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# Curcumin reduces the pathogenicity of *Streptococcus suis* serotype 2 in mice by targeting suilysin

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**Abstract**: **Background**: *Streptococcus suis* (*S. suis*) is a significant zoonotic pathogen, with serotype 2 (SS2) being the most prevalent. Suilysin (SLY), an essential toxin indicator for *S. suis*, is crucial in the infections caused by SS2. Consequently, an anti-virulence strategy targeting SLY presents a promising approach to combat SS2. **Objectives**: To investigate the effect of curcumin, a naturally occurring phenolic compound, on the hemolytic activity of SLY and the pathogenicity of SS2, and to assess its viability as a novel anti-virulence candidate for addressing SS2 infections. **Methods**: The antibacterial activity of curcumin against SS2 was assessed by determining its minimal inhibitory concentration (MIC) and monitoring bacterial growth curves. Its impact on hemolytic activity was tested using supernatants from SS2 cultures and purified recombinant SLY protein. Western blot was used to determine if curcumin affected the secretion level of SLY. The interaction between curcumin and SLY was predicted using molecular docking. Finally, the protective efficacy of curcumin was evaluated in a murine model of lethal SS2 infection. **Results**: Curcumin (<1,024 μg/mL) did not inhibit SS2 growth or viability. However, it significantly and dose-dependently inhibited the hemolytic activity of both SS2 culture supernatants and purified SLY. Molecular docking predictions indicated that curcumin engaged three domains of SLY (D1, D2 and D3) simultaneously, forming five hydrogen bonds with residues ASN-50, GLN-107 and LYS-190, thereby supporting its multidomain-binding capability. Furthermore, curcumin administration significantly reduced the mortality of SS2-infected mice *in vivo*. **Conclusion**: Substantial evidence is presented demonstrating that the pathogenicity of SS2 can be effectively attenuated by curcumin via inhibition of the hemolytic activity of SLY, which supports the potential utility of curcumin as a host-directed anti-virulence agent for SS2 infections.

**Keywords**: Anti-virulence; Curcumin; *Streptococcus suis* serotype 2; Suilysin

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## INTRODUCTION

*Streptococcus suis* (*S. suis*) is a Gram-positive bacterium found globally, leading to major economic losses in the pork industry and causing various diseases such as meningitis, septicaemia and arthritis in humans and pigs (Breitfelder *et al*., 2025; Guo *et al.*, 2021; Meng *et al.*, 2023). Based on capsular antigen differences, *S. suis* is categorized into 35 serotypes (1-34 and 1/2), with serotype 2 (SS2) being the most virulent and widespread (Dong *et al.*, 2023; Eiamphungporn *et al.*, 2025). Currently, the issue of drug resistance in *S. suis* is exacerbated by the misuse of antibiotics such as penicillin, amoxicillin, ampicillin, etc., so it is imperative to explore safer and more effective strategies to combat infection caused by *S. suis* (Lunha *et al.*, 2022).

Studies have shown that during infection, *S. suis* can secrete a variety of virulence factors that, while not essential for the pathogen's growth, are closely associated with its pathogenicity (Xie *et al.*, 2022). Suilysin (SLY), considered the toxin marker for *S. suis*, is the sole factor capable of activating and aggregating platelets (Li *et al.*, 2023). It has been reported that this factor can lyse erythrocytes to release hemoglobin, thereby promoting the host's inflammatory responses and facilitating bacterial infection (Fu *et al*., 2024). SLY belongs to the cholesterol-dependent cytolysin (CDC) family, divided into four structural domains (D1-D4). D4 of SLY can be the first to specifically recognize and bind the caholesterol on the cell membrane when *S. suis* invades the host. SLY monomers bound to cholesterol undergo oligomerization into a pre-pore through a series of conformational changes. Concurrently, the collapse of D2 facilitates the interaction

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between D1 and D4 of neighboring monomers, leading to the tilting and expansion of the pre-pore. Afterward, the two β-hairpins (TMH1 and TMH2) of D3 are embedded into the cell membrane, forming a β-barrel transmembrane structure that results in hydrophilic pores in the membrane, ultimately leading to cell lysis and death (Xu *et al.*, 2010). Previously, the researchers concluded that the strain of *S. suis* with a high level of SLY secretion was more virulent and invasive than the strain that secreted a low level of SLY or no SLY (Takeuchi *et al.*, 2014). Additionally, increasing the expression of the *SLY* gene at the transcriptional level can boost the virulence of *S. suis*. Compared with the wild strain, the SLY deletion strain was significantly less virulent and caused little or no mortality in infected mice (Gottschalk *et al.*, 2007; Lafrance *et al.*, 2015). Therefore, focusing on SLY in an anti-virulence strategy suggests a new avenue for *S. suis* infection treatment.

Curcumin is a naturally occurring phenolic compound primarily derived from turmeric (*Curcuma longa L.*). It exhibits a range of physiological effects, including anti-inflammatory, antioxidant, antitumor and antiviral properties (Liu *et al.*, 2024). Notably, curcumin has been reported to reduce the virulence of *Streptococcus pneumoniae* by effectively down-regulating the expression of NanA (Lu *et al.*, 2018). Additionally, it has been shown to inhibit the inflammatory effects induced by SS2 by significantly lowering the concentrations of pro-inflammatory cytokines (IL-6, TNF-α and IL-12) in splenocytes *in vitro*. These results indicated that curcumin could act as a possible antivirulent agent against SS2 (Wen *et al.*, 2014). However, whether curcumin can target the pivotal virulence factor SLY to exert its protective effect, independent of its anti-inflammatory properties, remains an open question. In the present study, it was discovered that curcumin directly interacted with SLY by engaging its three structural domains simultaneously, and this binding compromised the hemolytic function of SLY. Consequently, the pathogenicity of SS2 was significantly attenuated by curcumin in a murine in vivo model. Notably, this protective effect was achieved in the absence of direct antibacterial activity or a reduction in SLY secretion. Curcumin is thus positioned as a promising anti-virulence lead compound for combating *S. suis* infections.

## MATERIALS AND METHODS

### *Culture conditions of bacterial strain and preparation of curcumin*

The SS2 strain SC21 used in this experiment was isolated from infected sows in a pig farm in Sichuan province and was identified by the Center for Animal Experiment of Sichuan Academy of Chinese Medicine Sciences. The strain was cultured in fresh Tryptone Soya Broth (TSB; Beijing Noble Ryder Technology Co. Ltd., Beijing, China) with 5% fetal bovine serum (FBS; Thermo Scientific, New York, USA) at 37°C for 10 h with shaking and stored at 4°C.

Curcumin (purity >99%), purchased from Chengdu Push Bio-Technology Co. Ltd. (Chengdu, China), was dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions of 20,480 μg/mL for *in vitro* assays and 400 mg/mL for *in-vivo* administration, which were stored at -20°C. Work solutions for both applications were prepared by diluting the respective stock solutions in phosphate-buffered saline (PBS). Vehicle-matched DMSO controls at the highest percentage used across all curcumin doses were incorporated throughout the study.

### *Evaluating the minimal inhibitory concentration (MIC) for curcumin*

The MIC of curcumin for SS2 was assessed using a serial dilution method following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (Gu *et al.*, 2020). The final concentration of SS2 was adjusted to $5\times10^5$ CFU/mL via adding different doses of curcumin (1-2,048 μg/mL) into the bacterial cultures. After incubating the mixture at 37°C for 24 h, the curcumin concentration was considered the MIC when no bacterial growth was visible. The positive control (bacteria culture without curcumin), the negative control (TSB with 5% FBS) and the vehicle control (as described above) were included in parallel.

### *Assay of growth curves for SS2*

SS2 was cultured in TSB with 5% FBS until the $OD_{600}$ reached 0.3. This standardized inoculum was then aliquoted and different doses of curcumin (0, 2, 4, 8, 16, 32 and 64 μg/mL) were separately added to the bacterial cultures in aliquots. The mixture was incubated for 12 h at 37°C with shaking at 180 rpm and the $OD_{600}$ was measured at 1 h intervals.

### *Hemolysis assay of culture supernatant*

Curcumin was added in concentrations of 0, 2, 4, 8, 16 and 32 μg/mL to SS2 cultures with an $OD_{600}$ of 0.3 and the cultures were grown to the stationary phase ($OD_{600}$ = 2.5). Following a 2-minute centrifugation at 12,000 rpm and 4°C, the culture supernatants were transferred to the new tubes. 25 μL of defibrillated chicken erythrocytes (4%) (Guangzhou Hongquan Bio-tel, Guangzhou, China), 100 μL of cultural supernatant and 875 μL of PBS were gently mixed and incubated for 1 h at 37°C. Then, the mixture was centrifuged (3,000 rpm, 5 min) to determine the $OD_{543}$ of the supernatant. The control groups included 4% chicken erythrocytes in 1% Triton X-100 (positive control), in PBS (negative control) and in the highest DMSO concentration from the curcumin series (vehicle control, as specified above).

### *Western blotting*

A Western blot assay was used to determine the expression level of SLY in the co-culture system. Equal amounts of purified GFP protein, as an internal control (Zhan *et al.*, 2022), were added to the supernatants, which were from the co-culture system of bacterial culture with curcumin (0,

2, 4, 8, 16 and 32 μg/mL). After separation by 15% SDS-PAGE, the protein samples in the supernatant were transferred to nitrocellulose membranes, then incubated with mouse-derived antibody against SLY (1:1000 v/v dilution) and mouse-derived antibody against GFP (1:2000 v/v dilution) overnight at 4°C. The sections were washed four times with PBS and then incubated with HRP-conjugated sheep anti-mouse IgG (diluted 1:2000 v/v in 0.1% Evan's Blue) in the dark at 37°C for 2 h and the membranes were visualized using a fluorescence microscope (Nikon 55i, Tokyo, Japan).

***Molecular docking***

The 3D structure of SLY (Code: 3HVN) was downloaded from the X-ray crystal structure in the Protein Data Bank (PDB) (Protein Data Bank, 2010). 2D and 3D structures of curcumin (Compound CID: 969516) were taken from PubChem (PubChem, 2004). Molecular docking was performed using AutoDock Vina 1.2.4 software. The docking site for SLY was defined as a cubic structure centered at x = 25.505, y = 20.965 and z = 40.672 with a radius of 52 Å (1 Å = 0.1 nm) and docked against the curcumin molecule. Each docking run was repeated 100 times. During the molecular docking, all torsional bonds of curcumin were set to free rotation and the structure of SLY was set as rigid. A 2D analysis of the protein-ligand interaction was generated using Ligplot+ software.

***Expression and purification of SLY protein and hemolysis assay of SLY***

The sequence of SLY (GenBank: AY341263.1), whose signal peptide and stop codon had been removed, was amplified using the primers 5'-GGATCCGATTCCAAAC AAGATATTAATCA-3' (including a *Bam*H site) and 5'-AAGCTTTACTCTATCACCTCATCCGCAT-3' (including a *Hind* III site). The fragment that was amplified was ligated into the pCold-I plasmid (Novagen, Madison, USA) and then transformed into *Escherichia coli* (*E. coli*) BL21 (DE3) cells (Takara, Shiga, Japan). Subsequently, the expression of protein was detected by inducing the transformant with 1 mM IPTG for 8 h. $Ni^{2+}$ affinity chromatography (Bio-Rad, Hercules, CA, USA) was employed to harvest and purify the recombinant protein. The SLY protein's expression and purification were evaluated via 15% SDS-PAGE and its concentration was quantified using a BCA protein assay kit (Thermo Fisher, Shanghai, China).

The hemolytic activity of SLY was assessed following a 30-minute pre-incubation at 37°C of 50 μL purified SLY (1 μg/μL) with 50 μL of curcumin (0, 2, 4, 8, 16 and 32 μg/mL). The reaction mixture was then supplemented with 25 μL of defibrillated chicken erythrocytes (4%) and brought to a final volume of 1 mL with PBS, followed by a 10-minute incubation at 37°C. Other specific experimental operations were performed as described above.

***Mice infection test in-vivo***

A total of 30 eight-week-old female C57 BL/6J mice (weighing approximately 20 g each) from Vital River Laboratories in Chengdu, China, were randomly divided into three groups (n=10). Mice were inoculated intraperitoneally with $2.8 \times 10^{10}$ CFU of SS2 strain SC21 in 500 μL PBS, a dose equivalent to $5 \times LD_{50}$ established in the preliminary studies. An equal volume of PBS was injected into the mice that served as a control. Two hours after the injection, mice were subcutaneously administered 200 μL of either curcumin (200 mg/kg; Zhou *et al.*, 2017) or the vehicle control at a final DMSO concentration of 5% (v/v), as prepared above, with doses repeated every 8h. The survival of mice was monitored at 12h intervals for 72h.

***Statistical analysis***

All experiments were conducted in triplicate and statistical analyses and calculations were executed using GraphPad Prism (version 8.0). Data are presented as mean ± standard deviation (SD). For comparison between groups, unpaired Student's *t*-tests and one-way analysis of variance (ANOVA) were performed using SPSS software (version 22.0). Significance levels of $P<0.05$ and $P<0.01$ were indicated in the figures.

## RESULTS

***Effect of curcumin on the growth of SS2***

A key contributor to antibiotic resistance is the selective pressure from bactericidal agents. In this study, curcumin demonstrated no such activity against SS2, with an MIC >1,024 μg/mL and no impact on growth at sub-inhibitory concentrations (0-64 μg/mL). Given its lack of growth inhibition, curcumin is unlikely to drive the development of antibiotic resistance, thereby presenting a significant therapeutic advantage (Fig. 1).

***Inhibitory effect of curcumin on the hemolytic activity of culture supernatant***

As shown in fig. 2, approximately 82.67% hemoglobin was released when chicken erythrocytes were infected with culture supernatant without curcumin. With the increase of drug concentration, the hemoglobin release showed a gradual decrease. At a drug concentration of 32 μg/mL, the hemolytic activity in the supernatant dropped to 20.66%. The results suggested that curcumin could significantly inhibit SS2's hemolytic activity, which was negatively correlated with the concentration of curcumin.

***Effect of curcumin on the expression of SLY in bacterial supernatant***

There was no notable change in the expression level of secreted SLY protein in the bacterial supernatant after exposure to different curcumin concentrations. The exogenous addition of GFP was detected as an internal control using an anti-GFP antibody (Fig. 3). These results demonstrated that curcumin-mediated inhibition of the hemolytic capacity of SS2 culture supernatant didn't result from impaired secretion of SLY.

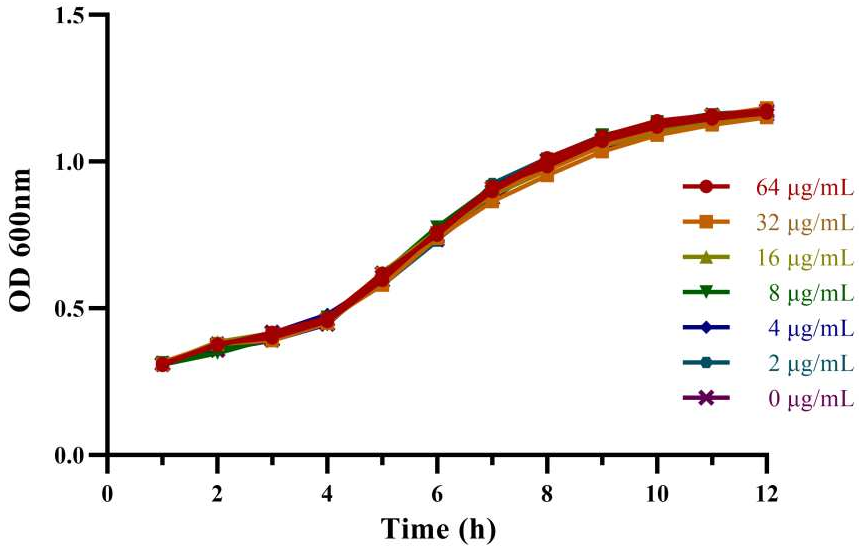


**Fig. 1**: Growth curves of SS2 treated with different concentrations of curcumin.

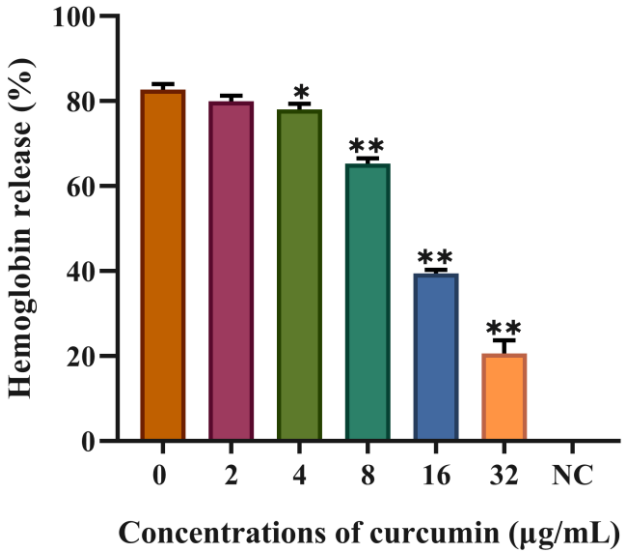


**Fig. 2**: Hemolytic activity in the supernatant of the curcumin and SS2 co-culture system. NC, negative control. * means $P<0.05$ and ** means $P<0.01$ compared with the curcumin-free group. The same as below.

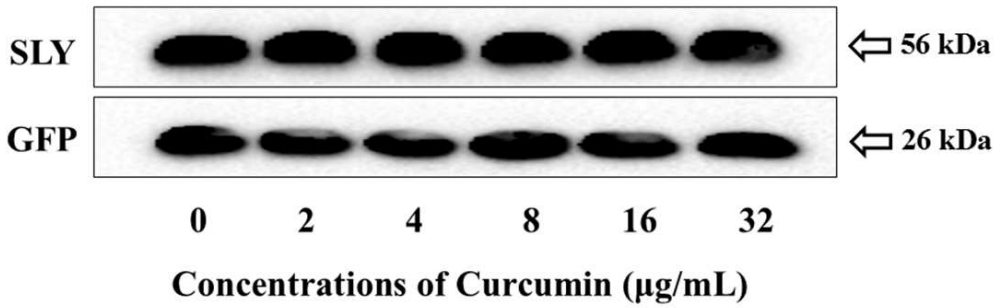


**Fig. 3**: Expression of secreted SLY in the supernatant of the curcumin and SS2 co-culture system. GFP was detected as an internal control.

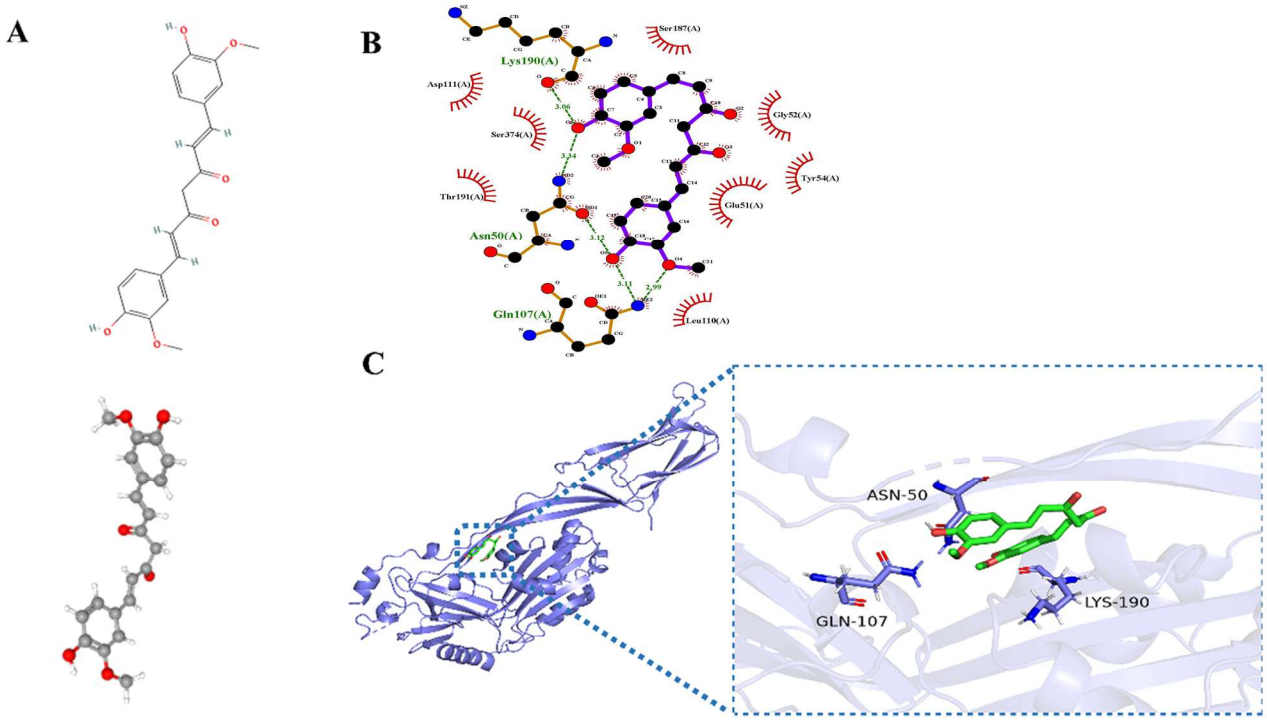


**Fig. 4**: The molecular docking conformation of curcumin with SLY. (A) 2D and 3D chemical structure of curcumin; (B) 2D analysis diagram of curcumin-SLY interactions; (C) 3D structure of curcumin-SLY complex and details of binding between curcumin and the residues of SLY.

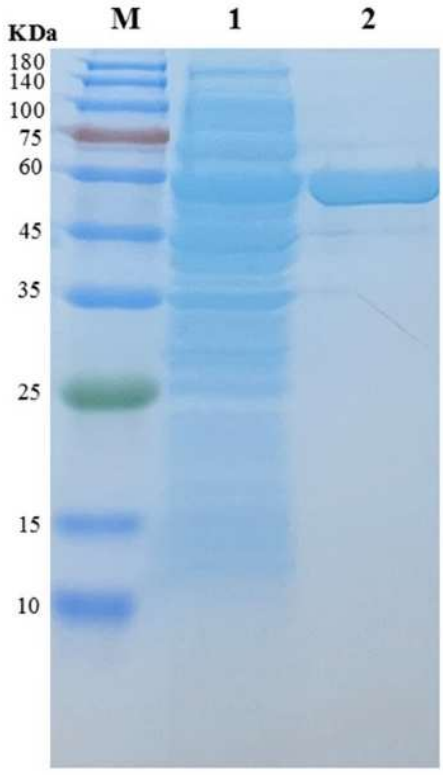


**Fig. 5**: Expression and purification of recombinant SLY**.** M, protein molecular weight marker; 1, lysate of IPTG-induced *E. coli* BL21 expressing recombinant SLY; 2, purified recombinant SLY

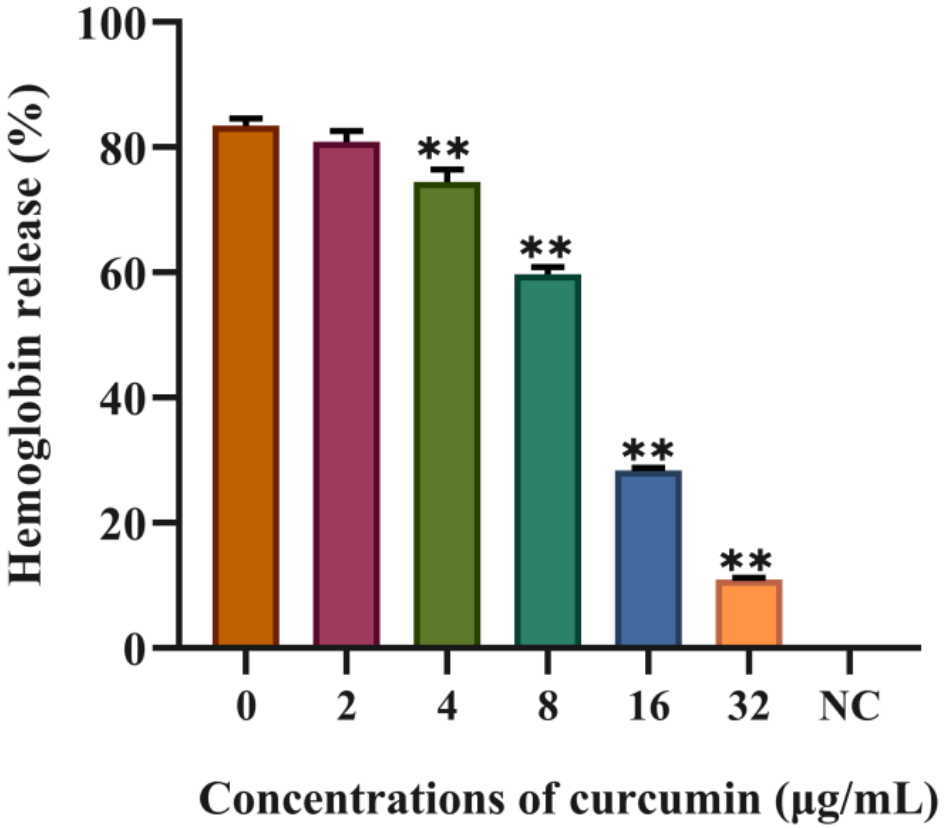


**Fig. 6**: The effect of curcumin on the hemolytic activity of purified SLY

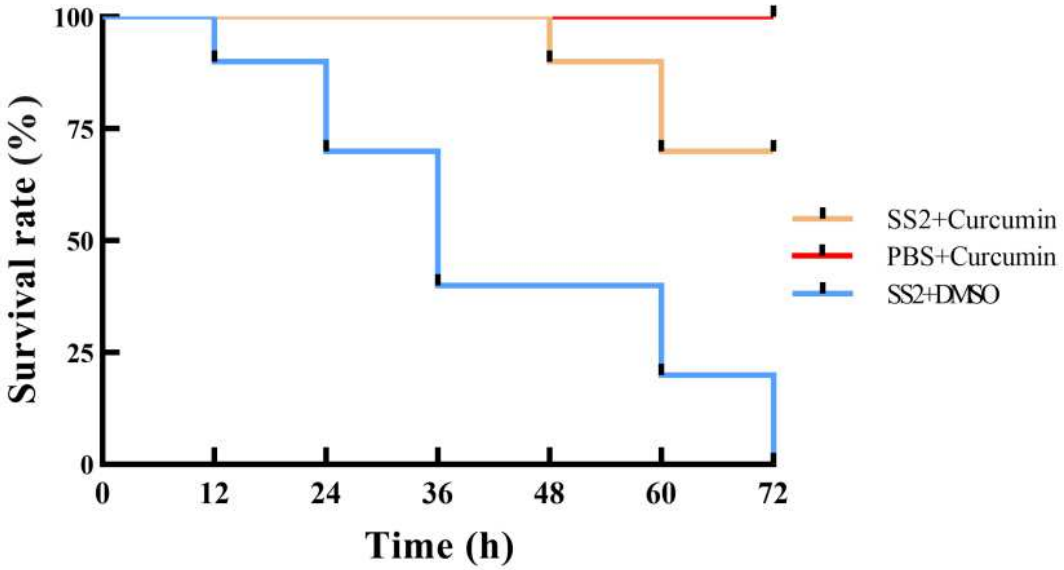


**Fig. 7**: The effect of curcumin on the survival rate of SS2-infected mice

***Prediction of the binding sites between curcumin and SLY***

As predicted by molecular docking, curcumin bound to three domains (D1, D2 and D3) of SLY. And there were five strong hydrogen bonds between curcumin and three amino acid residues (ASN-50, GLN-107 and LYS-190) of protein. The compact binding conformation, evidenced by a favorable binding energy of -6.0 kcal/mol, led us to suspect that this multi-domain interaction triggered conformational changes in SLY (Fig. 4).

***Expression and purification of SLY***

In *E. coli* BL21 (DE3), the recombinant SLY protein, featuring a 6 His-tag at its N-terminus, was successfully expressed. After purification, the molecular mass of SLY, yielding a single band, was approximately 50 kDa, which

was close to the expected value (Fig. 5). And it was found that the concentration of purified SLY is 1 μg/μL.

***Inhibitory effect of curcumin on the hemolytic activity of SLY***

The findings from the hemolytic activity assay of SS2 culture supernatant and Western blotting analysis lead us to hypothesize that curcumin may directly inhibit the hemolytic activity of SLY. After different concentrations of curcumin were incubated with purified SLY, the SLY-mediated hemolytic activity gradually decreased with the increase of the concentration of curcumin in a dose-dependent manner (Fig. 6), which was consistent with the result of curcumin on the hemolytic ability of culture supernatant. Taken together, the results indicated that curcumin directly inhibited the hemolytic activity of SLY.

***Effect of curcumin on pathogenicity of SS2 in-vivo***

To further investigate whether curcumin had the efficacy to reduce the toxicity of SS2, the model of SS2-infected mice was constructed in this study. As shown in fig. 7, all infected mice not inoculated with curcumin died within 72 h. However, the mortality of SS2-infected mice that received curcumin was 30%, significantly lower than that of curcumin-untreated mice. In addition, the time of death was delayed in curcumin-treated mice. It should be revealed that curcumin usefully reduced the pathogenicity of SS2 in mice.

## DISCUSSION

Currently, *S. suis* is treated clinically with antibiotics that target essential components of bacterial survival, such as DNA, proteins and cell membranes, to achieve a bactericidal effect. While this method effectively eliminates pathogenic bacteria, the overuse of antibiotics can lead to increased bacterial resistance due to prolonged survival pressure (Gao *et al.*, 2025; Han *et al.*, 2023). It has been demonstrated that virulence factors, which play a significant role in bacterial pathogenicity, generally do not pose a threat to the survivability of pathogens. Consequently, the anti-virulence strategy, which effectively reduces bacterial pathogenicity, exerts less selective pressure on bacteria, thereby minimizing the development of resistance (Xie *et al.*, 2022). SLY, a crucial virulence-associated factor of SS2, mitigates the adverse effects of the pathogen by reducing its hemolytic capacity, making it a prime candidate for combating SS2-related infections without impacting bacterial growth.

Clinical studies have demonstrated that herbal medicine and its compounds are generally safer than antibiotics and possess significant therapeutic effects against bacterial infectious diseases (Zhan *et al.*, 2022). In this study, it was discovered that the monomeric compound curcumin effectively suppressed the hemolytic activity of SS2 without affecting bacterial proliferation. As previously described, the hemolytic activity of *S. suis* is primarily mediated by SLY. Thus, the reduced hemolytic ability of SS2 may result from either decreased secretion of SLY or inactivation of SLY through direct binding of curcumin to the protein. However, the findings indicated that curcumin had a minimal effect on SLY expression in the supernatant of SS2, as determined by Western blot assays. Consequently, molecular docking methods were employed to investigate the structural interaction between curcumin and SLY. Molecular docking, a cutting-edge tool in medical research, has the potential to facilitate breakthroughs in new drug development (Pinzi and Rastelli, 2019). Several herbal monomers have been identified as targeting SLY to inhibit the hemolytic activity of SS2. Molecular docking analysis has confirmed that verbascoside localizes to D4 of SLY (Zhan *et al.*, 2022), myricetin is situated in the gap between D2 and D3 of SLY (Li *et al.*, 2019) and morin binds to D2 (Li *et al.*, 2017). Both piceatannol (Wang *et al.*, 2020a) and formononetin (Wang *et al.*, 2020b) are found at the junction of D2 and D4 of the protein. Although these compounds occupy different binding sites on SLY, they all inhibit the hemolytic activity of SLY and protect host cells from the pathogenic effects of SS2. This suggests that the bioactive function of SLY is closely linked to its spatial configuration. In contrast to the SLY binding regions identified in prior research, this study is the first to demonstrate that curcumin simultaneously binds to three distinct domains (D1, D2 and D3) of SLY. This finding suggested a novel mechanism of action for curcumin, characterized by multidomain engagement, which provided a more extensive binding spectrum compared to previously reported SLY inhibitors. It was therefore hypothesized that the high structural affinity of curcumin for SLY resulted in a conformational change that disrupted the hemolytic capability of the protein. *In-vitro* cellular assays further confirmed that curcumin directly inhibited SLY-mediated hemolytic activity.

Furthermore, *in-vivo* study demonstrated that the mortality rate of infected mice treated with curcumin was lower than that of mice not treated with curcumin, indicating that curcumin could effectively lessen the pathogenicity of SS2. Except for SLY, SS2 can also produce a number of virulence factors associated with its pathogenicity, including peptidoglycan, capsular polysaccharide (CPS), muramidase-released protein (MRP) and enolase (ENO), etc., all of which contribute to the bacterium's ability to invade hosts (Pian and Wang, 2025). Hence, it was inferred that curcumin was capable of inhibiting the hemolytic activity of SLY, but other virulence factors may still be attacking the host, which could explain why curcumin was unable to completely eliminate SS2 pathogenicity in mice. Consequently, future research should focus on the development of combination drugs targeting multiple virulence factors of SS2. In addition, the dosage of curcumin will also be further optimized.

Currently, the CDC family is known to comprise more than 40 members, exhibiting a high pairwise sequence identity (40%–70%) among them, including SLY, pneumolysin (PLY), listeriolysin O (LLO), perfringolysin O (PFO), streptolysin O (SLO) and so on. Additionally, these members share highly similar spatial structures and functional mechanisms, i.e., forming holes in the target cell membrane of the host to disrupt its integrity (Pramitasuri *et al.*, 2023). It has been reported that curcumin interacts with amino acid residues (VAL100 and LEU503) of LLO, altering its spatial conformation to inhibit its hemolytic activity and suppress the virulence of *Listeria monocytogenes* (*L. monocytogenes*) in mice (Zhou *et al.*, 2017). In conjunction with the present study, it was speculated that curcumin may serve as a broad-spectrum natural compound against the pathogenicity of all CDC members, a hypothesis that is intended to be further investigated.

## CONCLUSION

To sum up, curcumin did not alter the growth of SS2; however, it bound directly to SLY, significantly suppressing its hemolytic activity. Additionally, curcumin significantly reduced the pathogenicity of SS2 *in-vivo* in mice. This study demonstrates that curcumin is a reliable inhibitor of SS2 infection by targeting SLY, providing a theoretical basis for the further development of new drugs aimed at preventing and treating *S. suis* infection.

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***Authors' contributions***
Jiafei Zhan: Responsible for the conception of the study and drafting of the manuscript; Jieying Xia: Participated in the study design and coordination of research activities; Jiaxin Ma: Carried out mouse-related experiments; Yung-Fu Chang: Provided technical guidance and refined the manuscript; Han Dong, Tingting Cheng, Ziyi Xu, Yang Hong and Guoqiang Cheng: Conducted basic experimental work; Ning Wang: Performed statistical analyses; Tiezhu Chen: Supervised the overall experimental process; Kui Xu: Provided project funding support and oversaw project management. All authors read and approved the final manuscript.

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***Data availability statement***
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

***Ethical approval***
The animal experiments were conducted in accordance with the guidelines approved by the Animal Care Advisory Committee of the Institute of Laboratory Animals, Sichuan Academy of Medical Sciences (Approval No. DWSYLL-2024-184). All animal procedures used in this study were strictly carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, Bethesda, MD, USA) and recommendations of the ARRIVE guidelines (Percie du Sert *et al.*, 2020). This study was performed in adherence with the ARRIVE guidelines. See supplementary file for the ARRIVE checklist.

***Conflict of interest***
The authors declare no conflict of interest.

***Supplementary data***
https://www.pjps.pk/uploads/2026/05/SUP1779344302.pdf

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