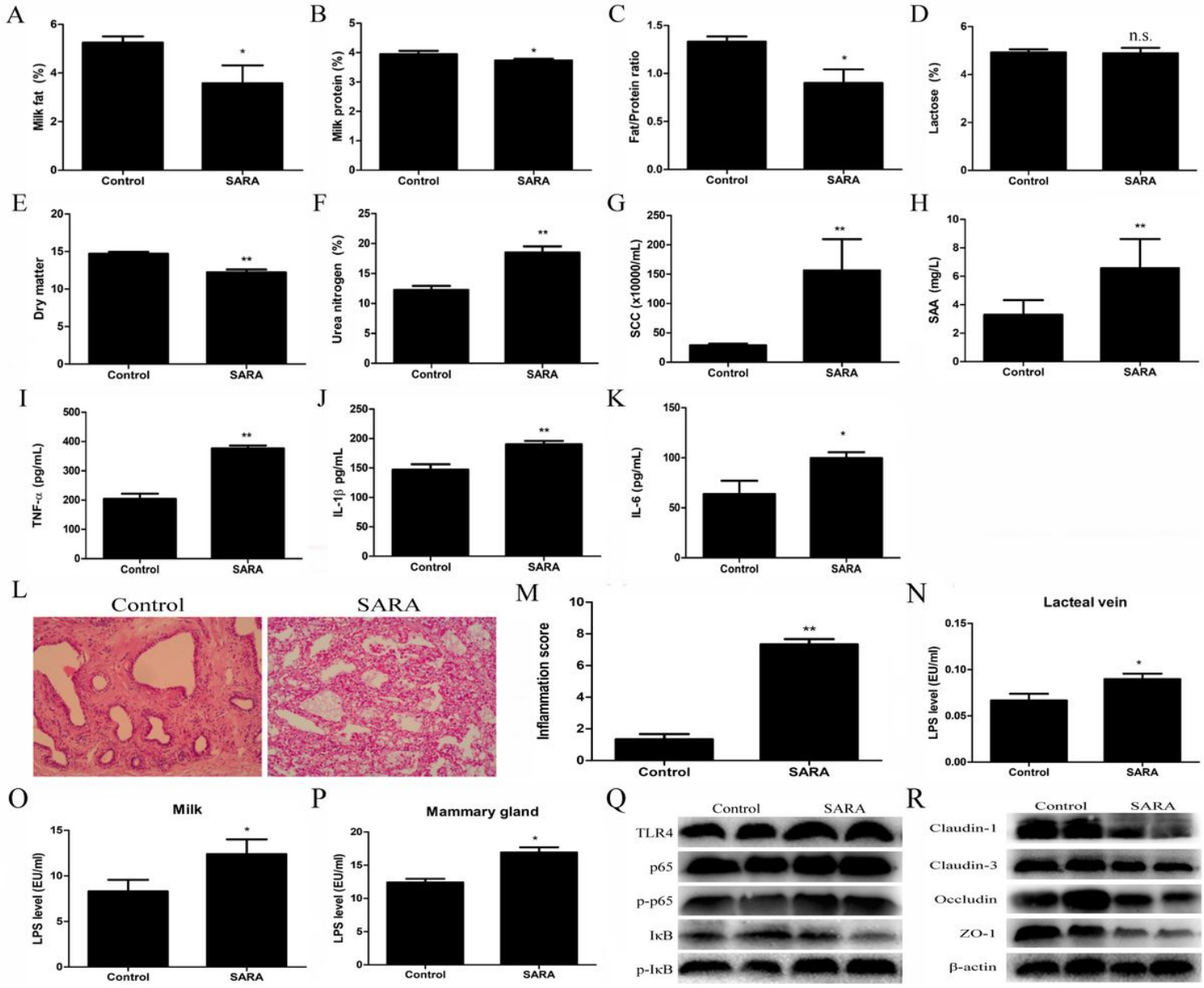

Figure 3

SARA induces the inflammatory response in the mammary gland by increasing blood-milk barrier permeability in cows. (A) Milk fat, (B) milk protein, (C) fat/protein ratio, (D) lactose, (E) dry matter, (F) urea nitrogen, (G) somatic cell count (SCC), (H) SAA level of milk between control and SARA cows. (I) TNF-α, (J) IL-1β, (K) IL-6 levels in the mammary glands of control and SARA cows. (L) Histological analysis and (M) inflammation score of the mammary gland between control and SARA cows. (N) LPS from lacteal veins, (O) milk, and (P) mammary glands of control and SARA cows. (Q) Western blots for quantification of TLR4, NF-κB p65, p-NF-κB p65, IκB, and pIκB protein levels in the mammary glands of control and SARA cows. (R) Western blots for quantification of Claudin-1, Claudin-3, Occludin, and ZO-1 in the mammary glands of control and SARA cows. $p < 0.05$ indicates a significant difference between the different groups.

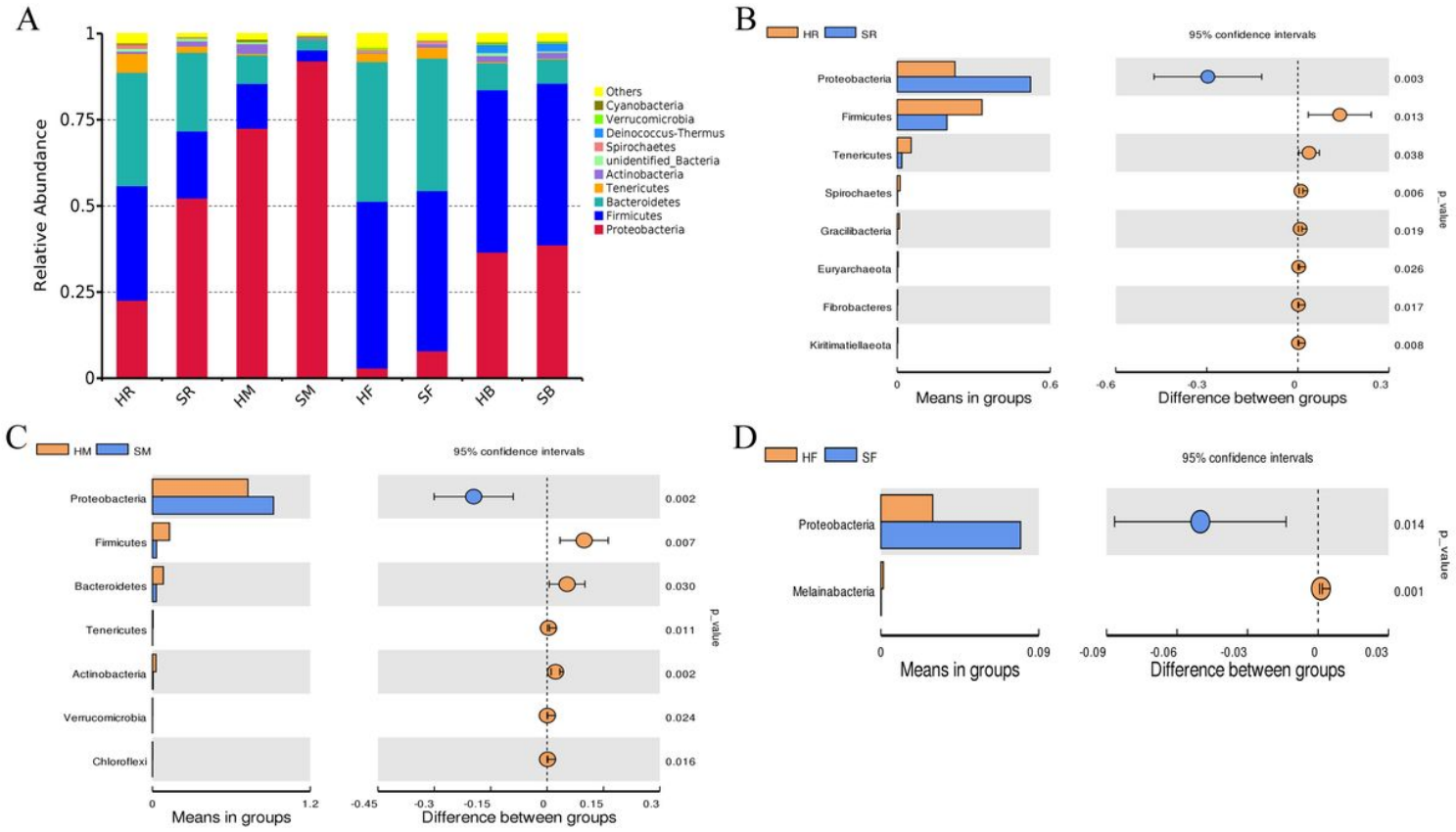

Figure 4

Comparisons at the phyluum level between control and SARA cows. (A) Relative abundance of the top 10 phyla of milk, rumen fluid, and feces bacteria between control and SARA cows. (B) T-test analysis of different species in rumen fluid (C) milk, and (D) feces at the phylum level between the control and SARA groups. $p < 0.05$ indicates a significant difference between the different groups.

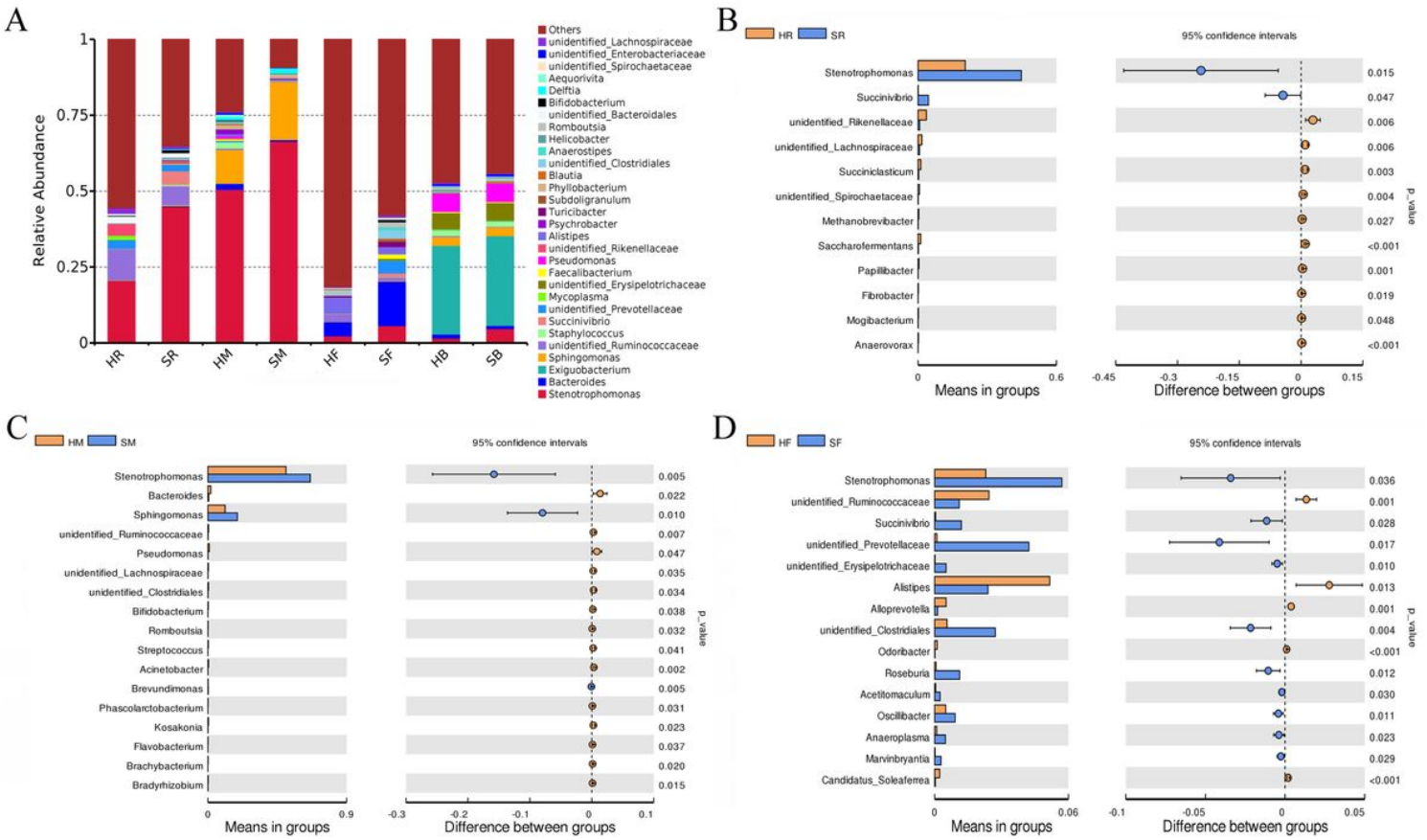

Figure 5

Comparisons at the genera level between control and SARA cows. (A) Relative abundance of the top 30 genera of milk, rumen fluid, and feces bacteria between control and SARA cows. (B) T-test analysis of different species in rumen fluid (C) milk, and (D) feces at the genera level between the control and SARA groups.$p < 0.05$ indicates a significant difference between the different groups.

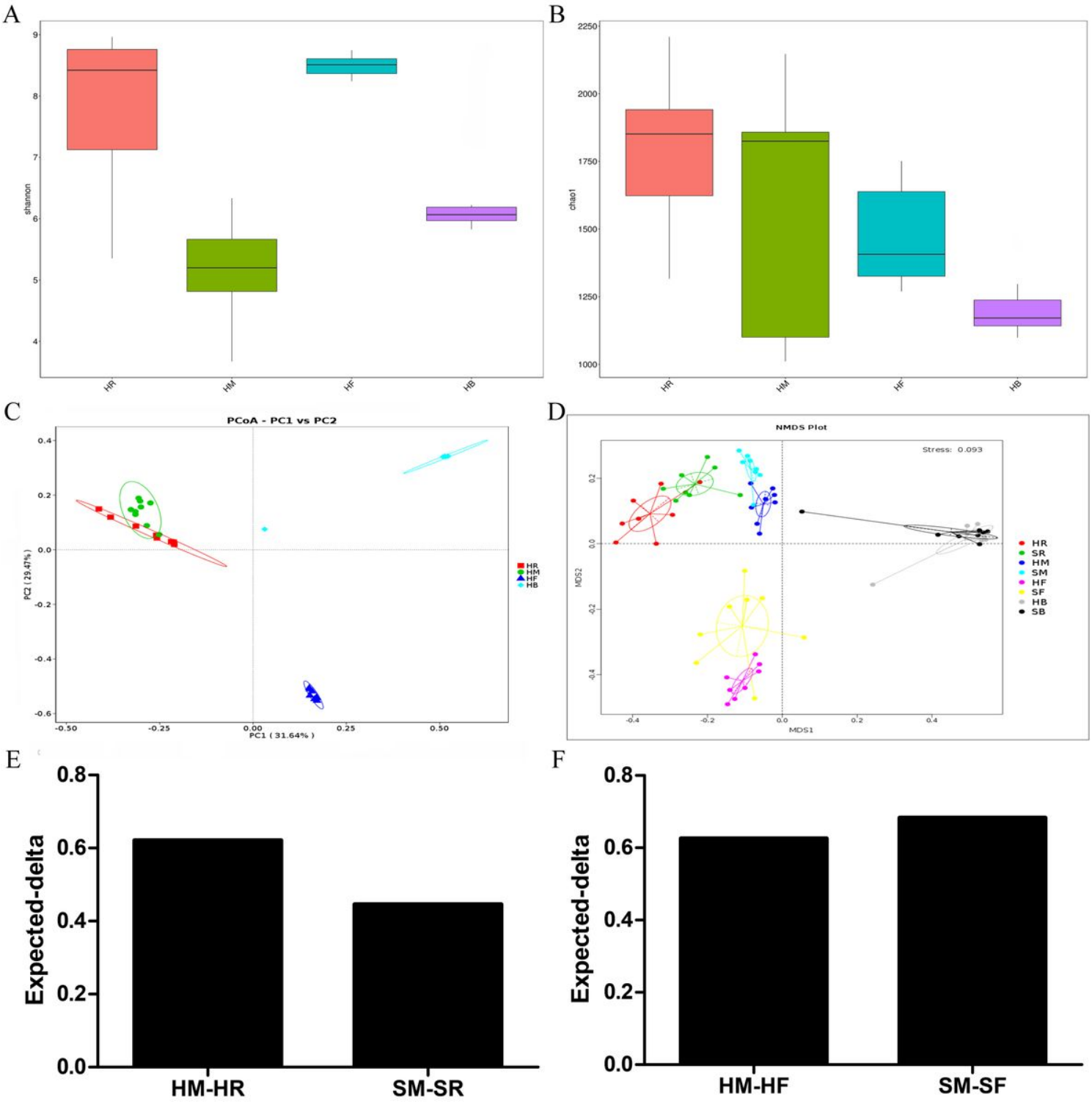

Figure 6

Similarity of bacterial community composition in milk, rumen fluid, feces, and blood in cows from the control and SARA groups. (A) Comparison of the microbiota diversity in term of shannon index among milk, rumen fluid, feces, and blood in control cows. (B) Comparison of the microbiota richness in term chao1 among milk, rumen fluid, feces, and blood in control cows. (C) PCoA based on the Bray-Curtis similarity among milk microbiota, rumen fluid microbiota, feces microbiota, and blood microbiota in control cows. (D) NMDS plot based on Bray-Curtis dissimilarity of milk microbiota, rumen fluid

microbiota, feces microbiota, and blood microbiota between control and SARA cows. MRPP analysis microbiota community dissimilarity of (E) milk and rumen fluid, and (F) milk and fecal between control and SARA cows. $p < 0.05$ indicates a significant difference between the different groups.

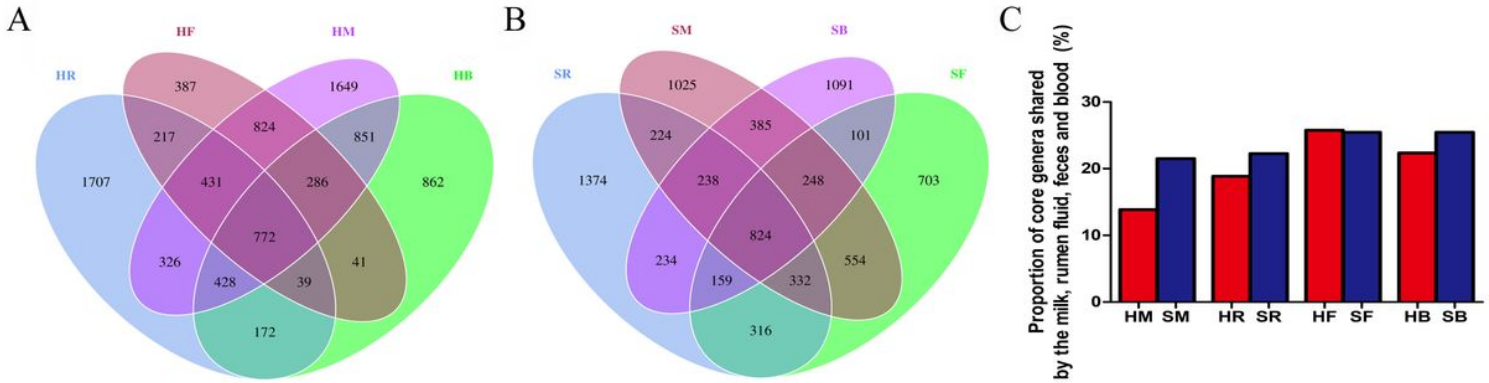

Figure 7

Stenotrophomonas from the rumen maybe a factor to induce inflammation in the mammary gland in cows suffering from SARA. (A-B) Venn diagram showing the number of core genera in rumen fluid, milk, feces, and blood samples from control and SARA cows. (C) Total relative abundance proportion of core genera shared by milk, rumen fluid, feces, and blood.

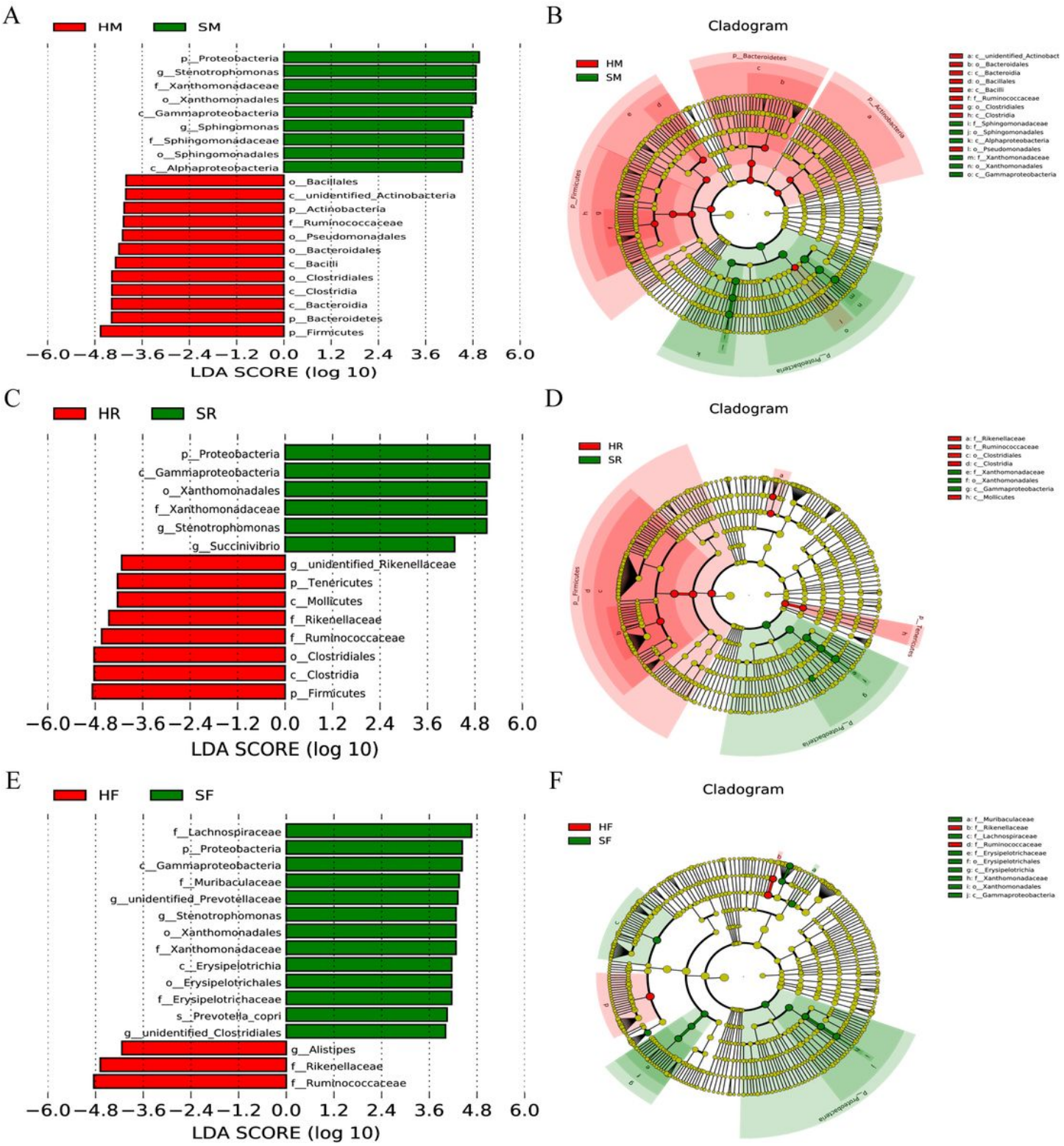

Figure 8

LEfSe analysis the mocribiota from milk, rumen fluid, feces, and blood between control and SARA cows. Linear discriminant analysis (LDA) score derived from LEfSe analysis, showing the biomarker taxa (LDA score of >4 and a significance of $p < 0.05$ calculated by the Wilcoxon signed-rank test) in milk (D-E), rumen fluid (F-G), and feces (H-I) from control and SARA cows.

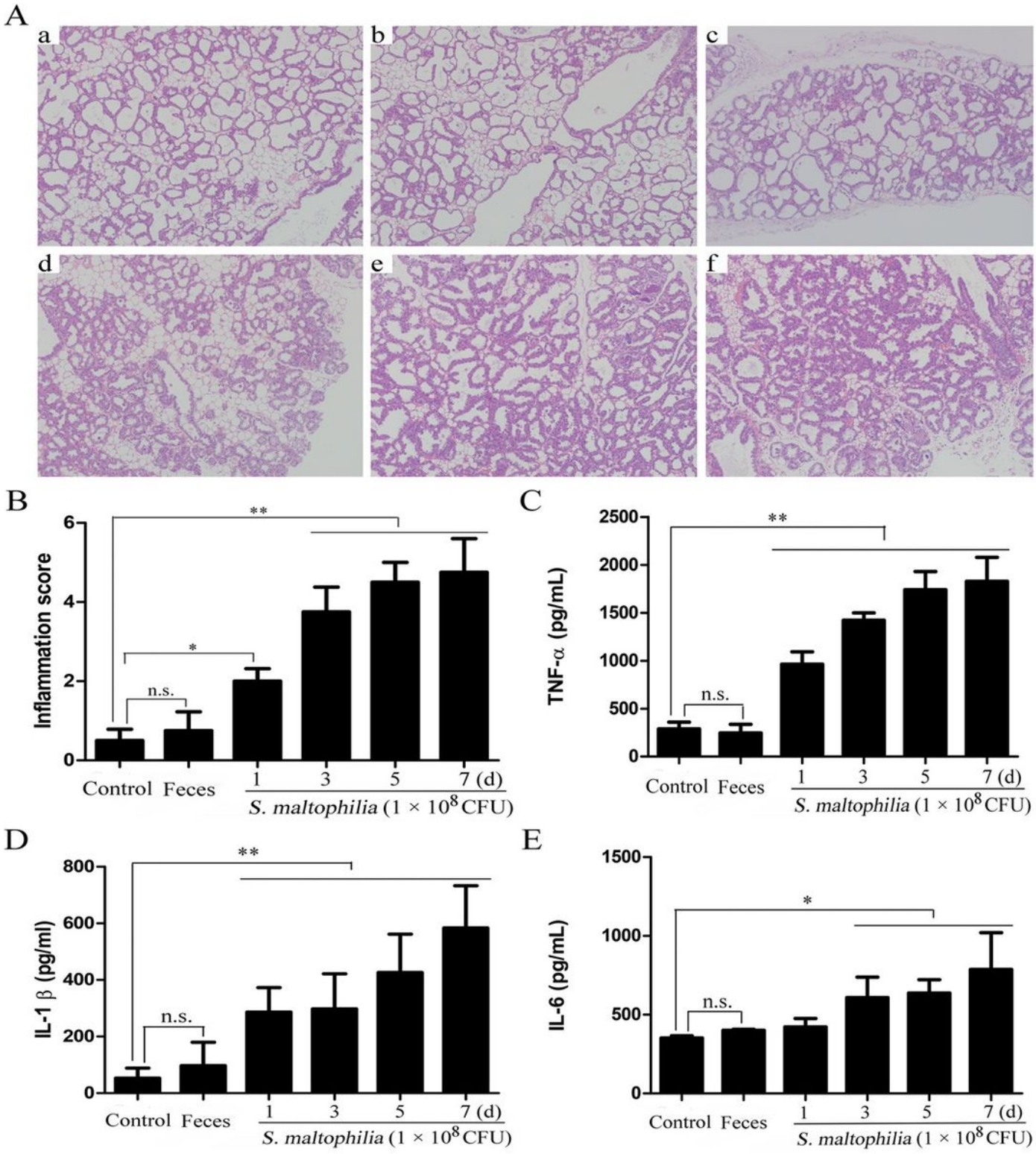

Figure 9

Treatment of Stenotrophomonas. maltophilia (S. maltophilia) induced mastitis in mice. S. maltophilia (1 × 108 CFU/200μL PBS) were administered to mice by oral gavage for 1 d, 3 d, 5 d, and 7 d consecutive days (once a day). In addition, fecal samples from each of the treatment of S. maltophilia was collected, mixed, suspended with sterile saline solution, and gently smeared on the mammary gland surface by swabs for consecutive 7 day. (A) Histological analysis and (B) inflammation score of the mammary

gland among different treatment group mice. (C) TNF-α, (D) IL-1β, and (E) IL-6 levels in mammary gland among different treatment group mice. $p < 0.05$ indicates a significant difference between the different groups.

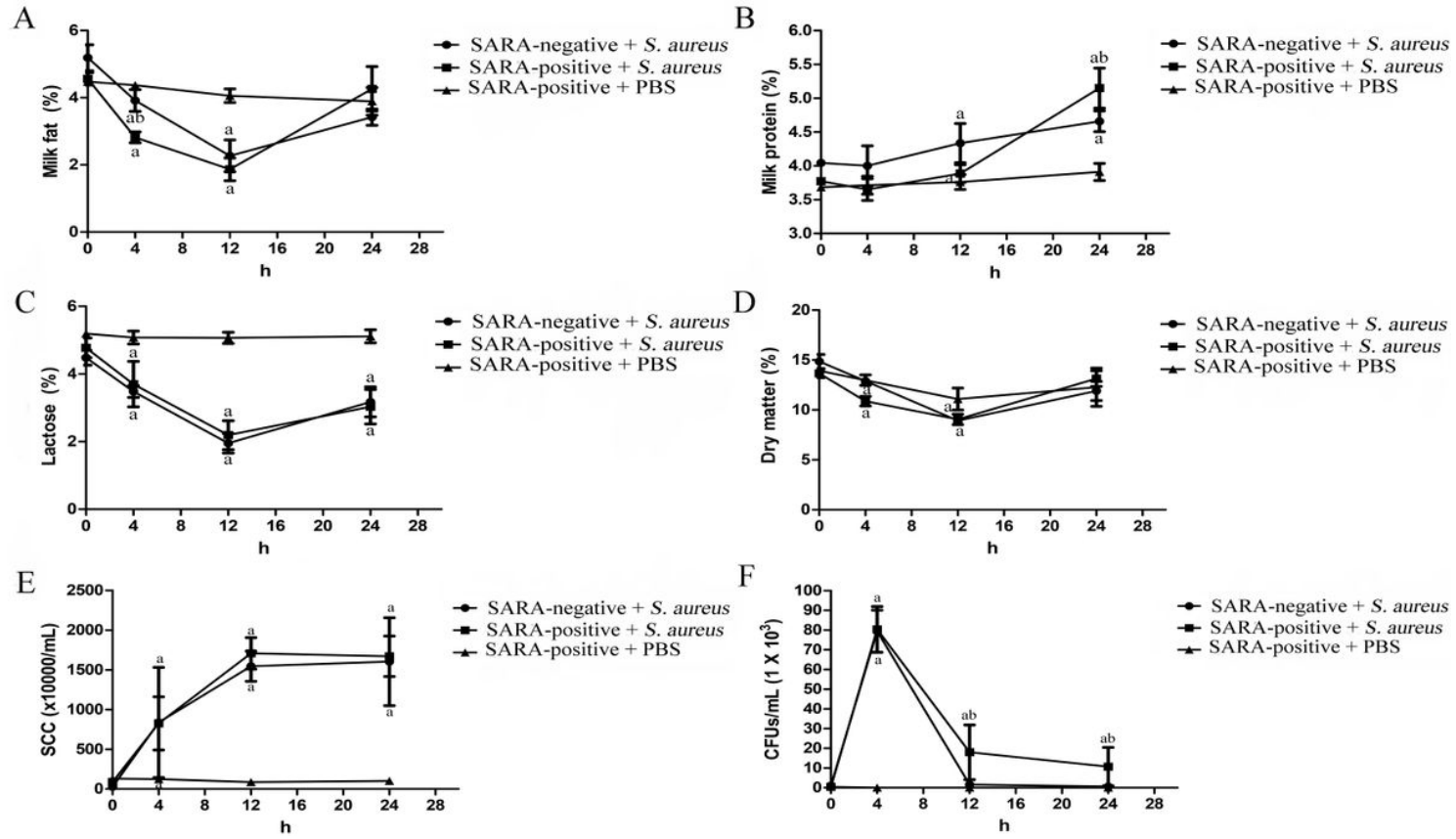

Figure 10

Increased severity to S. aureus mastitis during cows suffering from SARA. (A) milk fat, (B) milk protein, (C) lactose, (D) dry matter, (E) SCC, and (F) numbers of S. aureus in milk of lactating cows with control or SARA and receiving either S. aureus or a placebo infusion detected at 0, 4, 12, and 24 h after the S. aureus infusion. $p < 0.05$ indicates a significant difference between the different groups

Supplementary Files

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