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Reuterin-producing *Limosilactobacillus reuteri* with glycerol metabolism ameliorates *Staphylococcus aureus*-induced intestinal inflammation and dysfunction without gut microbiota dependent

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ABSTRACT

Limosilactobacillus reuteri is a vertebrate symbiont that is widely appreciated as being of significant ecological importance for human health. As a unique feature, *L. reuteri* converts glycerol to the antimicrobial compound reuterin using enzymes encoded in its propanediol-utilization operon and evolves with host-driven diversification. Reuterin-producing *L. reuteri* HLRE13 was selectively isolated from poultry previously and confirmed to inhibit the growth of *Staphylococcus aureus in vitro*. However, it remains unclear whether *L. reuteri* HLRE13 retains these antagonistic properties when ingested in specific-pathogen-free mice. Here, we investigated the ameliorative effects and potential mechanisms of action of *L. reuteri* HLRE13 in combination with glycerol on *S. aureus*-induced infection phenotypes in mice. Firstly, our results confirmed that *L. reuteri* HLRE13 effectively inhibited the intestinal colonization of *S. aureus* CMCC26003; Secondly, *L. reuteri* HLRE13 combined with glycerol could alleviate the intestinal tissues damage caused by *S. aureus* through increasing the expression of ZO-1, Occludin, and MUC-2, ameliorate the intestinal systemic inflammatory response, and maintain the balance of gut microbiota by increasing the relative abundance of *Lactobacillus* and reducing the relative abundance of *Staphylococcus*. Furthermore, the colonization resistance was also found on *L. reuteri* HLRE13 combined with glycerol against *S. aureus* in pseudo germ-free mice, and they exerted the similar effects on alleviating intestinal damage and improving immune function. Combining these results, we speculate that reuterin-producing *L. reuteri* antagonize *S. aureus* in mice without the gut microbiota-dependent manner. Overall, our findings will provide a theoretical foundation for the scientific cognition of *L. reiteri* in maintaining intestinal health by producing reuterin.

1. Introduction

Lactic acid bacteria (LAB) are considered potential "green" antibiotics and have a long been used history on improving gut health[1]. *Limosilactobacillus reuteri* is a widely developed LAB that inhabits the gastrointestinal tracts of vertebrates (such as chickens, pigs, birds, and humans)[2-3], which rely on the biofilm formation to colonize host[4]. The multilocus sequence analysis have demonstrated that *L. reuteri* have evolutionarily adapted to the gut of specific hosts and can be categorized into 6 lineages. For example, *L. reuteri* in lineage VI are mainly derived from poultry and humans[2]. Several studies have shown that *L. reuteri* is highly resistant to gastric acid and bile[5], and could prevent diarrhea, relieve stress, regulate gut microbiota and the immune system[6-8]. It also inhibits the proliferation of pathogens through the production of short-chain fatty acids and bacteriocins[9].

Glycerol acts as an external hydrogen receptor for *L. reuteri* and is intracellularly dehydrated to 3-hydroxypropionaldehyde (3-HPA)

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by coenzyme vitamin B_{12} and glycerol dehydratase. Under anaerobic conditions, 3-HPA can be further reduced to 1,3-propanediol by the coenzyme NAD^+ and oxidoreductase[10]. The complex dynamic mixed product with 3-HPA monomer as the center of transformation is the broad-spectrum antimicrobial reuterin[11], namely, the antimicrobial system consisting primarily of 3-HPA, 3-HPA dimer, 3-HPA hydrate, acrolein, and 3-hydroxypropionic acid. Metabolism of glycerol to produce reuterin is a typical feature of *L. reuteri* carrying the *pdu* gene cluster, and strains in lineage VI have a relatively greater capacity to metabolize glycerol to produce reuterin than strains in other lineages[12]. Reuterin exhibits strong bacteriostatic activity and high stability, making it a promising candidate for use as a food preservative[13]. Vimont et al.[14] demonstrated that increasing the concentration of reuterin from 0.138 to 138 mmol/L in a yogurt system significantly inhibited the growth of fungi. Furthermore, reuterin has been shown to possess anti-inflammatory, anti-infective, and anti-cancer properties[15].

Staphylococcus aureus, a commensal pathogen widely distributed in nature, is a major cause of bacterial skin and mucosal infections[16]. Toxins produced by it can cause food spoilage and staphylococcal food-borne diseases[17]. *S. aureus* is a zoonotic bacterium, and consumption of food contaminated with it can cause diarrhea, vomiting or toxic shock and some gastrointestinal diseases[18]. *S. aureus* infections are typically treated with antibiotics. However, the overuse and misuse of antibiotics has led to the emergence of methicillin-resistant *S. aureus*, which poses a severe threat to food safety and human health[19]. Therefore, alternative non-toxic antimicrobial agents with limited side effects are needed to prevent *S. aureus* infections.

Our group previously reported that *L. reuteri* HLRE13, a member of lineage VI isolated from a health chicken, is tolerant to gastric acid and bile, adheres to intestinal epithelial cells, and ferments glycerol to produce 230 mg/L reuterin, which significantly inhibited proliferation of *S. aureus* CMCC26003 during milk fermentation[20]. As a continuation of our work, the aims of the present study were to further determine whether *L. reuteri* HLRE13 in the presence of glycerol can alleviate pathogenetic effects triggered by colonized *S. aureus* and whether this process is mediated by the gut microbiota.

2. Materials and methods

2.1 Bacterial strains and growth conditions

L. reuteri HLRE13 used in this study was isolated from the feces of healthy chicken[20]. It was cultured in De Man, Rogosa and Sharp (MRS) broth (Solarbio, Beijing, China) at 37 °C for 20 h under anaerobic conditions. *Staphylococcus aureus* CMCC26003 was cultured in Luria-Bertani (LB) medium (Solarbio, Beijing, China) at 37 °C for 12 h under aerobic conditions. All experimental strains were stored in the State Key Laboratory of Food Science and Resources.

2.2 Animals and experimental design

Female BALB/c mice (6 weeks) were purchased from Hunan Slake Jingda Laboratory Animal Company (Hunan, China). All animals were housed in an animal facility at (23 ± 1) °C with a 12 h light/dark cycle in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and carried out with protocol approved by the Animal Ethics Committee of Nanchang University (No. 0064257).

Animal experiments 1: Mice were randomly assigned to 4 different groups (n = 6): 1) Control group: mice were orally gavaged with 200 μL phosphate buffer saline (PBS); 2) Sa group: mice were orally gavaged with 200 μL PBS for three days, followed by 200 μL *S. aureus* CMCC26003 for 1 week, the *S. aureus* CMCC26003 was cultured aerobically for 12 h and centrifuged at 8 000 × g for 5 min, then the strain pellet was washed twice with 1 × PBS and the concentration was adjusted to 5.0×10^8 CFU/mL with PBS for immediate use; 3) Sa-Lr group: mice were orally gavaged with 200 μL *L. reuteri* HLRE13 (1×10^9 CFU/mL) for 3 days, followed by a mixture of *L. reuteri* HLRE13 and *S. aureus* CMCC26003 for 1 week, the *L. reuteri* HLRE13 was cultured anaerobically for 20 h and centrifuged at 8 000 × g for 5 min, then the strain pellet was washed twice with 1 × PBS and the concentration was adjusted to 1.0×10^9 CFU/mL with PBS for immediate use; 4) Sa-Lr-gly group: mice were orally gavaged with 200 μL *L. reuteri* HLRE13 containing 2.5 mol/L glycerol for 3 days, followed by a mixture of *L. reuteri* HLRE13 and *S. aureus* CMCC26003 containing 2.5 mol/L glycerol for 1 week.

Animal experiments 2: An antibiotic cocktail (ABX, consisting of metronidazole (1.0 g/L; Sigma, Germany), vancomycin (0.5 g/L; Sigma, Germany), neomycin (1.0 g/L; Yuanye, Shanghai, China), and ampicillin (1.0 g/L; Yuanye, Shanghai, China)) was gavaged with 200 μL for 7 consecutive days as previous report[21]. Subsequently, the ABX treated mice were randomly assigned to 2 different groups (n = 6): 1) ABX-Sa group: mice were orally gavaged with 200 μL PBS for 3 days, followed by *S. aureus* CMCC26003 (5×10^8 CFU/mL) for 1 week; 2) ABX-Sa-Lr-gly group: mice were orally gavaged with 200 μL *L. reuteri* HLRE13 (1×10^9 CFU/mL) containing 2.5 mol/L glycerol for 3 days, followed by a mixture of *L. reuteri* HLRE13 and *S. aureus* CMCC26003 containing 2.5 mol/L glycerol for 1 week.

2.3 Quantification of S. aureus burden in mice feces during gavage

Fresh fecal samples were collected and weighted. Fecal samples were then diluted in 1 mL PBS and homogenized. To determine the content of *S. aureus* in feces, serial dilutions of the homogenized samples were plated on Baird-Parker plates (Hopebio, Qingdao, China) and the plates were incubated at 37 °C for 24 h before enumeration. The content of *S. aureus* CMCC26003 were calculated after normalization to the weight of each fecal sample.

2.4 Histological analysis of mice cecum tissue

Cecal tissues were collected and fixed in 4% neutrally buffered formaldehyde (Servicebio, Wuhan, China) and embedded in paraffin (Servicebio, Wuhan, China) according to standard histological procedures. About 5 μm thick sections were mounted on glass slides and then stained with hematoxylin and eosin (H&E)[22]. Images were evaluated blinded to the experimental group and the cecum was scored for the following markers: inflammation, epithelial erosion and edema. Each marker was scored from 0 to 3, and the final scores were summed up to a total of a maximum of 9 per sample. Furthermore, the prepared cecum sections were stained with periodic acid-schiff (PAS)

to observe and quantify the number of goblet cells[23]. The number of goblet cells in the cecal crypt was calculated using Image Pro Plus software (version 6.0).

2.5 RNA isolation and relative gene expression analysis

Total RNA was extracted from cecum tissue by using RNAiso Plus (Takara, Japan) and transcribed into cDNA using PrimeScript™ reagent kit with gDNA Eraser (Takara, Japan) according to the manufacturer's protocol. RT-qPCR was performed using SYBR® Premix Ex *Taq*TM II (Takara, Japan), and the primer sequences are listed in Table 1. The *β*-actin gene was used as a standard internal control. The $2^{-\Delta\Delta CT}$ method was used to express the relative expression of each gene as a fold change[24].

2.6 Immunofluorescence assessment

Cecum sections were permeabilized with 0.25% Triton X-100 (Servicebio, Wuhan, China) according to the previous method[25]. After blocking with 5.0% FBS (Servicebio, Wuhan, China), the tissue was incubated with the primary antibodies of ZO-1 and Occludin (Servicebio, Wuhan, China). After rinsing, the sections were incubated with fluorescence-labeled secondary antibodies. All images were viewed and captured using an inverted fluorescence microscope. The relative fluorescence intensity of ZO-1 and Occludin was analyzed using ImageJ software (version 1.53c).

2.7 Determination of serum biochemical indexes

Eyeballs were removed for blood collection after mice were anesthetized and centrifuged at 4 000 × *g* for 5 min. The supernatant was collected and stored at –80 °C. Serum secretory immunoglobulin A (sIgA) levels were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits from Shanghai Yansheng Industrial Co., Ltd (Shanghai, China). Myeloperoxidase (MPO) levels in serum were estimated using commercial kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All experimental procedures were performed according to the manufacturer's protocols.

2.8 Microbial composition analysis of cecal contents in mice

Genomic DNA was extracted from the cecal contents. The 16S rRNA gene amplicon was sequenced on the Illumina MiSeq platform, and the primer sequences was derived from the V3-V4 high variant region of the bacterial 16S rRNA gene (338F: 5′-ACTCCTACGGGAGGCAGCAG-3′, 806R: 5′-GGACTACHVGGGTWTCTAAT-3′). Bioinformatic analysis of the gut microbiota was carried out using the Majorbio Cloud platform (https://cloud.majorbio.com). Based on the OTUs information, *α*-diversity indices and partial least-squares discriminant analysis (PLS-DA) were calculated with Mothur v1.30.1[26]. The linear discriminant analysis (LDA) effect size (LEfSe) (http://huttenhower.sph.harvard.edu/LEfSe) was performed to identify the significantly abundant taxa of bacteria among the different groups[27].

2.9 Statistical analysis

The data were analyzed using GraphPad Prism 8.0 software (GraphPad Software Inc., La Jolla, CA). All research results were expressed as the mean ± standard deviation (SD). The data were subjected to a one-way ANOVA with Dunnett's multiple-comparison test or two-way ANOVA with Tukey's multiple-comparison test, and the differences were considered statistically significant at $P < 0.05$.

3. Results

3.1 L. reuteri HLRE13 reduced S. aureus colonization in the gut

To confirm the potential of *L. reuteri* HLRE13 to reduce *S. aureus* colonization *in vivo*, the interaction of *L. reuteri* HLRE13 with *S. aureus* CMCC26003 was evaluated in mice (Fig. 1A). As shown in Fig. 1B, *S. aureus* CMCC26003 had no significant effect on the body weight of mice. At 6 h post infection, the content of *S. aureus* CMCC26003 was significantly higher ($P < 0.000\ 1$) in the Sa group (7.41×10^7 CFU/g) than in the Sa-Lr group. Thereafter, *S. aureus* CMCC26003 levels decreased in both groups, but were consistently higher in the Sa group than in the Sa-Lr group (Figs. 1C and D). Meanwhile, fecal *S. aureus* CMCC26003 levels increased from day 5 to day 9 in the Sa group (5.62×10^6 to 2.29×10^7 CFU/g), but tended to decrease significantly in the Sa-Lr group (6.31×10^7 to 1.32×10^5 CFU/g, $P < 0.000\ 1$), and were lower in the Sa-Lr group than in the Sa group except for the day 5 which had a little bit fluctuation (Fig. 1E). Overall, *L. reuteri* HLRE13 effectively inhibited *S. aureus* CMCC26003 colonization in the intestinal tract of mice, which was consistent with the results of the *in vitro* study.

Table 1
Information of oligonucleotide primers used for RT-qPCR.

Target gene	Primer sense (5'-3')	Primer antisense (5'-3')
TLR2	CAGCTTAAAGGGCGGGTCAGAG	TGGAGACGCCAGCTCTGGCTCA
TLR4	TTCAGAGCCGTTGGTGTATC	CCCATTCCAGGTAGGTGTTT
MyD88	CCTGCGGTTCATCACTAT	GGCTCCGCATCAGTCT
IFN-γ	CATGAAAATCCTGCAGAGCC	GGACAATCTCTTCCCCAGCC
IL-12	CCTCCACTGTGCTGGTTTTAT	TCAGCAACATGCTCCAGAAG
IL-10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
Occludin	ACAGTGTTGGCTCCCTGTTC	ATCGTTTACCTTGCCAGCCA
ZO-1	CTGAGGTGTAAGATGGAAGCG	CAACTGTCCATAGTGCAATGG
MUC-2	ACGATGCCTACACCAAGGTC	TGATCTTTACATGTTCCCA
β-Actin	TGACGTTGACATCCGTAAAGACC	CTCAGGAGGAGCAATGATCTTGA

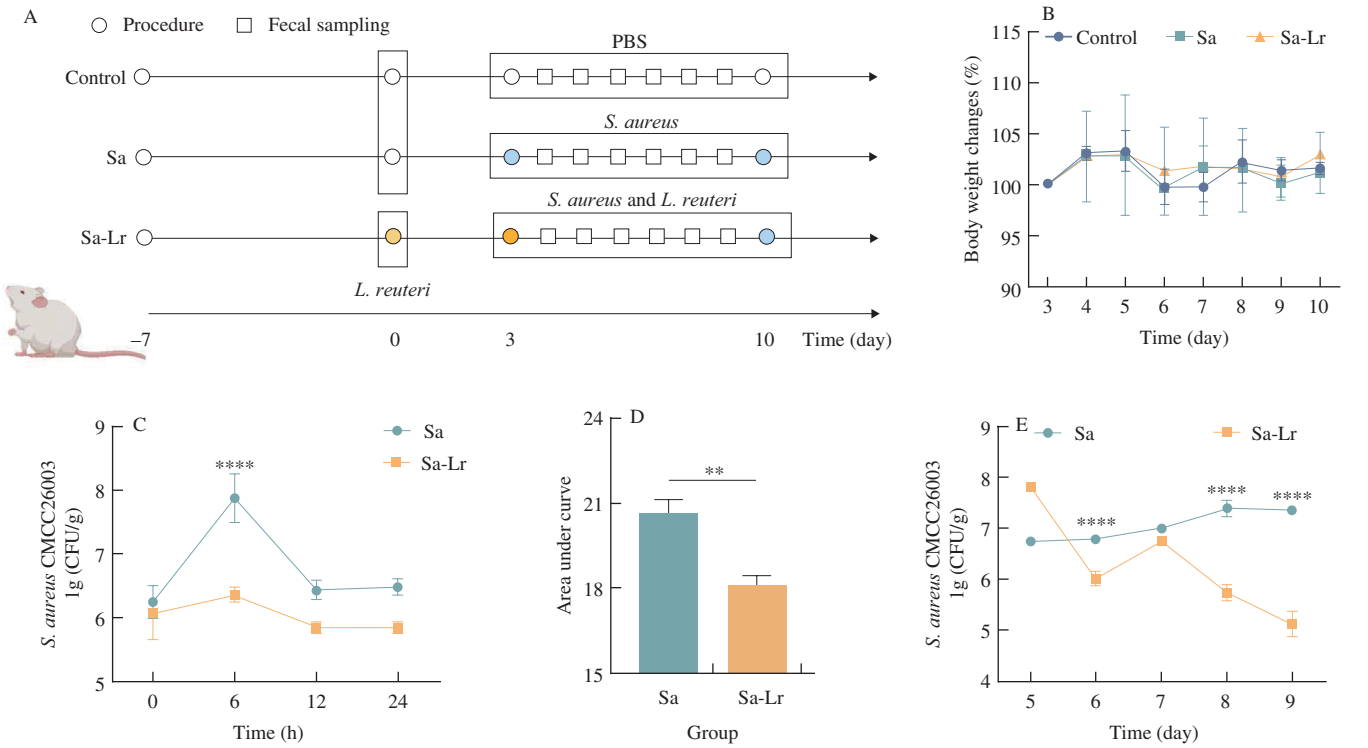

Fig. 1 *L. reuteri* HLRE13 inhibited *S. aureus* CMCC26003 colonization *in vivo*. (A) Animal experimental design ($n = 6$). (B) Body weight changes of mice. (C) Dynamic content of *S. aureus* in fecal samples over 24 h. (D) The area under curve of *S. aureus* CMCC26003 colonization over 24 h. (E) Dynamics content of *S. aureus* in mice feces during gavage. Data are presented as the mean ± SD. $^{**}P < 0.01$, $^{****}P < 0.000\ 1$.

3.2 L. reuteri HLRE13 attenuated S. aureus-induced intestinal damage and increased mucin expression with the presence of glycerol

Although *L. reuteri* HLRE13 can produce the broad-spectrum antibacterial reuterin from glycerol[20], the effects of *L. reuteri* HLRE13 and glycerol on *S. aureus*-induced structural damage to intestinal tissues remain unclear. Therefore, intestinal damage and changes in goblet cells proportions induced by *S. aureus* CMCC26003 and the ameliorative effect of *L. reuteri* HLRE13 coupled with glycerol were evaluated (Fig. 2A). H&E staining of cecal tissue showed that gavage with *S. aureus* resulted in rearrangement and shortening of the intestinal villi along with partial loss of the lamina propria as compared to the Control group (Figs. 2B-C and S1). However, the villi of the cecal mucosa were longer, denser, and more uniformly arranged, and the overall tissue structure was significantly improved in the Sa-Lr and Sa-Lr-gly groups compared with the Sa group ($P < 0.000\ 1$), with superior improvements in the Sa-Lr-gly group compared with the Sa-Lr group. These results indicated that *L. reuteri* HLRE13 combined with glycerol effectively alleviated *S. aureus*-induced structural damage to cecal tissue.

The main function of intestinal goblet cells is to synthesize and secrete mucin, which is important for protecting the structure of intestinal epithelial cells and maintaining intestinal homeostasis[28]. PAS staining revealed that *S. aureus* significantly reduced the proportion of goblet cells in the cecum ($P < 0.001$), while *L. reuteri* treatment prevented this trend and significantly restored the number of goblet cells in the Sa-Lr-gly group (Figs. 2B and D, $P < 0.01$). Additionally, *MUC-2* expression was similarly improved by *L. reuteri* HLRE13 with glycerol (Fig. 2E, $P < 0.01$). Collectively, these results confirmed that *L. reuteri* HLRE13 significantly alleviated *S. aureus*-induced damage to the cecum, restored the number of goblet cells, and upregulated *MUC-2* expression with the presence of glycerol.

3.3 Immunofluorescence staining revealed that L. reuteri HLRE13 with glycerol increased the expression of tight junction proteins in the mouse cecum

S. aureus infection disrupted the intestinal barrier and induced tissue damage in mice. Immunofluorescence staining of cecal tissues revealed that *S. aureus* CMCC26003 decreased ZO-1 expression and significantly reduced Occludin expression ($P < 0.05$). The protein expression levels of ZO-1 ($P < 0.05$ and $P < 0.01$, respectively) and Occludin ($P < 0.01$) were significantly enhanced in the Sa-Lr and Sa-Lr-gly groups (Figs. 3A and C). Meanwhile, *L. reuteri* HLRE13 significantly increased the relative expression levels of *ZO-1* and *Occludin* (Figs. 3D and E, $P < 0.000\ 1$ and $P < 0.01$, respectively). These results indicated that *L. reuteri* HLRE13 with glycerol enhanced the expression of tight junction proteins in the cecum, thereby improving intestinal barrier function in mice.

3.4 L. reuteri HLRE13 combined with glycerol effectively regulated the expression of inflammatory factors and improved the immune function in mice

Toll-like receptors, specifically TLR2 and TLR4, are critical for regulating the interaction between intestinal barrier function and mucosal immunity[29]. Therefore, the relative mRNA expression levels of *TLR2*, *TLR4*, and *MyD88* in the mouse cecum were quantified. The results showed that

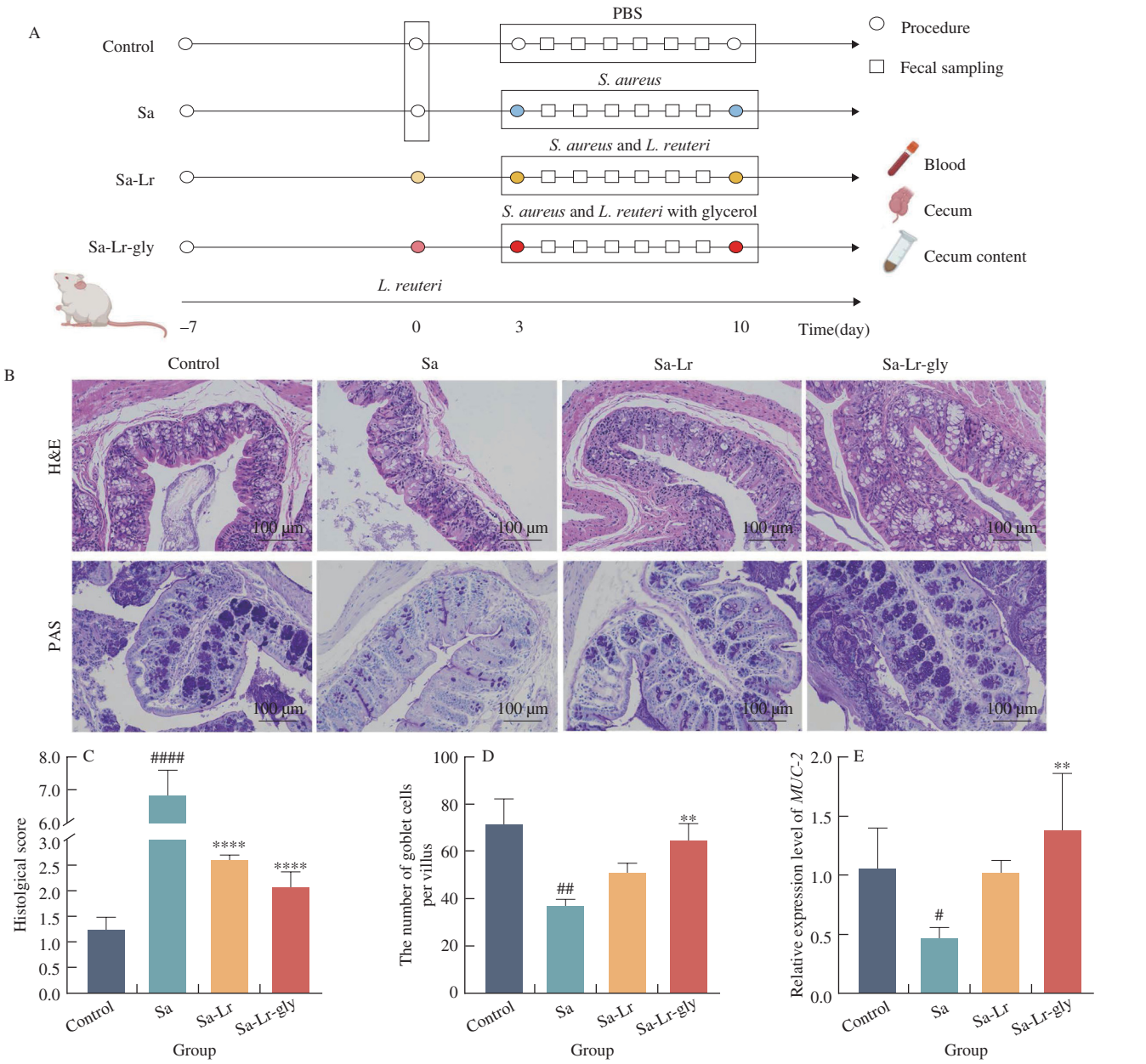

Fig. 2 *L. reuteri* HLRE13 alleviated intestinal tissues changes and increased MUC-2 expression with the presence of glycerol. (A) Animal experimental design ($n = 6$). (B) Representative images of cecum by H&E and PAS staining. Scale bar is 100 μm. (C) Histological score of the cecum representative images from each group of mice. (D) Number of goblet cells in the cecum of mice. (E) Relative expression level of *MUC-2* in cecum. Data are presented as the mean ± SD. $^{**}P < 0.01$, $^{****}P < 0.000\ 1$ *vs*. Sa group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#\#}P < 0.000\ 1$ *vs*. Control group.

S. aureus CMCC26003 significantly inhibited the expression of *TLR4* and *MyD88* ($P < 0.05$), while *L. reuteri* HLRE13 alone or coupled with glycerol enhanced the expression of *TLR2*, *TLR4*, and *MyD88*. Similarly, the expression levels of the pro-inflammatory factors *IFN-γ* and *IL-12* were significantly increased, while the expression of the anti-inflammatory factor *IL-10* was significantly decreased ($P < 0.01$) in the cecum of mice infected with *S. aureus* (Fig. 4A). In contrast, the mRNA expression levels of *IFN-γ* and *IL-12* were significantly decreased ($P < 0.05$), while the expression of *IL-10* was significantly increased ($P < 0.000\ 1$) after intervention with *L. reuteri* HLRE13 alone or coupled with glycerol (Fig. 4A). These results demonstrated that *L. reuteri* HLRE13 alone or coupled with glycerol activated pathogen recognition by upregulating *TLR2*, *TLR4*, and *MyD88*, which rendered immune cells more susceptible to invasion by *S. aureus*.

MPO is a well-established marker of central trophoblast function and activation as well as the degree of inflammatory cell infiltration[30]. In this study, *S. aureus* CMCC26003 significantly increased MPO activity in mice ($P < 0.01$). This effect was reversed by treatment with either *L. reuteri* HLRE13 alone or coupled with glycerol ($P < 0.05$, $P < 0.000\ 1$; Fig. 4B). The antibody sIgA plays a decisive role in the innate immune response against pathogens by blocking adherence, invasion, and colonization of mucosal epithelia cells[31]. As shown in Fig. 4C, sIgA levels in mice infected with *S. aureus* were significantly decreased from 67.43 to 61.35 ng/mL ($P < 0.05$). However, treatment with *L. reuteri* HLRE13 coupled with glycerol significantly increased sIgA levels ($P < 0.001$), with a greater improvement than the Sa-Lr group. These findings indicated that *S. aureus* CMCC26003 reduced the immune response in mice, while *L. reuteri* HLRE13 coupled with glycerol mitigated this effect.

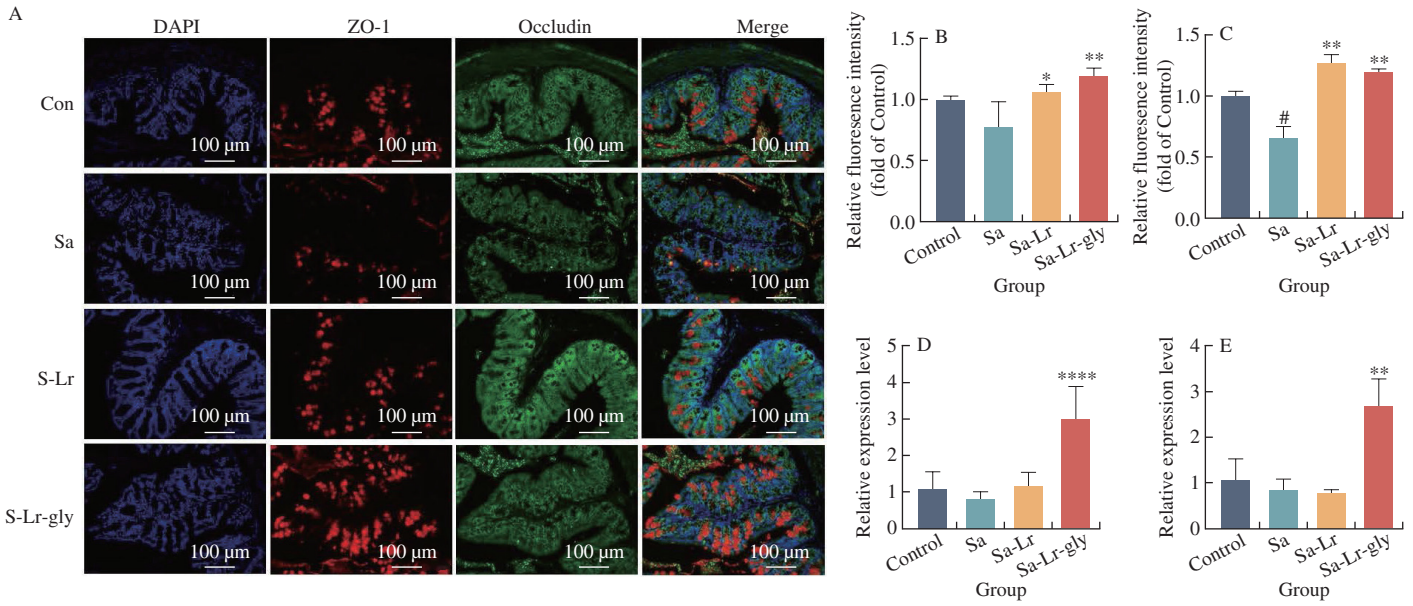

Fig. 3 *L. reuteri* HLRE13 combined with glycerol increased tight junction protein expression in the cecum. (A) Immunofluorescence staining of cecum tight junction proteins. Scale bar is 100 μm. Relative fluorescence intensity of (B) ZO-1 and (C) *Occludin*. Relative expression of (D) *ZO-1* and (E) *Occludin* in mice cecum. Data are presented as the mean ± SD. **P* < 0.05, ***P* < 0.01, *****P* < 0.000 1 *vs*. Sa group. #*P* < 0.05 *vs*. Control group.

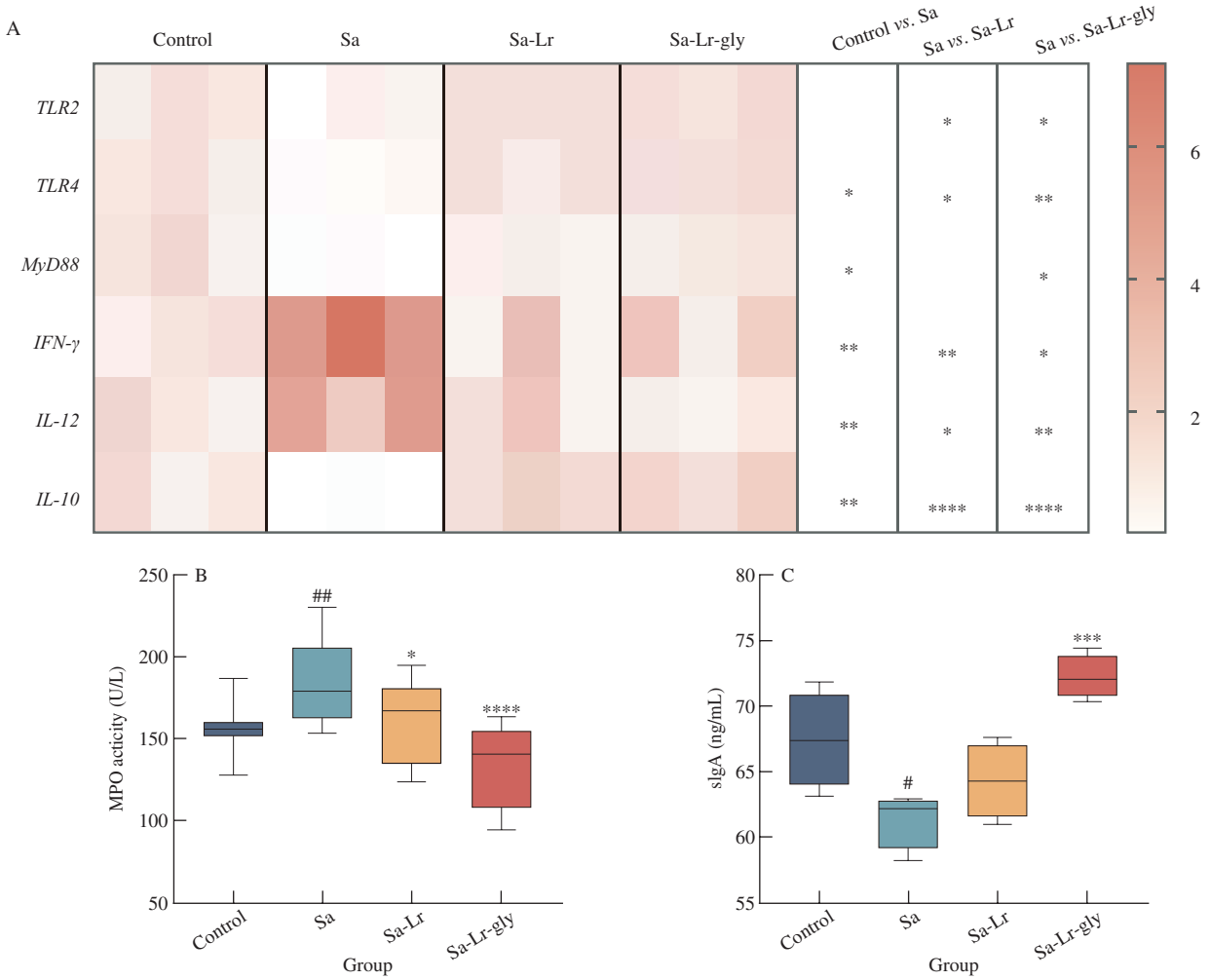

Fig. 4 *L. reuteri* HLRE13 with glycerol regulated expression of inflammatory factors and enhanced immune levels in mice. (A) Heatmap of relative mRNA expression of *TLR-2*, *TLR-4*, *MyD88*, *IFN-γ*, *IL-12* and *IL-10*. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.000 1. (B) MPO activity in serum. (C) sIgA concentration in serum. Data are presented as the mean ± SD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.000 1 *vs*. Sa group. #*P* < 0.05, ##*P* < 0.01 *vs*. Control group.

3.5 *L. reuteri* HLRE13 coupled with glycerol regulated the structural composition of the gut microbiota in mice

The dynamic interactions between the gut microbiota and the host immune system are critical for maintaining intestinal homeostasis and regulating inflammation. The results of 16S rRNA gene sequencing showed that the Chao and Shannon indices of the cecal flora were significantly decreased, and the Simpson index was significantly increased after infection of mice with *S. aureus* CMCC26003 ($P < 0.05$, Fig. 5A), suggesting decreased diversity of the gut microbiota. However, the intervention with *L. reuteri* HLRE13 with glycerol significantly increased the Ace, Chao, and Shannon indices, and significantly decreased the Simpson index ($P < 0.05$), indicating that *L. reuteri* HLRE13 combined with glycerol restored the altered diversity of gut microbiota in mice caused by *S. aureus* infection. Meanwhile, the results of PLS-DA showed that the samples from each group were well clustered and differentiated between groups, indicating structural differences in the gut microbiota (Fig. 5B).

The Firmicutes/Bacteroidetes (F/B) ratio may reflect the degree of inflammation to some extent. As shown in Figs. 5C and D, there was no significant difference in the relative abundance of Bacteroidetes in the gut microbiota of mice in each group, but the relative abundance of Firmicutes was significantly decreased in the group infected with *S. aureus* ($P < 0.001$) and significantly restored by intervention with *L. reuteri* HLRE13 with glycerol ($P < 0.05$). In particular, the F/B ratio was lowest for the group infected with *S. aureus* ($P < 0.001$, Fig. 5E). As shown in Fig. 5F, there were some differences in the composition of the gut microbiota at the genus level among the groups. Notably, *S. aureus* infection significantly increased the relative abundance of *Staphylococcus*, whereas *L. reuteri* HLRE13 treatment reversed this effect ($P < 0.01$). In contrast, *S. aureus* CMCC26003 infection significantly reduced the relative abundance of *Lactobacillus*, which was restored by intervention with *L. reuteri* HLRE13 and glycerol ($P < 0.05$).

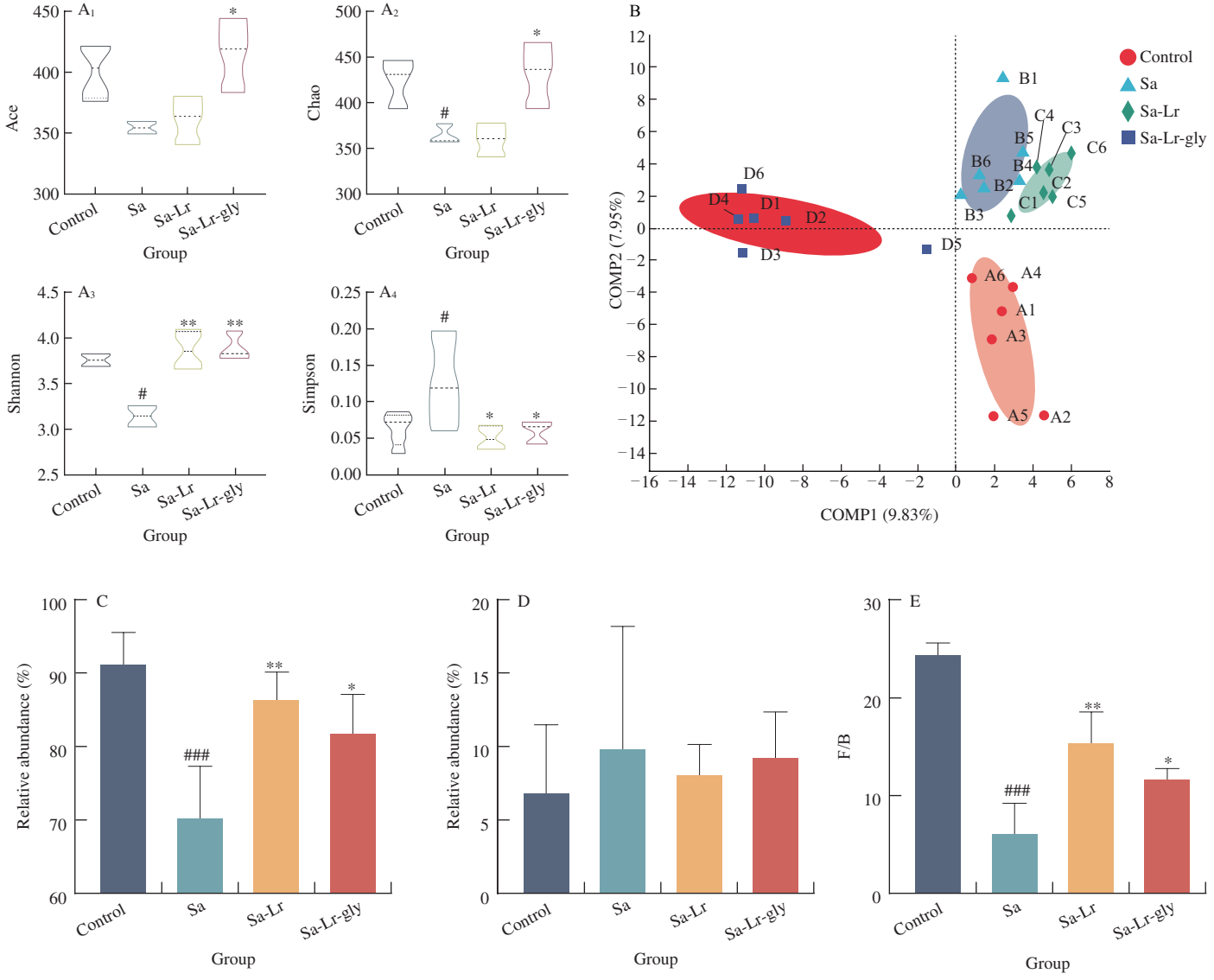

Fig. 5 Composition of gut microbiota was regulated by *L. reuteri* HLRE13 with glycerol. (A) α-Diversity (B) PLS-DA analysis. Relative abundance of (C) Firmicutes and (D) Bacteroidetes, and (E) F/B ratio. (F) Relative abundance of bacteria at the genus level. Data are presented as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs*. Sa group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs*. Control group.

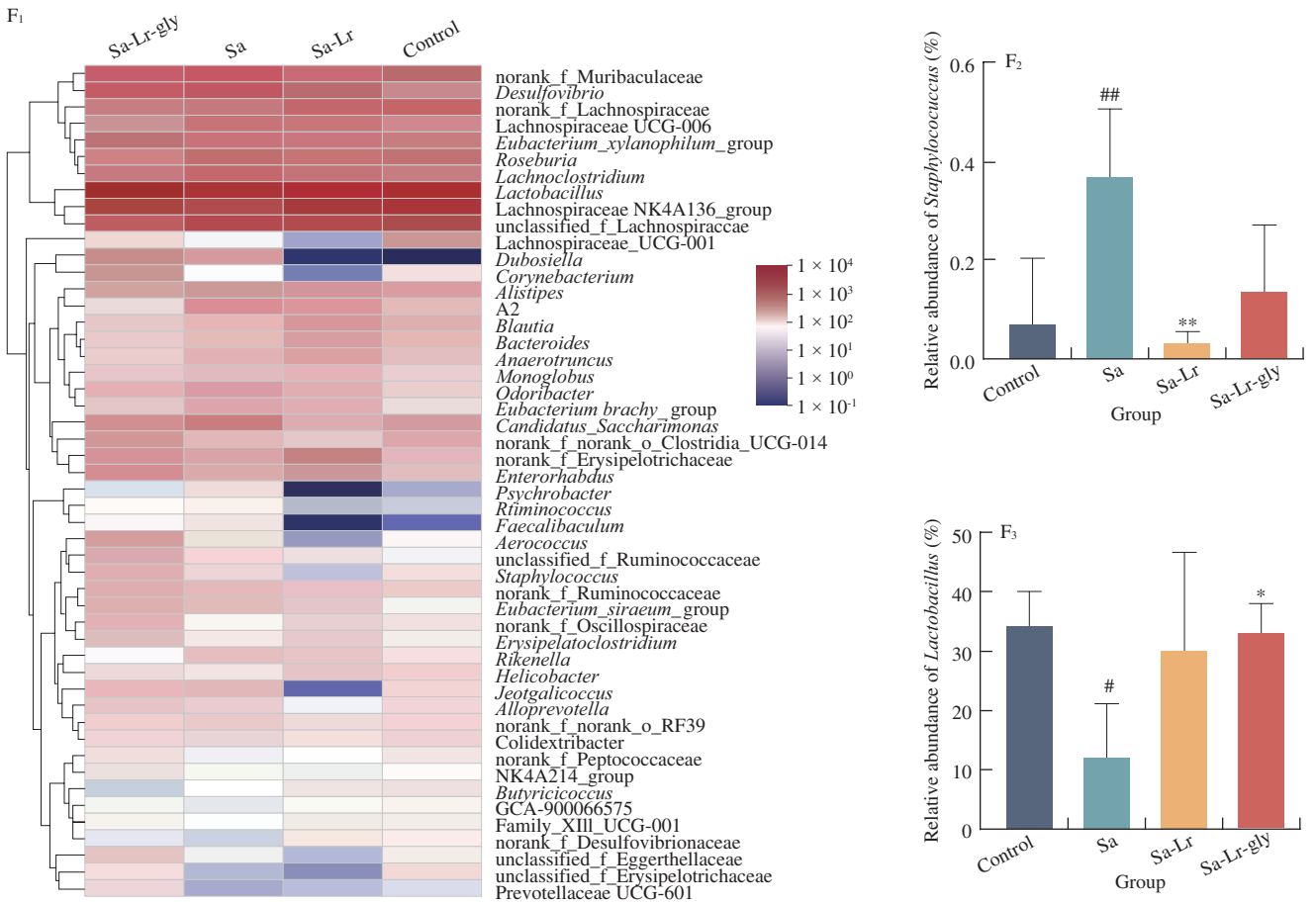

Fig. 5 (*Continued*)

3.6 *Species variability of the gut microbiota*

Further analysis of the differences in the gut microbiota between the groups was conducted. As shown in Fig. 6A, the abundance of *Lactobacillus* was significantly higher in the Sa-Lr group than in the Sa group (P = 0.045). Specifically, both *Lactobacillus* and *L. reuteri* were more abundant in the Sa-Lr-gly group than in the Sa group (Fig. 6B). In addition, discriminant analysis of multilevel species differences in the gut microbiota was analyzed by the LEfSe method. The results showed that the abundance of Desulfovibrionaceae, which are considered to be harmful intestinal bacteria, was highest in the Sa group, while the abundance of *Eubacterium*, a genus that plays a crucial role in the nutrient metabolism and maintaining intestinal homeostasis, was significantly higher in the Sa-Lr group, and the abundances of the probiotic genera *Dubosiella* and Ruminococcaceae, which ferment dietary fiber to produce energy, were highest in the

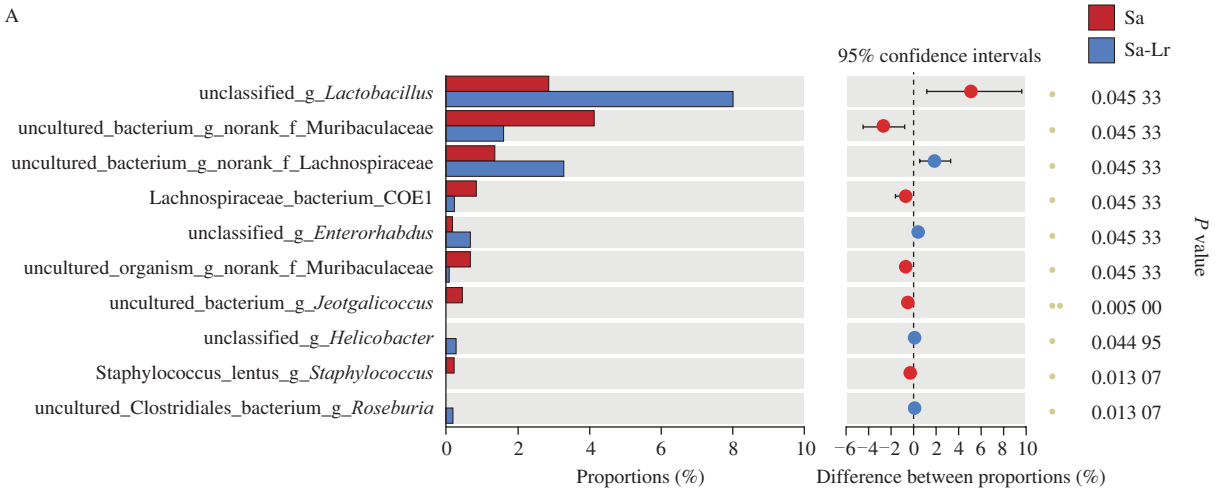

Fig. 6 Species variability analysis of gut microbiota. (A) Wilcoxon rank-sum test bar plot on species level of mice in Sa group and Sa-Lr group. (B) Wilcoxon rank-sum test bar plot on species level of mice in Sa group and Sa-Lr-gly group. (C) LDA of the most differential taxa between groups.

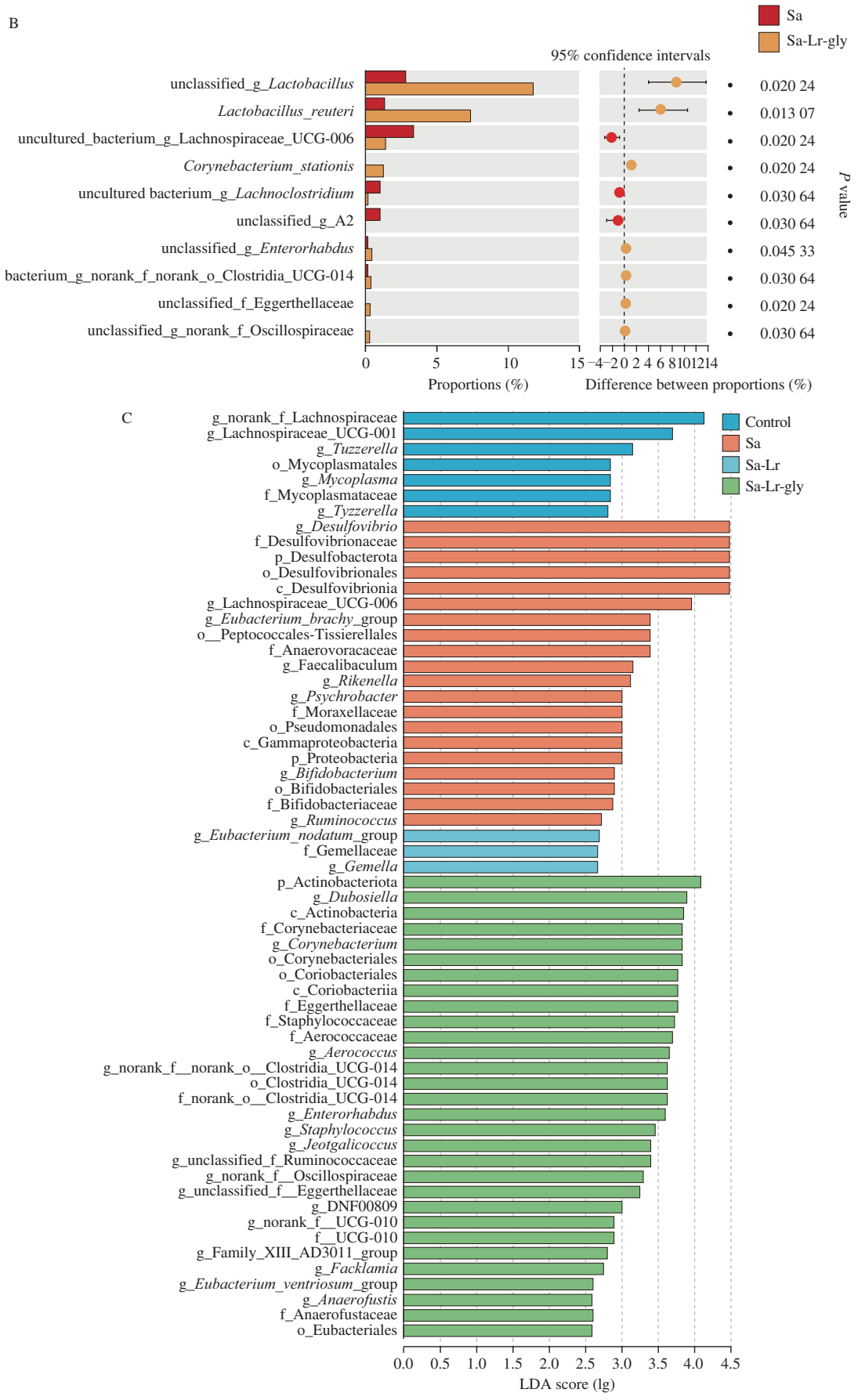

Fig. 6 (*Continued*)

Sa-Lr-gly group (Fig. 6C). These results indicated that *L. reuteri* HLRE13 with glycerol modified changes in the gut microbiota induced by *S. aureus* CMCC26003.

3.7 *L. reuteri HLRE13 with glycerol inhibited S. aureus colonization in ABX-treated mice*

To determine the effect of the intrinsic gut microbiota on the inhibition of *S. aureus* by *L. reuteri* HLRE13 with glycerol, mice were gavaged with broad-spectrum antibiotics to deplete gut commensal bacteria (Fig. 7A). Over a period of 7 days, body weight tended to increase in the ABX-Sa-Lr-gly group, but decreased in the ABX-Sa group (Fig. 7B, $P < 0.01$). In addition, the fecal content of *S. aureus* was consistently higher in the ABX-Sa group than in the ABX-Sa-Lr-gly group from day 14 to day 16 (Fig. 7C, $P < 0.05$), suggesting that *L. reuteri* HLRE13 with glycerol effectively inhibited the colonization of *S. aureus* in the absence of gut microbiota. Meanwhile, H&E and PAS staining of the cecum indicated that *L. reuteri* HLRE13 with glycerol alleviated the intestinal damage caused by *S. aureus* (Figs. 7D and S2). Furthermore, H&E staining showed that the cecal villi of the mice were disordered and exhibited structural damage along with inflammatory cell infiltration after gavage with *S. aureus*. In contrast, *L. reuteri* HLRE13 coupled with glycerol significantly restored the intestinal structure of mice, as evidenced by longer villi that were tightly and neatly arranged. PAS staining revealed that the number of goblet cells was significantly increased in the cecum of mice in the ABX-Sa-Lr-gly group as compared to the ABX-Sa group, suggesting that *L. reuteri* HLRE13 and glycerol promoted the production of goblet cells, which resulted in higher secretion of mucin to enhance intestinal barrier function. Also, the immune function of the mice was assessed by measuring MPO activity and sIgA content. As shown in Figs. 7E and F, *L. reuteri* HLRE13 coupled with glycerol significantly decreased MPO activity and significantly increased sIgA content in

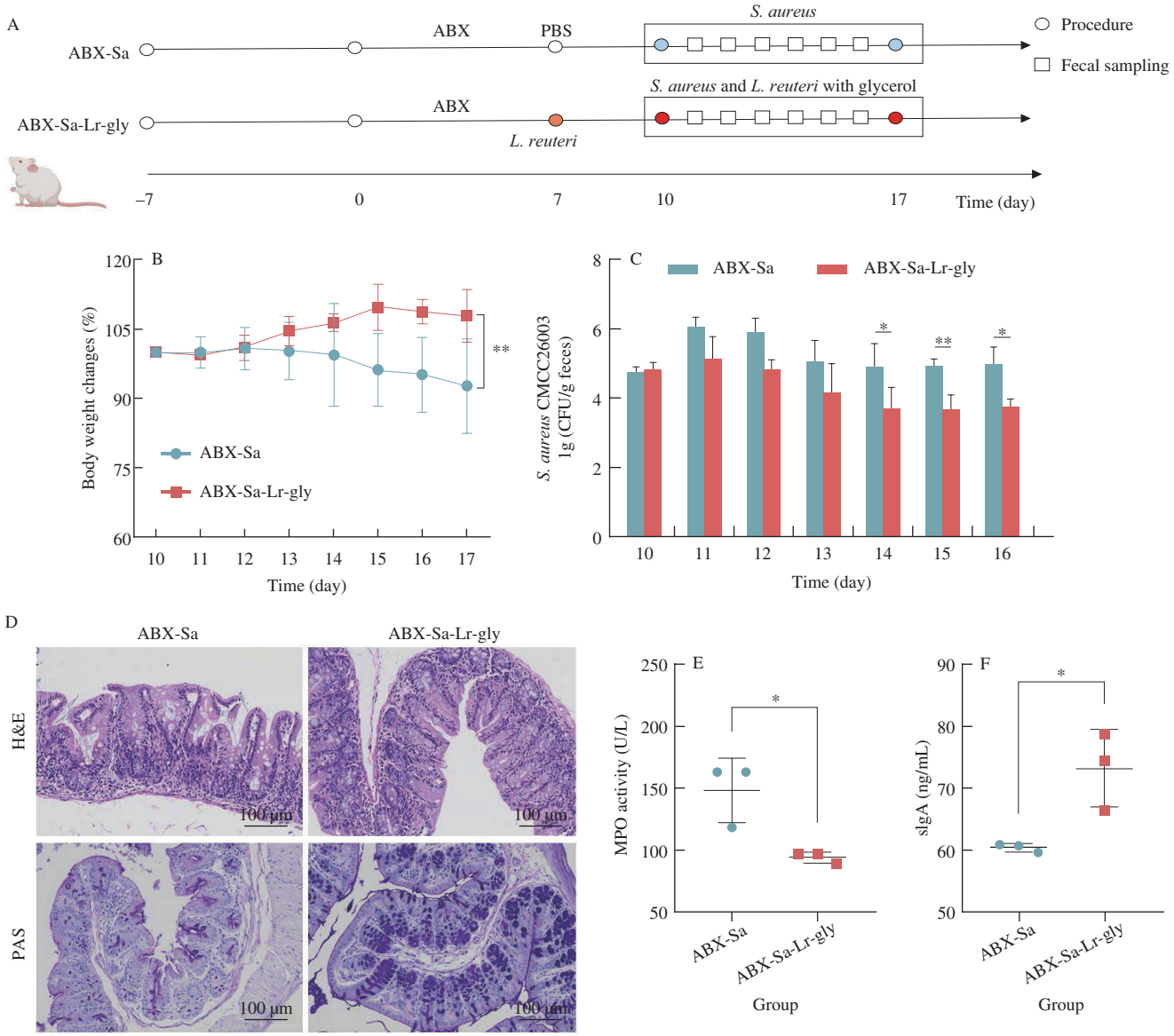

Fig. 7 *S. aureus* colonization was inhibited by *L. reuteri* HLRE13 with glycerol in ABX-treated mice. (A) Animal experimental design ($n = 6$). (B) Body weight changes. (C) Dynamics of *S. aureus* colonization in mice during gavage. (D) Representative images of cecum by H&E and PAS staining. Scale bar is 100 μm. (E) MPO activity in serum. (F) sIgA concentration in serum. Data are presented as the mean ± SD. *$P < 0.05$, **$P < 0.01$.

mice infected with *S. aureus* ($P < 0.05$). These results suggested that *L. reuteri* HLRE13 and glycerol improved immune function in mice. Taken together, *L. reuteri* HLRE13 with glycerol inhibited *S. aureus* colonization in ABX-treated mice, thereby ameliorating intestinal inflammation and improving immune function.

4. Discussion

S. aureus infection leads to inflammation and dysregulation of commensal bacteria in the gut, resulting in disease[32]. Antibiotics are widely used to treat bacterial infections, but bacterial resistance is currently a significant threat to global health and food security[33]. Probiotics are a promising strategy for the prevention and treatment of bacterial infections due to their ability to inhibit the growth of pathogenic microorganisms and regulate the gut microbiota. The present study investigated the effect of *L. reuteri* HLRE13, which can utilize glycerol to produce the broad-spectrum antibacterial reuterin, on the inhibition of *S. aureus* proliferation *in vivo*.

This study confirmed that *S. aureus* colonization in the gut tended to increase with the duration of gavage. The colonized *S. aureus* further induced tissue inflammation and reduced the number of goblet cells. Notably, treatment of *L. reuteri* HLRE13 with glycerol increased the rate of body weight change, alleviated intestinal inflammation, and increased the number of goblet cells, resulting in increased mucus secretion in the intestinal tract of mice. The fermentation of glycerol by *L. reuteri* HLRE13 might have produced reuterin like the previous report[34], which inhibited the proliferation of *S. aureus*, consistent with the finding that reuterin interferes with the metabolism of *Clostridium difficile*, thereby reducing its nutrient competition and virulence in the gut[35].

Tight junction proteins are essential for maintaining the integrity of the intestinal epithelial barrier. ZO-1 is an intracellular protein with the ability to act as a scaffolding protein[36], and Occludin is a transmembrane junctional protein that links tight junction membrane proteins to the cytoskeleton and various signaling proteins[37]. In this study, the expression levels of ZO-1 and Occludin were reduced in the Sa group, suggesting that *S. aureus* disrupted the expression of tight junction proteins in the intestinal tract, which may lead to a sensitized state, thereby increasing the risk of disease outbreaks[38]. In contrast, *L. reuteri* HLRE13 with glycerol significantly increased the expression of ZO-1 and Occludin, increased the number of goblet cells, and up-regulated mucin expression, suggesting that *L. reuteri* HLRE13 with glycerol repaired and strengthened the intestinal barrier to inhibit invasion and proliferation of *S. aureus*. Overall, *L. reuteri* HLRE13 combined with glycerol increased tight junction proteins expression in intestinal epithelial cells, decreased intestinal permeability, and promoted intestinal barrier function, thereby decreasing the ability of pathogenic bacteria to infect the intestinal tract.

The intestinal mucosa plays an important role in maintaining the integrity of the intestinal barrier[39]. As a hallmark of mucosal immunity, sIgA protects the intestinal environment from bacteria, viruses, and parasites[40]. MPO is an established marker of neutrophil proliferation and inflammatory severity[41]. In this study, *S. aureus* infection increased MPO activity and decreased sIgA content in mice serum, indicating the presence of inflammation *in vivo*. These results confirmed the increased mRNA expression of the pro-inflammatory factors *IFN-γ* and *IL-12*, and decreased expression of the anti-inflammatory factor *IL-10* in the cecum of infected mice. In contrast, treatment with *L. reuteri* HLRE13 with glycerol significantly decreased MPO activity and significantly increased sIgA levels, thereby restoring the levels of inflammatory factors. Collectively, these findings suggest that *L. reuteri* HLRE13 with glycerol may regulate the immune response *via* cytokine production.

Under normal conditions, the gut microbiota and barrier function act to maintain host homeostasis and health[42]. Although *S. aureus* did not cause severe diarrhea in mice, significant changes to the abundance and diversity of the gut microbiota occurred (Figs. 5A and B), indicating that *S. aureus* can disrupt the natural balance of the host's gut microbiota. Additionally, the intestinal F/B ratio was significantly increased in mice treated with *L. reuteri* HLRE13 and glycerol, consistent with a previous report[43]. Meanwhile, the relative abundance of *Staphylococcus* had decreased and that of *Lactobacillus* had increased by treatment with either *L. reuteri* HLRE13 alone or coupled with glycerol, confirming the inhibition of *S. aureus* colonization in the mouse intestine. Notably, the inhibitory effect of *L. reuteri* HLRE13 with glycerol on *S. aureus* colonization was observed in mice after antibiotic-induced depletion of the gut microbiota. In addition, *L. reuteri* HLRE13 coupled with glycerol alleviated intestinal damage and improved the immune response of infected mice. These findings suggest that the antagonistic effect of *L. reuteri* HLRE13 with glycerol on *S. aureus* may be mediated without the gut microbiota, likely due to the ability of *L. reuteri* to produce reuterin, which can directly inhibit colonization.

5. Conclusion

The present study demonstrated that reuterin-producing *L. reuteri* HLRE13 could effectively inhibit *S. aureus* colonization in the presence of glycerol in mice. In addition, the expression of tight junction proteins and mucins was increased to protect intestinal barrier function, while cytokine levels were adjusted to alleviate intestinal inflammation. Also, the abundance of *Lactobacillus* was increased and that of *Staphylococcus* was decreased to maintain the balance of the gut microbiota of infected mice. Interestingly, resistance to colonization persisted in pseudo germ-free mice. Collectively, *L. reuteri* HLRE13 with glycerol regulated the intestinal microbiota and immune response, which further improved the intestinal barrier and alleviated inflammation and dysfunction in the mouse intestinal tract, thereby effectively inhibiting the pathogenetic effects triggered by *S. aureus* (Fig. 8). This study provides a strategy for scientific recognition of the featured *L. reuteri* to alleviate *S. aureus* infections, as well as provide a theoretical basis for the functional development of *L. reuteri* HLRE13.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

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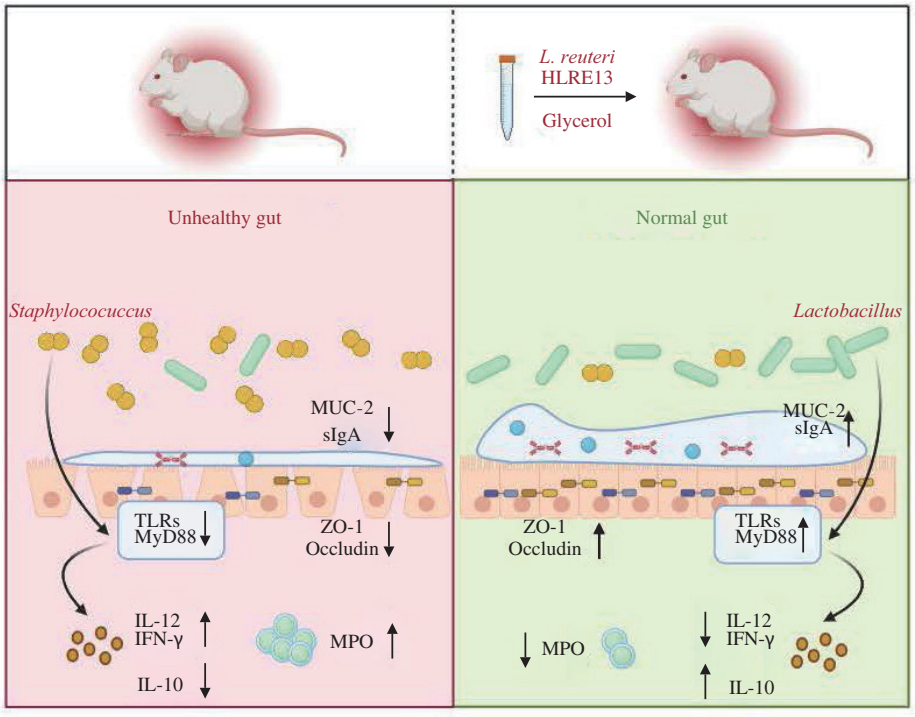

Fig. 8 The underlying mechanism of *L. reuteri* HLRE13 coupled with glycerol on inhibiting *S. aureus* colonization and mitigating intestinal damage and dysfunction.

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Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2024.9250360.

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