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Inhibition of suilysin activity and inflammation by myricetin attenuates *Streptococcus suis* virulence

Gen Li[a,1], Guizhen Wang[a,1], Xiaosa Si[a], Xueke Zhang[b], Wentao Liu[b], Li Li[a,*], Jianfeng Wang[a,*]

[a] Key Laboratory of Zoonosis, Ministry of Education, College of Veterinary Medicine, Jilin University, Changchun, China
[b] Heilongjiang Veterinary Drug and Feed Super Vision Institute, Haerbin, China

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ABSTRACT

Streptococcus suis (*S. suis*) is a gram-positive, zoonotic pathogenic bacterium that poses a serious threat to the pig industry and human health. This globally distributed pathogen can cause multiple diseases and fatal infections in both humans and animals. Suilysin (SLY) is an important extracellular secreted toxin regarded as an essential *S. suis* capsular type 2 (SS2) virulence factor and plays a key role in the infection and cytotoxicity of SS2. In addition, an excessive inflammatory response is also a serious hazard caused by SS2 infection. In this study, we demonstrated that the natural compound myricetin can inhibit the hemolytic activity of SLY and is effective at reducing the production of the inflammatory cytokines TNF-α and IL-1β and reducing inflammation by down-regulating the activation of P38. In addition, myricetin could effectively treat SS2 infections in vitro and in vivo. These findings may aid in the development of promising therapeutic candidates for treating SS2 infections.

1. Introduction

S. suis is one of the most important porcine pathogens worldwide and can cause a variety of diseases, including septicemia, endocarditis, arthritis and meningitis [1,2]. *S. suis* infections cause major economic losses every year and pose a major threat to the pig industry [3]. *S. suis* strains are currently divided into multiple serotypes based on capsular antigens [1,4], of which serotype 2 is the most widespread and virulent [5]. Two severe outbreaks of *S. suis* that occurred in 1998 and 2005 in China resulted in hundreds of infections, and caused panic within the population [6]. In recent years, sporadic cases are still frequently reported, especially in Southeast Asia [7–9]. Meningitis is one of the most serious diseases caused by *S. suis* and is also the most common clinical manifestation of such infections in humans [10]. Thus, increasing attention has been paid to *S. suis* in recent years.

To successfully infect the host, *S. suis* needs to adhere to and colonize the mucosa and/or epithelium, requiring versatile virulence factors [11]. Thus, *S. suis* infection can be effectively alleviated if the activity of these virulence factors is sufficiently inhibited. SLY is an extracellular secretory protein with pore-forming activity that belongs to the family of cholesterol-dependent cytolysin. This protein can be detected in most virulent strains and is regarded as an important factor associated with *S. suis* virulence [12]. In addition, in mice intraperitoneally injected with a strain exhibiting enhanced SLY secretion, greater virulence and lethality was observed compared to mice infected with a nonvirulent strain [13]. Recently, SLY was shown to have a crucial role in a mouse model of meningitis. In mice infected with a SLY knockout mutant or a hyposecretion strain, the mortality of mice and the inflammatory response in brain tissues were significantly reduced compared to those of mice infected with the wild-type strain secreting high level of SLY. In addition, the authors of this study also showed that people infected with the strain secreting high levels of SLY frequently had meningitis [14]. Thus, SLY may be a promising target to treat individuals infected with SS2, especially in the treatment of meningitis.

The inflammatory response induced by *S. suis* is also an important cause of the damage induced by *S. suis* and *S. suis* infection can also lead to a range of diseases that manifest inflammation such as arthritis and pneumonia [1]. MAPKs are important inflammation-related signaling pathways that can be activated by SS2 infection [15], resulting in an increase in the production of the inflammatory cytokines TNF-α and IL-1β [16]. Therefore, the effective control of inflammation is an important therapeutic strategy for the treatment of *S. suis* infection.

Myricetin is a natural flavonoid that can be extracted from the bark and leaves of bayberry and has a variety of biological functions, such as antioxidant [17] and anticancer activities [18]. Niu found myricetin can bind SLY to inhibit its activity through biological assays, and molecular simulations [19]. These exciting results suggest that myricetin has the potential to be a leading compound targeting SLY.

* Corresponding authors.
E-mail addresses: lilitonghappy@163.com (L. Li), wjf927@jlu.edu.cn (J. Wang).
[1] These authors contributed equally to this work.

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Unfortunately, the authors did not conduct in vitro and in vivo *S. suis* infection studies. Thus, additional studies are still needed to develop this molecule as a potential drug for the treatment of *S. suis* infection. In this study, we confirmed that myricetin inhibits the oligomerization of SLY without affecting bacterial growth. Furthermore, we showed that myricetin can reduce the inflammation induced by *S. suis* and further confirmed the therapeutic effect of myricetin against *S. suis* in vitro and in vivo. Taken together, these findings provide a detailed basis for the use of myricetin as a lead compound for the development of a novel treatment for *S. suis* infection.

2. Materials and methods

2.1. Bacterial culturing

The SS2 ZY05719 strain used in this study was a gift from Professor Hongjie Fan at the Nanjing Agricultural University. The myricetin (purity > 99%) was obtained from meilun Bio Co., Ltd. (Dalian, China). Bacteria were grown in THB containing 5% FBS and 2%yeast extract at 37 °C for 12 h (1:50) into THB containing 5% FBS and 2%yeast extract with different doses of myricetin (0, 4, 8, 16, and 32 μg/mL). Bacteria were further cultured to the logarithmic or stationary phase as needed. Bacteria were subsequently collected by centrifugation (3000 rpm, 5 min), washed twice with PBS to remove residual medium and diluted in either DMEM without FBS or PBS for infection.

2.2. Myricetin anti-S. suis activity assay

THB containing 5% FBS and 2%yeast extract was used to dilute the SS2 culture to a density of 5×10^5 CFU/mL. The MIC (minimal inhibitory concentration) of myricetin against SS2 was measured using the procedures established by the Clinical & Laboratory Standards Institute guideline M31-A3 (JL Watts) [20]. Growth curves were measured to determine the growth kinetics of SS2 in the presence of myricetin. SS2 cultures in the lag phase were portioned into equal amounts (10 mL), and different doses of myricetin were added to final concentrations of 0, 4, 8, 16, and 32 μg/mL. The samples were cultured, and the OD_{600} of each sample was measured every 30 min for 5 h.

2.3. Hemolysis assay

SS2 was cultured for 12 h and diluted in THB supplemented with 5% FBS and 2% yeast extract (1:50). Subsequently, myricetin was added to the cultures at different concentrations (0, 4, 8, 16, and 32 μg/mL), and the cultures continued to an OD_{600} of 2.5–2.6. The bacterial culture supernatants were collected by centrifugation. A reaction system with a final volume of 1 mL was established by mixing together 100 μL of culture supernatant, 25 μL of defibrinated rabbit blood and 875 μL PBS. The reactions were incubated at 37 °C for 30 min. A sample incubated with 2.5 vol% Triton X-100 was used as a positive control, and a sample without bacterial culture supernatant was used as a negative control. After incubation, each sample was centrifuged, and the OD_{543} of the collected supernatants was measured.

The SLY protein was purified and the inhibition of myricetin against purified SLY protein was measured as described in our previous study [21].

2.4. Western blotting assay

The culture supernatants described above were mixed with Laemmli SDS sample buffer to a final volume of 20 μL, boiled for 8 min and then separated by 12% SDS-PAGE. The proteins were transferred onto polyvinylidene fluoride (PVDF) membranes, which were blocked with milk for 2 h. Then, PVDF membranes were incubated with an antibody specific for SLY (1:800) [21] for 2 h at room temperature, washed 3 times with TBST and subsequently incubated with a secondary rabbit antibody (1:10000). Proteins were detected using Amersham ECL western blotting detection reagents.

2.5. Semi-quantitative RT-PCR (SQ-RTPCR) assays

The Semi-quantitative RT-PCR assays were performed as our previous study [22]. The total RNA in SS2 cells co-cultured with myricetin were extracted by RNeasy Mini kit and the RNA were reverse transcribed into cDNA by TaKaRaRNAPCR kit according to the manufacturer's instructions. The product was electrophoresed by 1% agarose gels, and the group of 16SrDNA was as control.

2.6. Oligomerization assay

SLY was preincubated with myricetin at 37 °C, and the induction of protein oligomerization was assessed using 6% SDS-PAGE according to protocols described in our previous studies [21,23].

2.7. Cell infection assays

To assess the inflammatory response of the host cells infected by SS2, J774 macrophages were cultured at 37 °C with 5% CO_2 in complete DMEM containing 10% FBS and seeded at a density of 2.0×10^6 cells/well in 6-well culture dishes overnight. Next, the cells were infected with SS2 at a MOI (multiplicity of infection) of 8 and incubated with different concentrations of myricetin for 5 h. The cell supernatants were collected by centrifugation at 4 °C, and the levels of the inflammatory cytokines TNF-α and IL-1β were assessed by ELISA kits according to the manufacturer's instructions. To assess the levels of p38/p-p38, total proteins were extracted from cells using M-PER mammalian protein extraction reagent (Thermo), and a western blotting assay was performed as described above using primary antibodies against p38/p-p38 (1:1000) and β-actin (1:1000) and a secondary rabbit or mouse antibody diluted 1:10000.

To assess SS2-mediated cytotoxicity, J774 cells were infected with SS2 (MOI = 12) for 5 h and then treated with or without myricetin (32 μg/mL), after which images of the infected cells stained with live/dead (green/red) reagent were collected by laser confocal microscopy. The LDH in the cell-free supernatants was measured using a cytotoxicity detection kit, and cells treated with DMEM with no FBS or with 2.5 vol % Triton X-100 were used as the negative and positive control groups, respectively.

2.8. Animal experiments

To evaluate the effect of myricetin in vivo, we developed a mouse model of infection for *S. suis*. Six-week-old female C57BL/6J mice were obtained from the experimental animal center of Jilin University. All animal experiments complied with animal ethics regulations and were approved by the Jilin University institutional animal care committee. Mice that were intraperitoneally injected with 2×10^7 CFU of *S. suis* received 100 mg/kg myricetin or DMSO (control group) 2 h after infection and then at 8-h intervals. The mice treated with or without myricetin were killed 72 h after infection, and the dissected livers and spleens were homogenized. The organ homogenates were serially diluted and spread onto THB plates to count the number of resulting colonies.

2.9. Statistical analysis

All experimental data ($n \geq 3$) are expressed as the mean ± SD. GraphPad Prism 5.0 was used for statistical analysis using Student's *t*-test. Significance levels of $P < 0.05$ and $P < 0.01$ are indicated in the figures.

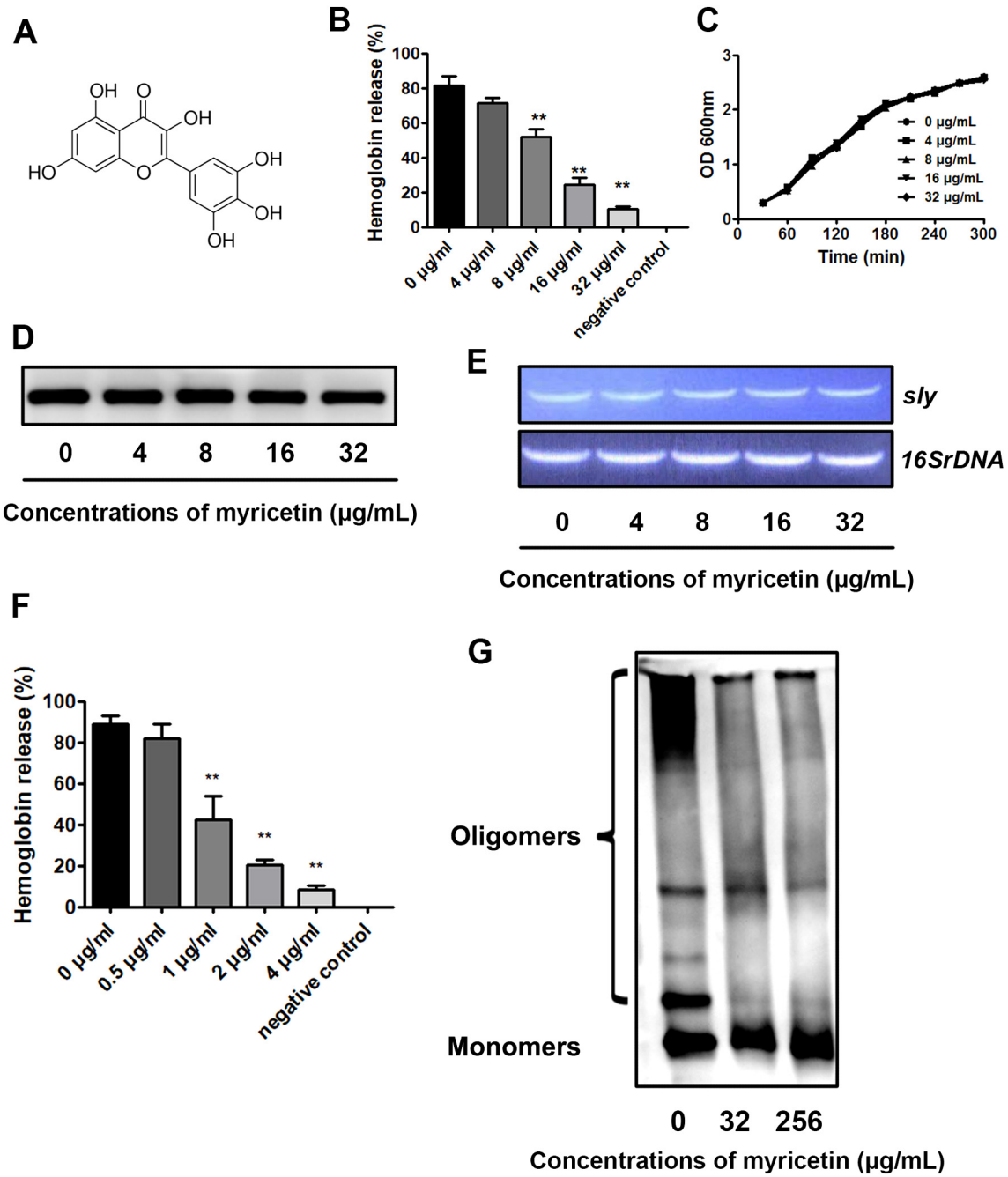

Fig. 1. Inhibition of SLY oligomerization by myricetin. A, The chemical structure of myricetin. B, The inhibition of the hemolytic activity of SS2 bacterial supernatants by myricetin. The supernatants of SS2 cultured with myricetin were collected by centrifugation and used in a hemolysis assay. The percent hemolysis was compared with that of the positive control group, which was set as 100%. C, SS2 was cocultured with different doses of myricetin, and the growth kinetics of each sample was assessed by measuring the OD_{600} every 30 min. D, SLY secretion in the supernatants of SS2 cultured with various doses of myricetin was measured by western blotting. E, The gene expression of sly and 16SrRNA in SS2 co-cultured with myricetin was assessed by semi-quantitative PCR. F, The inhibition of the hemolytic activity of purified SLY by myricetin. G, The purified SLY protein was treated with myricetin, and SLY oligomerization was determined by western blotting. *indicates $P < 0.05$, and ** indicates $P < 0.01$compared with the myricetin-free sample (two-tailed Student's *t*-test).

3. Results

3.1. Myricetin inhibits SLY oligomerization and activity

As shown in Fig. 1B, the hemolytic activity of the culture supernatant of SS2 grown in the presence of 0, 4, 8, 16, and 32 μg/mL of myricetin (Fig. 1A) was significantly decreased compared to the samples lacking myricetin. Based on other people's research on the concentration of myricetin and our previous studies in screening of the effective concentrations for inhibitors [21,23–25], 32 μg/mL was used as the upper limit value. Then, we further explored whether this inhibitory effect was related to SLY expression or bacterial growth. In addition, the MIC of the myricetin on SS2 was > 256 μg/mL and the growth curve of bacteria was not visibly affected by myricetin treatment (Fig. 1C), which suggested that myricetin did not inhibit the viability of SS2. However, no significant difference in the expression of SLY in the SS2 supernatants cocultured with various concentrations of myricetin was observed (Fig. 1D) and the expression of *sly* gene was also not affected by various concentrations of myricetin (Fig. 1E). Consistent with the results of a previous study by Niu [19], our findings

suggested that the inhibition of the hemolytic activity of SS2 culture supernatant and purified SLY treated with myricetin was due to a direct inhibition of SLY activity by myricetin (Fig. 1F). Furthermore, through molecular modeling results, Niu proposed that this inhibition may result from a decrease in SLY oligomerization caused by myricetin, although experimental verification of this proposed mechanism was lacking [19]. Therefore, in this study, western blotting was performed to test this hypothesis. In agreement with the hypothesis, the number of oligomers was significantly reduced after the addition of myricetin, suggesting that myricetin treatment could inhibit the oligomerization of SLY (Fig. 1G). Taken together, our results indicated that myricetin neutralized the hemolytic activity of SLY by inhibiting its oligomerization.

3.2. Myricetin decreases the production of inflammatory mediators in host cells

TNF-α and IL-1β are crucial inflammatory mediators whose levels are often evaluated to assess inflammation. Thus, we measured the effects of myricetin on the production of TNF-α and IL-1β to assess the anti-inflammatory effects of myricetin. SS2 infection noticeably stimulated the expression of TNF-α and IL-1β in infected J774 cells. However, treatment with myricetin significantly decreased the levels of TNF-α and IL-1βin cells infected with SS2 (Fig. 2).

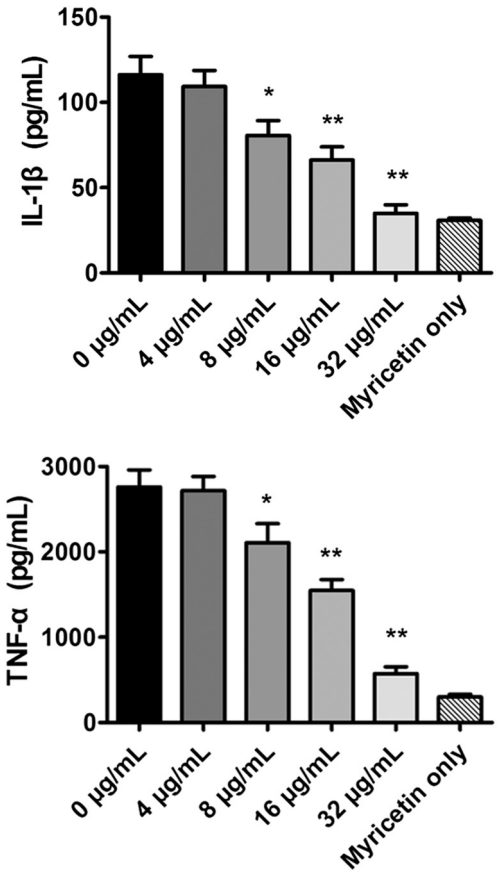

Fig. 2. Inhibitory effect of myricetin on the SS2-induced production of inflammatory cytokines. Mouse J774 macrophage-like cells were infected with SS2 at an MOI of 8 in the presence of various concentrations of myricetin (0, 4, 8, 16, and 32 μg/mL) for 5 h and the cells only treated with myricetin were as control. Cell supernatants were collected by centrifugation, and the production of IL-1βand TNF-α in the supernatants of the infected cells was detected by an ELISA. *indicates $P < 0.05$, and ** indicates $P < 0.01$compared with the myricetin-free sample (two-tailed Student's t-test).

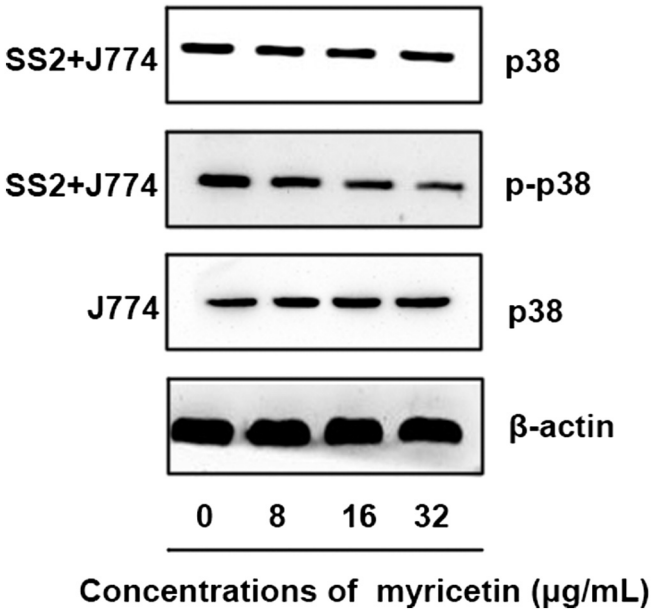

Fig. 3. Inhibitory effect of myricetin on the P38 pathways in SS2-induced inflammation. Mouse J774 macrophage-like cells were only treated with myricetin or infected with SS2 at an MOI of 8 in the presence of various concentrations of myricetin (0, 4, 8, 16, and 32 μg/mL) for 5 h. Total protein was extracted from lysed cells, and protein samples were analyzed by western blotting.

3.3. Suppression of inflammation by myricetin is associated with attenuation of P38 activation

P38 pathway is an inflammation-related factor that is important in the regulation of inflammatory mediators. To identify the mechanism by which myricetin attenuates inflammation, we assessed the expression of P38 in J774 cells infected with SS2 and treated with various concentrations of myricetin (0, 8, 16, and 32 μg/mL) by western blotting. The treatment of SS2-infected J774 cells with myricetin did not influence the expression of p38, but the activation of p38 was downregulated in cells infected with SS2 (Fig. 3). In addition, myricetin treatment did not influence the expression of p38 (Fig. 3) and the activation of p38 in cells only treated with myricetin was not observed. Previous study has shown SLY was associated with the activation of p38 pathway [26]. Thus, our results suggested the inhibition of p38 pathway by myricetin might be partly achieved by reducing SLY activity. Although our data do not support a direct cause-effect relationship between inhibition of P38 activation by myricetin and its anti-inflammatory activity, our results demonstrate that the two phenomena are closely related.

3.4. Myricetin reduces cell damage induced by SS2

To further test the potential therapeutic effect of myricetin in vitro, we used a live/dead (green/red) assay to qualitatively detect the protective effect of myricetin on SS2-infected cells. After myricetin treatment, the number of dead cells (stained red) was significantly reduced, and the state of the cells was improved (Fig. 4A and B). Additionally, the drug concentrations used in this study were not toxic to the J774 cells (Fig. 4C). We also measured the release of LDH into the cell supernatants to quantitatively analyze the therapeutic effect of myricetin on infected cells. Consistent with the above results, only 15.13% and 8.13% of infected cells treated with 16 and 32 μg/mL myricetin died, a much lower cell death rate than that observed in the untreated group of 38.61% (Fig. 4D). These results showed that myricetin can effectively alleviate SS2-mediated cell injury.

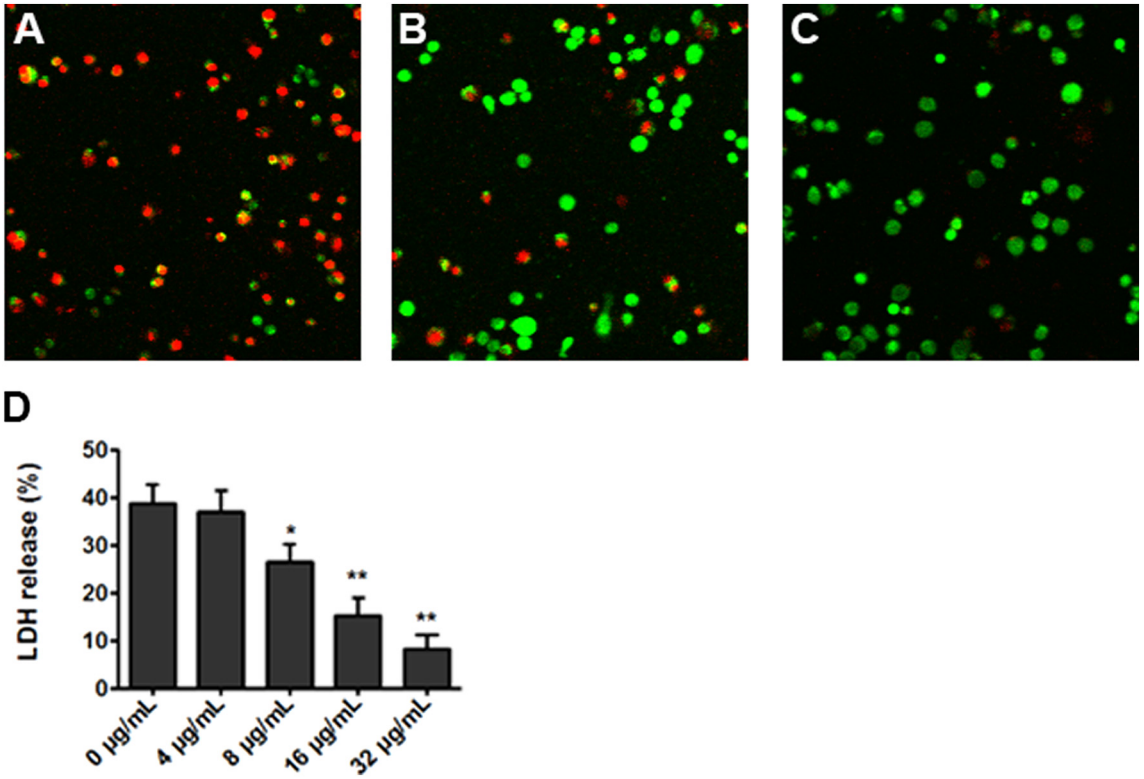

Fig. 4. The protective effect of myricetin against SS2 infection in vitro. The J774 macrophage-like cells infected with SS2 were treated with or without myricetin for 5 h. Next, the cells were stained with a live (green)/dead (red) reagent, and images were collected by confocal laser scanning microscopy. A, The cells infected with SS2. B, The cells infected with SS2 in the presence of 32 μg/mL myricetin. C, The cells treated with 32 μg/mL myricetin. D, The release of LDH into the supernatants of the coculture system with or without myricetin was detected by a cytotoxicity detection kit. *indicates $P < 0.05$, and ** indicates $P < 0.01$compared with the myricetin-free sample (two-tailed Student's t-test). (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

3.5. Myricetin reduces the bacterial burden in mice infected with SS2

A mouse model of SS2 infection was established to assess whether myricetin can exert its therapeutic effect in vivo. Following the treatment of mice with myricetin, the bacterial burden in the liver and spleen of each mouse was determined. As shown in Fig. 5, the bacterial colonization in the livers and spleens of the myricetin-treated mice was much lower than that observed in the control mice without myricetin. Taken together, our results indicated that myricetin can attenuate SS2 virulence in vivo.

4. Discussion

The drug resistance of *S. suis* has become increasingly problematic, and *S. suis* resistance against multiple antibiotics, such as gentamycin, penicillin, tetracyclines and macrolides, has been reported worldwide

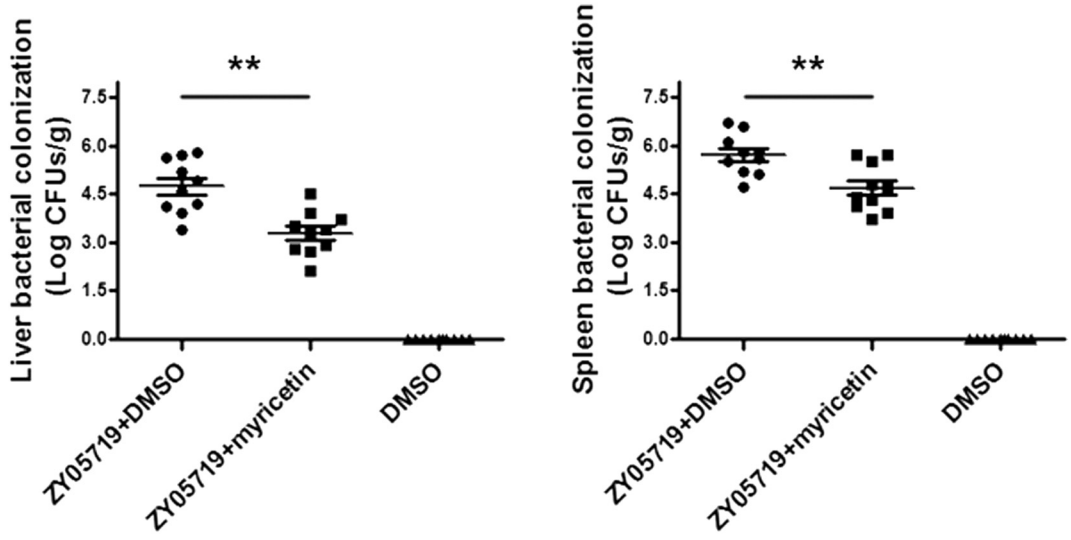

Fig. 5. The inhibition of the bacterial burden in mice by myricetin. Mice were intraperitoneally injected with 2×10^7 CFU of SS2 and treated with myricetin or DMSO 2 h after infection and then at 8-h intervals. All the mice were killed 72 h after infection; the organs (liver and spleen) were homogenized, and the number of colonies was calculated after serial dilution. *indicates $P < 0.05$, and ** indicates $P < 0.01$compared with the myricetin-free sample (two-tailed Student's t-test).

[27]. These findings indicate that the identification of new alternative treatment strategies or drugs is urgently needed. However, the emergence and development of anti-virulence strategies might be a promising and effective means for facing the challenge posed by worldwide bacterial resistance against drugs [28]. The core of this strategy involves prevention of the virulence factors of bacteria from interfering with bacterial infections without affecting bacterial growth to significantly reduce the selection pressure placed upon bacteria [29], which would potentially slow the emergence of drug resistance and prolong the life expectancy of antibiotics.

SLY plays an important role in the promotion of *S. suis* infection [13]. Thus, the inhibition of SLY might be a potential treatment for *S. suis* infection that does not prevent bacterial growth. In addition, *S. suis* infection can induce a series of serious inflammatory reactions, which are important aspects of the disease [1]. Therefore, reducing inflammation is important for the treatment of diseases related to *S. suis* infection.

A recent study showed that the natural compound myricetin can directly bind to SLY to suppress its hemolytic activity. Molecular simulations have predicted that myricetin binds to gaps between domains 2 and 3 of SLY, which play key roles in the binding process [19]. These results provide a preliminary theoretical basis for the possible effects of myricetin on *S. suis* infection. In this study, we further investigated the effect of myricetin on SS2 infection in vitro and in vivo. Consistent with previous studies, myricetin significantly inhibited the hemolytic activity of SLY, and we confirmed that myricetin can inhibit the oligomerization of SLY. Furthermore, we demonstrated that myricetin does not influence bacterial growth, which suggested that the survival pressure of the drug on SS2 is not conducive to the emergence of bacterial resistance. We subsequently assessed the efficacy of myricetin against SS2 infections and observed that myricetin exerted significant anti-inflammatory effects, which indicated that myricetin can affect SS2-induced inflammation in addition to its inhibition of SLY. Moreover, myricetin exerted an obvious protective effect against SS2 infection in vitro and in vivo. Our findings further elucidated the protective role of myricetin in SS2 infections and provide theoretical support for the use of this molecule as a lead compound for the development of novel clinical applications for the treatment of SS2 infection. It has also been demonstrated that the natural compound myricetin attenuates the lipopolysaccharide-stimulated activation of mouse bone marrow-derived dendritic cells through suppression of the IKK/NF-κB and MAPK signaling pathways, which suggests that myricetin might be a potential candidate for a wide range of applications [30].

Myricetin can be widely extracted from many plants and obtained inexpensively [31]. Molecular simulations previously revealed the mechanism through which myricetin interacts with SLY and validated its potential binding sites, and these findings might provide a basis for future modifications of the chemical structure of myricetin to enhance its ability to bind to SLY and thereby further improve its efficacy. Our research showed that myricetin has no antimicrobial activity, and thus, the bacteria remains in the host body after inhibiting SLY hemolysin activity. Methods for the removal of these pathogens or for helping the body more effectively eliminate these pathogens remain to be developed. In addition, whether myricetin combined with antibiotics can improve the therapeutic effect or slow the emergence of bacterial resistance is the topic of our next study. Our research does not fully elucidate the effect of myricetin on hosts of major natural infections, such as pigs, and this topic will be addressed in our next study. In addition, the identification of suitable doses of myricetin, specifically the doses that should be used to increase its absorption and those that would allow its passage through the blood-brain barrier to enhance its dual anti-SLY and anti-inflammatory effects, should be the focus of further clinical research. Moreover, a recent study found that anther natural compound fisetin could also inhibit the hemolytic activity of suilysin [32], but whether these two drugs utilize the same mechanism or whether the combination of the two drugs could yield a better therapeutic effect would be an interesting question to address in future research.

Author contributions

J.F.W., L.L. and G.L. conceived and designed the experiments. G.L., G.Z.W, X.S.S., X.K.Z. and W.T.L. performed the experiments. J.F.W. and L.L. contributed reagents/materials/analysis tools. J.F.W. and G.L. wrote the paper.

Conflict of interest

The authors have declared that no conflicts of interests exist.

Acknowledgments

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