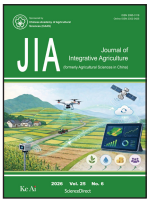
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RESEARCH ARTICLE

Targeting ThyA: Investigating the mechanisms of 5-FU-induced inhibition of biofilm formation and virulence in *Streptococcus suis* through LuxS/AI-2 quorum sensing

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Highlights

- 5-fluorouracil (5-FU) reduced biofilm formation and virulence of *Streptococcus suis* through LuxS/AI-2 quorum sensing system.
- 5-FU may regulate the LuxS/AI-2 quorum sensing system by influencing *thyA*.
- 5-FU can alleviate *S. suis* infection in the mouse infection model.

Abstract

Streptococcus suis is a significant zoonotic agent affecting both human and pig health and poses a substantial public health concern. The pathogenicity of *S. suis* is intricately linked to its ability to form biofilms and express virulence factors, which are regulated by the LuxS/AI-2 quorum sensing (QS) system. Herein, we uncover a novel therapeutic avenue by demonstrating that 5-fluorouracil (5-FU), an FDA-approved anti-cancer agent, effectively mitigates biofilm formation and attenuates the virulence of *S. suis*. Mechanistically, we observe a significant reduction in capsular polysaccharide and extracellular polysaccharide production upon 5-FU treatment, elucidating a potential mechanism for biofilm weakening. Additionally, 5-FU down-regulates virulence traits, diminishing *S. suis*'s ability to adhere to host cells and evade phagocytosis. Crucially, our study identifies the thymidylate synthase regulatory gene *thyA* as a key mediator of 5-FU's effects on the LuxS/AI-2 QS system. Virtual molecular docking and gene knockout experiments provide compelling evidence that 5-FU modulates the LuxS/AI-2 QS system by targeting *thyA*. *In vivo* experiments further validate the therapeutic potential of 5-FU, showcasing a significant reduction in bacterial load and mitigation of tissue damage in a mouse model. In conclusion, our investigation unveils 5-FU as a potent disruptor of *S. suis*'s biofilm formation and virulence, offering a promising avenue for the control of this devastating pathogen.

Keywords: *Streptococcus suis*, 5-fluorouracil, LuxS/AI-2 quorum sensing system, anti-biofilm, *thyA*

1. Introduction

Streptococcus suis is an important zoonotic pathogen that can cause a wide range of diseases in both humans and pigs, which in severe cases can lead to death and can also cause significant economic losses to the pig industry (Gottschalk *et al.* 2010; Wang *et al.* 2022). As a zoonotic pathogen, *S. suis* is capable of causing various diseases in humans through direct contact with infected pigs or contaminated environments (Yongkiettrakul *et al.* 2019). Although most cases of *S. suis* usually occur among pig farmers, slaughterhouse workers and cooks who have direct or indirect contact with pigs (Dutkiewicz *et al.* 2017). In fact, in some countries, a considerable number of human cases have occurred in diets containing raw pork, blood and other related products. Biofilm formation represents a key virulence strategy employed by *S. suis*, contributing to its ability to persist and thrive in diverse environments (Kerdsin

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et al. 2011, 2018; Takeuchi *et al.* 2012; Huong *et al.* 2014). The intricate structure of the biofilm enhances the bacterium's ability to recover, making infections difficult to treat and leading to persistent infections (Wang *et al.* 2014).

The process of biofilm formation involves a variety of complex regulatory mechanisms, among which the quorum sensing (QS) system is the focus of current research (Han *et al.* 2017). The autoinducer-2 (AI-2) quorum sensing pathway encoded by the *luxS* gene, functions universally in both gram-positive and gram-negative bacteria (Zhao *et al.* 2018). AI-2 is a furanone-derived signaling molecule that mediates the regulation of gene expression and bacterial physiological behavior within or between species, and is the only non-specific QS signaling molecule identified to date and is present in more than 500 bacterial species. AI-2 involved QS systems have been found to play an important role in many biochemical processes of bacteria, for example, the LuxS/AI-2 QS system not only mediates the growth and development, biofilm formation and pathogenicity of *Haemophilus parahaeus* (Zhang *et al.* 2019) but also participates in the susceptibility of *S. suis* to fluoroquinolones by regulating the efflux pump *SatAB*. Many studies have reported that the gene of *luxS* influences the formation of biofilms, such as *Bifidobacteria* (Sun *et al.* 2014) and *Lactobacillus rhamnosus* (Lebeer *et al.* 2008). In addition, it has been reported that the exogenous addition of AI-2 signal molecule can promote biofilm production in *Pseudomonas aeruginosa* PA01 (Li *et al.* 2015), *Helicobacter pylori* (Anderson *et al.* 2015), *Staphylococcus epidermis* RP62A (Xue *et al.* 2015), etc. Another study found that AI-2 not only significantly promoted the ability of avian pathogenic *Escherichia coli* to form the biofilm, but also weakened the transcription level of virulence genes, fibrocyte adhesion and invasion ability. Previous research by our group has found that LuxS/AI-2 QS system plays an important role in coordinating various virulence factors including biofilm formation in *S. suis* (Wang *et al.* 2013).

At present, preventing infection by controlling biofilm formation or weakening bacterial virulence has become a new research hotspot. Intervention of QS system to inhibit biofilm formation, reduce virulence factor production and improve antimicrobial susceptibility are promising anti-infective treatments. In this context, we selected 5-fluorouracil (5-FU), a drug approved by the U.S. Food and Drug Administration (FDA) and licensed for cancer treatment in 1962, to explore its potential as an inhibitor of *S. suis* biofilm and the QS system. (Wilson *et al.* 2014). And numerous studies have since shown that 5-FU also shows effective anti-biofilm or anti-quorum sensing activity against a variety of Gram-positive and Gram-negative bacteria. It was found that 5-FU is an effective population quencher, which can inhibit the production and release of AI-2 by MRSA, *Staphylococcus epidermidis*, *E. coli* and *Vibrio harveyi*, and effectively reduce the adhesion of MRSA and block the formation of biofilms *in vitro* (Sedlmayer *et al.* 2021). Another study showed that 5-FU can inhibit the formation of *P. aeruginosa* biofilm, eliminate QS phenotypes, and reduce virulence (Garcia-Contreras *et al.* 2013). However, the regulation of 5-FU on LuxS/AI-2 QS system of *S. suis* has not been reported in detail.

Since 5-FU is an inhibitor of thymidylate synthase (encoded by the *thyA* gene) and since folate metabolism is involved in the remethylation of homocysteine to methionine, it is inevitable to investigate the effect of *thyA* on the LuxS/AI-2 QS system (Singh *et al.* 2006). Previous studies have shown that *cvfC* operons are capable of participating in the production, detergent resistance, and virulence of *Staphylococcus aureus* in host animals by expressing *thyA* (Ikuo *et al.* 2010). Another study revealed that inactivation of the thymidylate synthase *thyA* affects the morphology of nontypeable *Haemophilus influenzae* (NTHi), reduces its virulence, and indirectly affects resistance to TxS (Rodriguez-Arce *et al.* 2017). Similarly, Kriegeskorte *et al.* (2014) found that mutation of *thyA* results in the inactivation of the TS protein, causing significant impacts on the physiology and virulence of *S. aureus*. Furthermore, a mutant strain of *Salmonella enterica serovar Typhimurium* with a *thyA* mutation showed severely impaired growth and survival in macrophage-like cell line P338D1 and HEp-2, with reduced virulence in BALB/c mice (Kok *et al.* 2001). Therefore, exploring the impact of *thyA* on the regulation of biofilm formation and virulence by the LuxS/AI-2 QS system in *S. suis* is particularly important.

This study aims to elucidate the inhibitory properties of 5-FU on biofilm formation and pathogenicity in *S. suis* through an exploration of its role in the LuxS/AI-2 QS system. And the ultimate goal of the research is to provide novel insights into new potential therapeutic targets of *S. suis* and develop more effective anti-*S. suis* drug.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The wild-type strain employed in this study originated from a pig infected during the *S. suis* outbreak in Jiangsu Province, China in 1998, while the Δ*luxS* strain and Δ*thyA* strain were meticulously constructed and stored within our laboratory. All bacterial strains were cultured in Tryptic Soy Broth (TSB; Oxoid, Britain) or on Tryptic Soy Agar (TSA; Oxoid, Britain) plates at 37°C, harvested during the mid-exponential phase, and preserved for future use. 5-FU was procured from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China).

2.2. Bacterial growth curve

To evaluate the inhibitory effect of 5-FU on *S. suis*, the growth curves were plotted by determining the number of viable bacteria in the culture medium at different time points. *S. suis* (10^6 CFU mL^{-1}, 100 μL) at logarithmic growth stage were inoculated into 10 mL of sterile TSB medium and 5-FU at concentrations of 0, 2.5, 1.25 and 0.625 μg mL^{-1} were added to the bacterial suspension, respectively. Then the culture solution was incubated at 37°C, 180 rpm min^{-1} for 12 h. The colonies were counted according to the previous study, and 100 μL bacterial solution was uniformly coated on a TSA plate every 2 h. The culture solution was left to incubate at 37°C for 24 h, and the growth curves of the *S. suis* were plotted by the plate counting method.

2.3. Biofilm formation assay

The assessment of *S. suis* biofilm formation utilized the crystal violet semi-quantitative staining method outlined by Dhouib *et al.* (2021). The wild-type strain, Δ*luxS* strain, and Δ*thyA* strain were introduced into 96-well polystyrene microfiber plates containing 200 μL of TSB medium. Subsequently, 5-FU was added at final concentrations of 0, 2.5, 1.25, and 0.625 μg mL^{-1} for each strain, respectively. The above bacterial solutions were incubated at 37°C for 24 h. Following the removal of the culture medium and free-floating bacteria, a double wash was performed. Subsequently, 95% methanol was added for fixation, allowing for a 20-minute incubation period. The cell walls were then stained with 0.1% crystal violet for 15 min, followed by two gentle washes and drying. Finally, the biofilms were lysed in 95% ethanol for 30 min. The optical density (OD) values were determined at 595 nm using a full-spectrum microplate analyzer (Synergy2, Biotek, Vermont, NY, USA).

2.4. AI-2 activity assay

The *S. suis* wild-type strain, Δ*luxS* strain, or Δ*thyA* strain were diluted to 10^5 CFU mL^{-1} and different concentrations of 5-FU were added to the wild-type strain. The bacterial cultures were incubated at 37°C for 12 h and subsequently centrifuged at 12,000×g, 4°C for 10 min at various time points to collect supernatants. The supernatant from *E. coli* DH5α was utilized as a negative control. Then the supernatant was filtered using a membrane filter with a pore size of 0.22 μm. The activity of the culture supernatant was detected by using the reporter strain *V. harveyi* BB170. *V. harveyi* BB170 was incubated in AB medium at 28°C overnight, and 90 μL of diluted culture solution of *V. harveyi* BB170 was incubated with 10 μL of AI-2 supernatant at 28°C for 6 h in the dark. The bioluminescent value of the samples was measured by Promega Luminometer at 490 nm wavelength. The results are presented as the ratio of the luminescence value for each experimental group to the luminescence value of *E. coli* DH5α.

2.5. Confocal laser scanning microscopy (CLSM)

To assess the morphology of *S. suis* biofilm under 5-FU (0.625 μg mL^{-1}), CLSM was employed. The *S. suis* biofilm was cultured at 37°C for 24 h, and cell slices were positioned in a 6-well cell culture plate. The biofilm adhering to the cell slide was delicately cleansed with sterile phosphate buffer saline (PBS). Staining was executed using a backlit live/dead bacterial viability kit comprising Syto9 and propyl iodide (PI). Following staining, a wash with distilled water was performed, and the samples were dried at room temperature. Biofilm observations were made using confocal laser scanning microscopy (Zeiss LSM CLSM). The 488 nm laser induced fluorescence in live bacteria, emitting a green glow, whereas the 543 nm laser triggered red fluorescence in bacteria with compromised membranes.

2.6. Scanning electron microscope (SEM)

The effects of 5-FU on the morphology and structure of *S. suis* were analyzed by SEM (Rasool *et al.* 2016). The *S. suis* culture underwent treatment with varying concentrations of 5-FU (0 or 0.625 μg mL^{-1}) and was then incubated at 37°C for 12 h. Subsequently, all samples were subjected to centrifugation at 8,000×g for 10 min and underwent a thorough washing with PBS. The bacterial cells were immobilized using 2.5% glutaraldehyde for 2 h, followed by dehydration with gradient alcohols (20%, 30%, 50%, 60%, 70%, 80%, 90%, and 100%) at room temperature for 20 min. Finally, gold was sprayed on the surface of the bacterial cells using a sputtering coater (8 mA, 4 min), and the samples were examined by SEM (JSM6380LV, Jeol, Tokyo, Japan) at ×5,000 magnification.

2.7. Extracellular polysaccharide (EPS) formation assay

The effect of 5-FU on EPS formation of *S. suis* was determined by sulfuric acid-phenol method (Yan *et al.* 2023). The bacteria were cultured using the previous method, then 1 mL of the culture was centrifuged at 12,000×g, 4°C for 10 min, and the supernatant was taken and filtered with a 0.22 μm aqueous filter. Then 3 mL of pre-cooled ethanol was added and treated at 4°C for 24 h. After centrifugation, the aqueous phase was discarded, and the resulting precipitate (EPS) was resuspended in 1 mL of distilled water. EPS was measured as described previously. The absorbance was measured by ultraviolet visible spectrophotometer (wavelength 490 nm).

2.8. Capsule polysaccharide (CPS) formation assay

Transfer the cultured *S. suis* bacterial solution in a 1:100 ratio to fresh TSB medium supplemented with 0.625 μg mL^{-1} 5-FU, and incubate at 37°C for 24 h. The culture was centrifuged at 10,000×g and bacterial cells were collected, then resuspended in 0.1 mol L^{-1} glycine buffer while adding lysozyme and incubated at 37°C for 6 h. Subsequently, centrifuge the supernatant and add protease K to incubate at 55°C for 2 h. Add $CaCl_2$ and stir for 1 h, then add 25% (v/v) anhydrous ethanol and let it stand at 4°C for 2 h. Centrifuge to obtain the supernatant. Finally, 80% (v/v) cooled anhydrous ethanol was added and stored overnight at 4°C. CPS were collected by centrifugation. The quantification of CPS was carried out using the phenol sulfuric acid method as described earlier (Li *et al.* 2021). The standard curve is drawn with galactose as the criterion, and the measurement equation of the standard curve is y=0.00951x+0.01659 (R^2=0.9961).

2.9. Disintegration of mature biofilm assay

The mature biofilm was first cultured, and the logarithmic *S. suis* solution was inoculated into a 96-well polystyrene culture plate containing 200 μL TSB medium and cultured at 37°C for 48 h. The culture medium was absorbed and replaced with fresh TSB medium with final concentrations of 0, 2.5, 1.25 and 0.625 μg mL^{-1} 5-FU, respectively, and treated for 12 h. Then, the biofilm formation was quantified according to Section 2.3.

2.10. RNA extraction and quantitative reverse transcription polymerase chain reaction

Total *S. suis* RNA was extracted according to the previously

described steps (Li *et al.* 2022). The wild-type strain, the Δ*luxS* strain and the Δ*thyA* strain were co-cultured with different concentrations of 5-FU (0 or 0.625 μg mL^{-1}) to the logarithmic growth stage, and the bacteria were collected after centrifugation at 8,000×g for 5 min. Total bacterial RNA was extracted using TRIzon reagent (Conway Biotechnology Co., Ltd., Beijing, China). RNA purity was determined with A260/A280. Then reverse transcription was performed using the M5HiPer One-step RT-PCR Kit (Mei5bio, Beijing, China). Fluorescence quantitative PCR assay was performed using 2×M5HiPer SYBR PremixEs Taq (Mei5bio, Beijing, China). The 16S rRNA gene serves as the internal control gene. PCR procedure involves an initial denaturation at 95°C for 30 s, followed by denaturation at 95°C for 10 s, annealing at 60°C for 30–60 s, repeated for 40 cycles. The relative expression genes were analyzed by $2^{-\Delta\Delta Ct}$ method.

2.11. Bacterial adherence and phagocytosis assays

Adhesion and phagocytosis experiments were performed using human laryngeal epithelial cell HEp-2 and mouse macrophage RAW264.7, respectively (Liu *et al.* 2019). In brief, the cultured bacterial solution was inoculated into monolayer cells of 24-well plates, incubated at 37°C and 5% CO_2 for 2 h, and then cleaned the holes 3 times to remove nonadherent bacteria. The cells were broken with pre-cooled ultra-pure water, and the lysate was diluted and coated on TSA agar plate, and cultured at 37°C for 24 h for colony counting. To determine the anti-phagocytosis of 5-FU, penicillin (5 μg mL^{-1}) and gentamicin (100 μg mL^{-1}) were added to kill extracellular fine bacteria. After incubation for 1 h, the pores were cleaned 3 times to remove antibiotics, and the cells were lysed in the same way and counted on the TSA agar plate.

2.12. *Galleria mellonella* larvae infection assay

The *G. mellonella* larvae were infected with *S. suis* strains (Velikova *et al.* 2016), and survival curves were drawn to evaluate the effects of 5-FU on the virulence of *S. suis*. The bacterial suspension was diluted with PBS to 1×10^6 CFU mL^{-1} and mixed with 20 μL and 0.625 μg mL^{-1} 5-FU, and the larvae were inoculated. The larvae were placed in an incubator at 37°C, the time of death was monitored, and the survival rate was recorded every 12 h. The infection experiment was conducted with PBS injection as a negative control, and the toxicity of 5-FU alone to the larva pluton was detected.

2.13. Molecular docking assay

We performed molecular docking experiments on 5-FU and LuxS proteins and ThyA proteins using the methods described previously. The chemical structure of 5-FU was retrieved from the PubChem database (PubChem CID: 3385). The structure of the *S. suis* HA9801 LuxS protein was derived from earlier studies in our laboratory (PDB DOI: https://doi.org/10.2210/pdb4XCH/pdb), while the *S. suis* ThyA protein was constructed using the Untitled Project (Quality evaluation results of the ThyA model of *S. suis* are presented in Appendices A and C. The binding affinity of 5-FU to LuxS and ThyA proteins was assessed using Autodock software.

2.14. Protein expression and polyclonal antibody preparation

The generation of mice polyclonal antibodies was previously detailed (Zhang *et al.* 2023). A set of specific primers (*luxS*-F/*luxS*-R) was designed for amplifying the *luxS* gene (Appendix B). The resulting product was digested with appropriate enzymes and subsequently inserted into the expression vector pET28a (Wang *et al.* 2023). *E. coli* BL21, transformed with pET28a-*luxS*, was induced at 37°C for 4 h with the addition of 0.5 mmol L^{-1} isopropyl-beta-D-thiogalactopyranoside (IPTG). Following induction, bacterial pellets were sonicated, and centrifugation was employed to eliminate insoluble debris. The recombinant protein underwent purification *via* affinity chromatography on a nickel-nitrotriacetic acid column. The target protein was isolated through SDS-PAGE, and its concentration was determined using the BCA Protein Assay Kit (Sangon Biotech (Shanghai) Co., Ltd., China). For Western blotting, polyclonal antiserum against LuxS was elicited in mice through three immunizations with 50 μg of purified protein and 50% Freund's incomplete adjuvant. Polyclonal antibodies against the other seven genes (*thyA*, *glyA*, *pfS*, *folA*, *metE*, *metK*, *trmL*) were prepared in the same way.

2.15. Western blot analysis

Western blot assay was used to verify the effects of 5-FU against folate cycle and methyl cycle (Peng *et al.* 2019). The whole bacterial protein was prepared as described previously. First, the cultured *S. suis* wild-type strain, the wild-type strain added with 5-FU, and the Δ*thyA* strain were calibrated to the same optical density. After centrifugation at 12,000×g 4°C, and the bacterial particles were resuspended in lysis buffer (containing 100 μg mL^{-1} lysozyme and 1 μg mL^{-1} PMSF) followed by 20 min of sonication to obtain total protein. The Western blot procedure adhered to the previously outlined scheme. Proteins from the cell lysate were separated using SDS-PAGE protein electrophoresis and transferred onto a PVDF membrane. The PVDF membrane was initially blocked with 5% skim milk, then incubated with a primary antibody (homemade), and subsequently with a secondary antibody (goat anti-mouse IgG). Finally, the appropriate amount of luminescent liquid (liquid A and liquid B 1:1 configuration) was absorbed onto the PVDF membrane, and the ECL luminescent instrument was exposed and the image was collected.

2.16. Animal experiments

For the evaluation of 5-FU's therapeutic efficacy in mice model, *S. suis* was cultured overnight at 37°C, and subsequently diluted with sterile PBS to a concentration of 5×10^6 CFU mL^{-1}. Mice were initially peritoneally inoculated with 200 μL of either the wild-type strain or Δ*luxS* strain. Following this, 5-FU (20 μg g^{-1}) was administered through tail vein injection, while PBS was injected into the untreated control groups. The first treatment commenced 2 h post-infection, followed by twice daily administrations. After two days, all mice were euthanized, and aseptically obtained brain, lung, liver, and spleen tissues were subjected to

bacterial count and histopathological section observation. Homogenized tissue was cultured on TSA plates to measure bacterial load.

2.17. Statistical analysis

One-way ANOVA was used for determining statistical significance and calculated using GraphPad Prism 8.0. $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. 5-FU reduces *S. suis* virulence and inhibits biofilm formation

The virulence of *S. suis* was assessed using a model of infection of *G mellonella* larvae, and the survival curve showed that 5-FU significantly improved the survival of *G mellonella* larvae in *S. suis* infection (Fig. 1-A and B). In addition, we detected the effects of 5-FU on the adhesion and phagocytosis of *S. suis* on HEp-2 cells and RAW264.7 cells respectively, and the results showed that the adhesion and phagocytosis of *S. suis* treated with 5-FU were significantly down-regulated (Fig. 1-C and D).

Prior research has indicated that the adhesion, gene expression, and virulence of *S. suis* exhibit variations between biofilm and planktonic states, suggesting a potential reliance of virulent strains on biofilm formation for pathogenicity. Therefore, we examined the influence of 5-FU on *S. suis* biofilm formation. We determined the antimicrobial capacity of 5-FU against *S. suis*, with minimal inhibitory concentration (MIC) and minimal inhibitory concentration (MBC) values of 5 and 10 μg mL^{-1}, respectively. Crystal violet staining and growth kinetics analysis showed that even at concentrations not inhibiting bacterial growth (0.625 μg mL^{-1}, 1/8 MIC), 5-FU still significantly inhibited biofilm formation by *S. suis* (Fig. 2-A). Utilizing CLSM and SEM, images depicted that the wild-type strain *S. suis* developed a dense, mature biofilm characterized by a three-dimensional structure comprising both bacteria and extracellular matrix. In contrast, *S. suis* treated with 5-FU exhibited more dispersed bacterial cells, lower aggregation, and a weaker biofilm matrix (Fig. 1-B and C). The disintegration effect of 5-FU on mature biofilm was also significant (Fig. 2-D).

As the biofilm matrix primarily consists of polysaccharides, we examined the impact of 5-FU on the formation of CPS and EPS by *S. suis*. The results showed a significant decrease

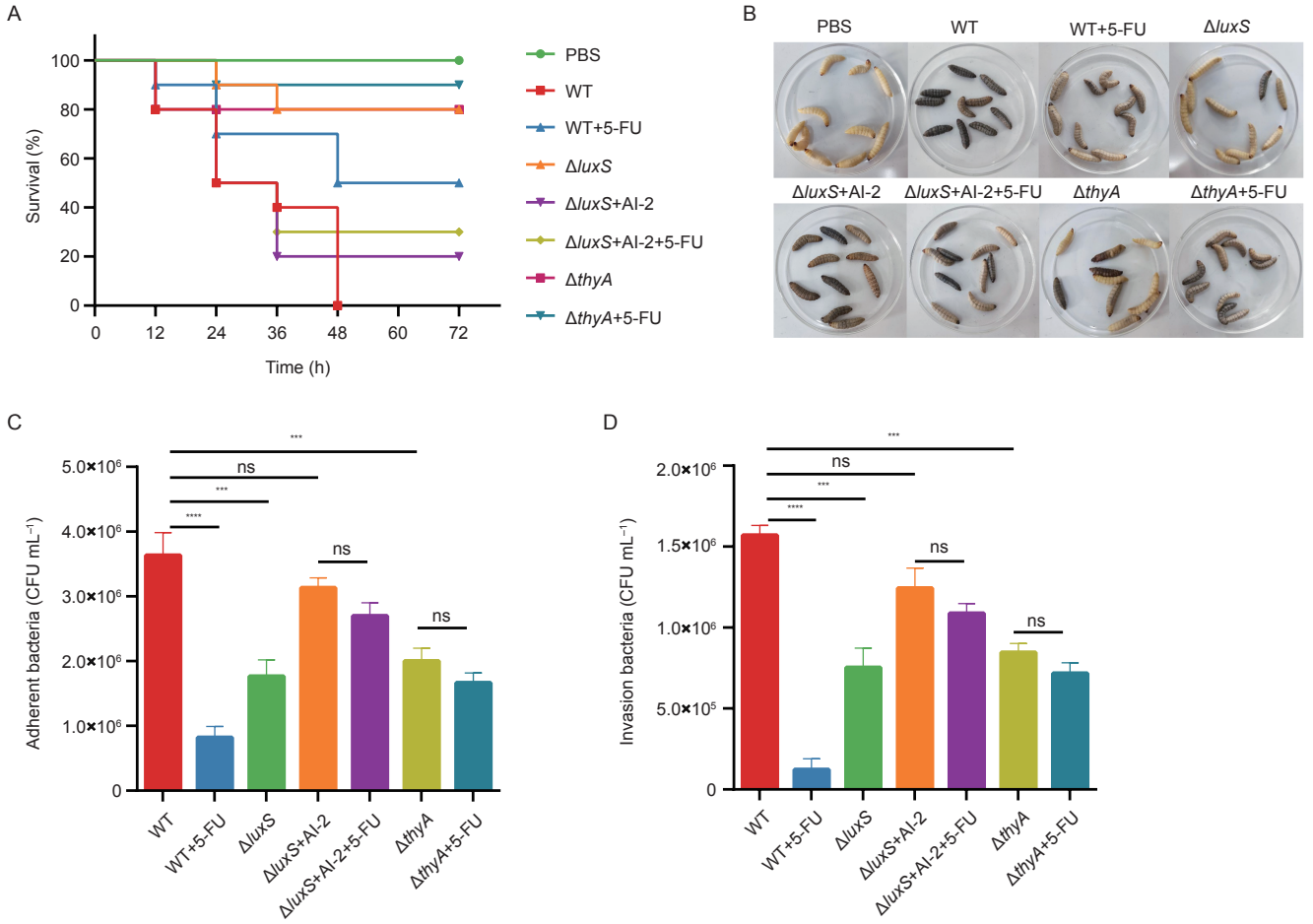


Fig. 1 Modulation of the virulence of *Streptococcus suis* by 5-fluorouracil (5-FU). A, survival curves of *Galleria mellonella* larvae inoculated with *S. suis* with 5-FU. B, larvae with melanization enhanced by bacterial 48 h post-infection. C, bacterial adhesion assay. The number of bacteria that adhered to the surface after incubation with HEp-2. D, bacterial anti-phagocytosis assay. The number of bacteria that are engulfed after incubation with RAW264.7. Data are mean±SD of three biological replicates. *** and **** indicate significant differences at $P<0.001$ and $P<0.0001$ (one-way ANOVA test), respectively. ns, did not show a significant difference.

in CPS and EPS production in 5-FU-treated *S. suis* (Fig. 2-E and F). Subsequently, quantitative experiments were conducted on genes associated with *S. suis* adhesion and virulence. 5-FU significantly downregulated the expression of *gapdh*, *ef*, *sly*, *fbps*, *mrp*, *gor*, and *ccpa* genes in *S. suis* (Fig. 2-G).

3.2. 5-FU exerts anti-virulence and anti-biofilm functions *via* the LuxS/AI-2 QS system

Previous research indicated that *S. suis* regulates biofilm development, maturation, expression of virulence factors, pathogenicity, and antibiotic resistance through the LuxS/AI-2 QS system. Therefore, we assessed the QS inhibitory activity of 5-FU by using a *V. harveyi* fluorescence reporter strain to test the impact of 5-FU on AI-2 signal molecule production by *S. suis*. The results showed that 5-FU significantly reduced AI-2 signal molecule levels in *S. suis* at different time points (Fig. 3).

To elucidate the mechanism by which 5-FU affects *S. suis* biofilm and virulence, we monitored the biofilm formation of Δ*luxS* strains. The Δ*luxS* strain exhibited significantly lower biofilm formation than the wild-type strain, and the addition of exogenous AI-2 signal molecules to the Δ*luxS* strain increased biofilm formation to the wild-type strain level. However, 5-FU did not exhibit a significant inhibitory effect on biofilm formation in the Δ*luxS* strain containing exogenous AI-2 signal molecules (Fig. 2-A). CLSM and SEM images vividly showed the impact of 5-FU on the biofilm formation capacity of the Δ*luxS* strain, wherein the Δ*luxS* strain with exogenous AI-2 signal molecules formed a dense, mature biofilm similar to the wild-type strain, while 5-FU treatment did not cause significant damage. However, the biofilm formed by the Δ*luxS* strain was similar to that of the wild-type strain treated with 5-FU, with more dispersed bacterial cells, lower aggregation, and a weaker biofilm matrix (Fig. 2-B and C). CPS and EPS production levels (Fig. 2-E and F) indicated no significant differences between the Δ*luxS* strain and the wild-type strain treated with 5-FU. qPCR results showed that the expressions of *gapdh*, *ef*, *sly*, *fbps*, *mrp*, *gor*, and *ccpa* genes in Δ*luxS* strain were significantly lower than those in wild-type strain, similar to those in wild-type strain treated with 5-FU, but 5-FU had no significant down-regulation effect on the expression of adhesion and virulence-related genes in Δ*luxS* strain (Fig. 2-G).

Virulence experiments revealed similar outcomes, with the *G. mellonella* larvae infected with the Δ*luxS* strain showing a high survival rate (80 %), indicating low virulence of the Δ*luxS* strain, but higher virulence of Δ*luxS* strain supplemented

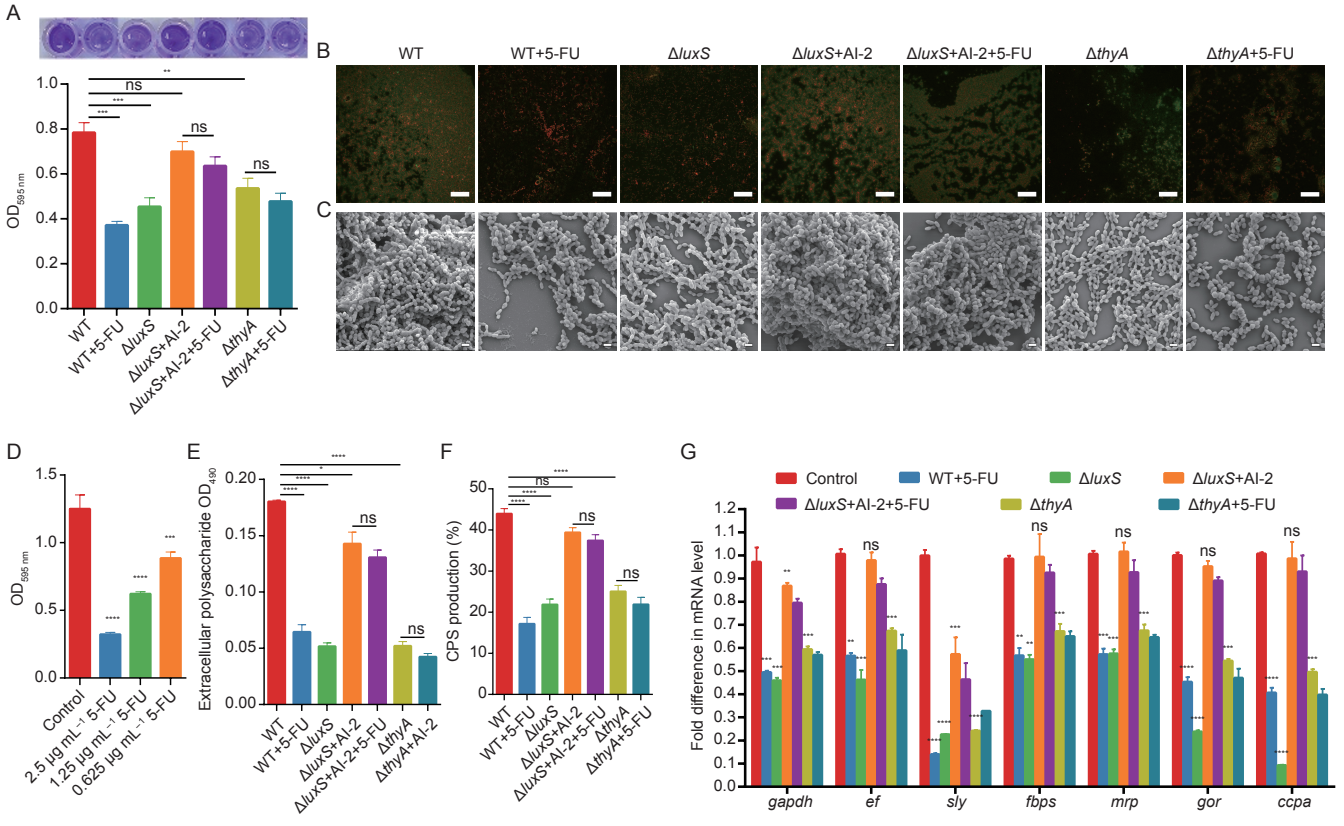


Fig. 2 Modulation of the biofilm of *Streptococcus suis* by 5-fluorouracil (5-FU). A, wells dyed with crystal violet and biofilm formation of *S. suis* was quantified by crystal violet staining. B, confocal laser scanning microscopy of *S. suis* biofilms; scale bars: 20 μm. C, scanning electron microscopy of *S. suis* biofilms; scale bars: 5 μm. D, destruction of mature biofilm of *S. suis* by 5-FU. E, effect of 5-FU on *S. suis* extracellular polysaccharide production. F, effect of 5-FU on *S. suis* capsular polysaccharide production. G, expression of *gapdh*, *ef*, *sly*, *fbps*, *mrp*, *gor*, and *ccpa* genes in *S. suis*. OD, optical density; CPS, capsule polysaccharide. Data are mean±SD of three biological replicates. *, **, ***, and **** indicate significant differences at $P<0.05$, $P<0.01$, $P<0.001$ and $P<0.0001$ (one-way ANOVA test), respectively. ns, did not show a significant difference.

with AI-2 signaling molecules, and 5-FU did not change its virulence (Fig. 1-A and B). Additionally, adhesion and phagocytosis results indicated weaker capabilities in the Δ*luxS* strain, which were increased by exogenous AI-2 signal molecules. However, the impact of 5-FU on adhesion and phagocytosis in the Δ*luxS* strain was not significant (Fig. 1-C and D). In summary, these results strongly suggest that 5-FU can affect the biofilm and virulence of *S. suis* through the LuxS/AI-2 QS system.

3.3. 5-FU affects the LuxS/AI-2 QS system of *S. suis* through *thyA*

To understand the interaction between 5-FU and LuxS proteins, we conducted virtual docking experiments using Autodock and visualized the results using Pymol. The results showed that the docking effect between 5-FU and LuxS protein was not significant, with a binding score of -3.52 (Fig. 4-B). Knowing that 5-FU is an inhibitor of thymidylate synthase and considering its role in the upstream regulation of methionine cycle, we hypothesized that the inhibition of 5-FU on the LuxS/AI-2 QS system might be influenced by the regulation of the thymidylate synthase-regulating gene, *thyA*. Therefore, we subsequently conducted virtual molecular docking to test whether 5-FU could bind to the ThyA protein. The ThyA protein is constructed using SWISS-MODEL, and the construction process and results are shown in Appendix A. The results of molecular docking between 5-FU and ThyA protein indicate that 5-FU could interact with the active pocket of ThyA protein, and their binding score can reach -4.62. 5-FU can interact with the HISA:186 active site residue of ThyA protein through Conventional Hydrogen Bond or Halogen (Fluorine), with bond distances of 2.23 Å and 3.34 Å, respectively. 5-FU interacts with the active site residue TYR A:213 through a Carbon Hydrogen Bond with a bond distance of 2.56 Å. In addition, 5-FU can also interact with active site residues ALA A:43 and ALA A:45 through Pi-Alkyl bonds, with bond distances of 5.14 Å and 3.18, respectively (Fig. 4-C).

To further confirm whether the inhibition of 5-FU on the LuxS/AI-2 QS system is influenced by thymidylate synthase, we conducted gene knockout experiments targeting *thyA* (The result of gene knockout was not listed). Biofilm formation tests indicated that the biofilm formation capacity of the Δ*thyA* strain was significantly lower than that of the wild-type strain, and 5-FU did not exhibit a significant inhibitory effect on the biofilm formation capacity of the Δ*thyA* strain (Fig. 2-A). CLSM and SEM images also showed the same results (Fig. 2-B and C). CPS and EPS production levels (Fig. 2-E and F) indicated that the Δ*thyA* strain had lower CPS and EPS production than the wild-type strain, with no significant change in CPS and EPS production in the Δ*thyA* strain treated with 5-FU. The qPCR results showed that the expressions of *gapdh*, *ef*, *sly*, *fbps*, *mrp*, *gor* and *ccpa* in Δ*thyA* strain were significantly decreased compared with those in wild-type strain, but 5-FU had no significant

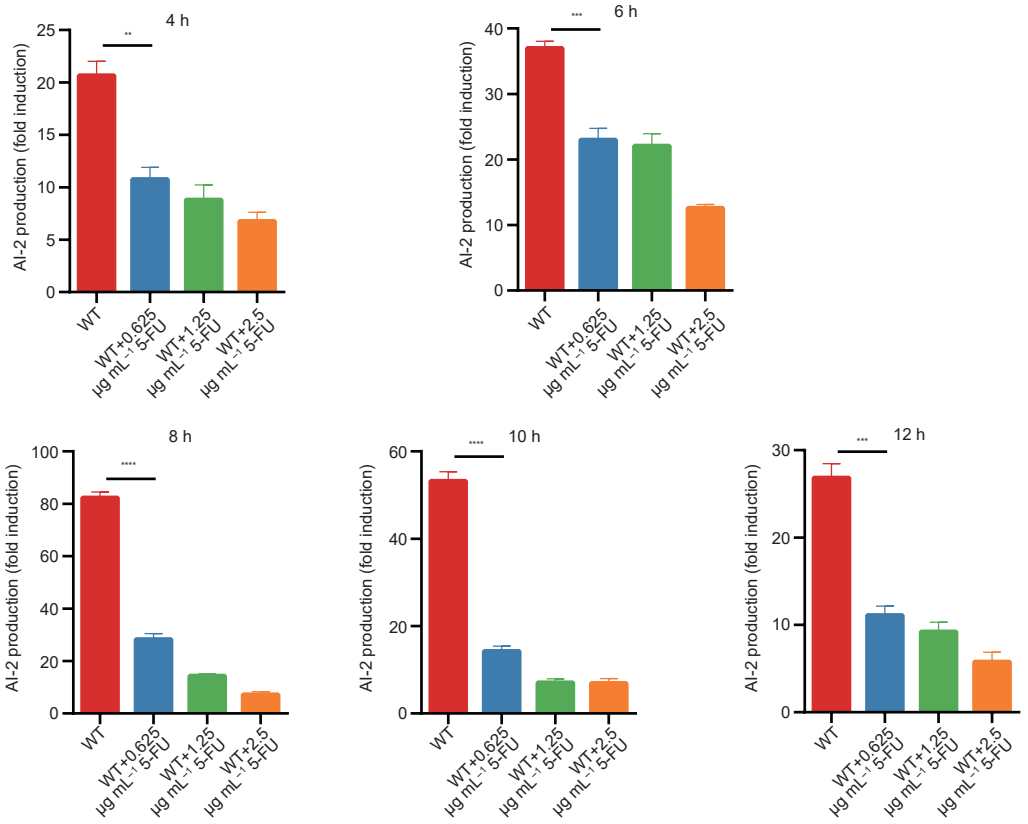


Fig. 3 Production of AI-2 signaling molecules in *Streptococcus suis* inhibited by 5-fluorouracil (5-FU). AI-2 production of *S. suis* by different concentrations of 5-FU (0, 0.625, 1.25, 2.5 μg mL^{-1}). Data are mean±SD of three biological replicates. **, ***, and **** indicate significant differences at P<0.01, P<0.001 and P<0.0001 (one-way ANOVA test), respectively.

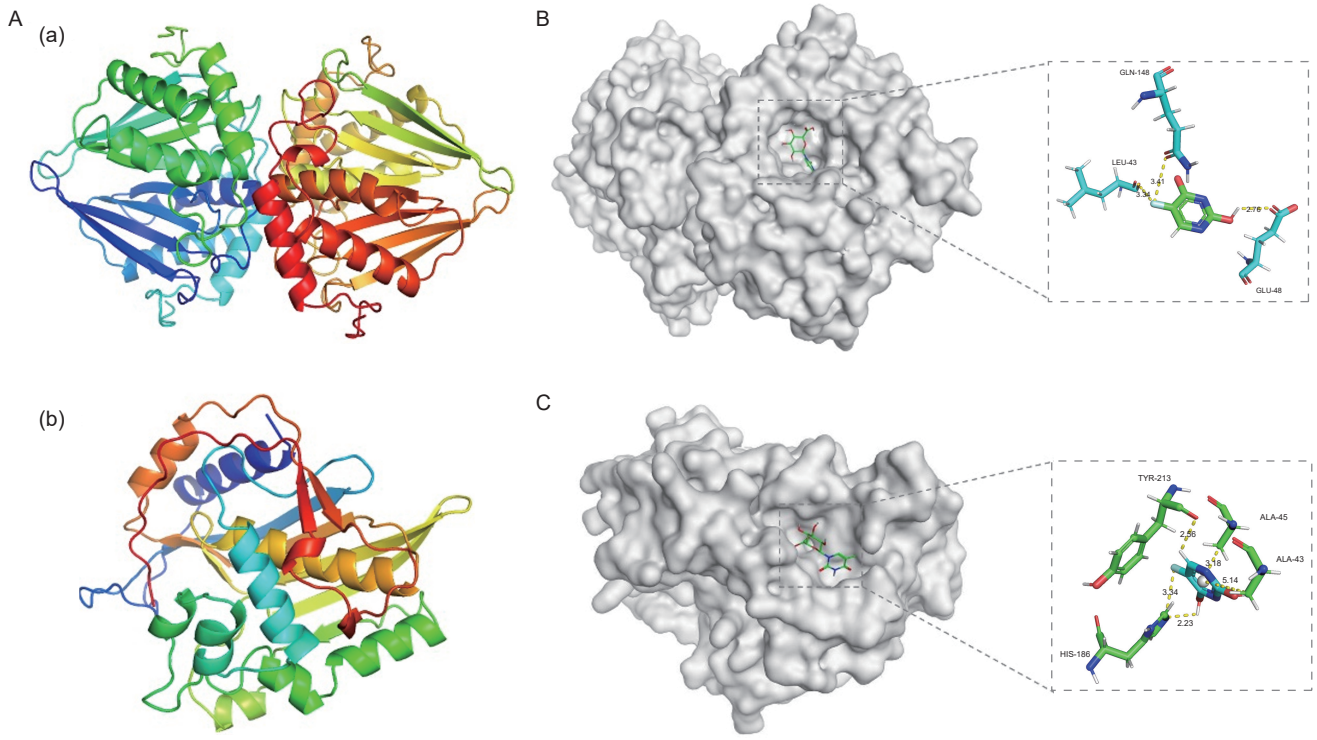


Fig. 4 Virtual molecular docking results. A, the 3D structure of proteins. (a), the 3D structure of LuxS protein; (b), the 3D structure of ThyA protein. B, molecular docking of 5-FU and LuxS. GLU, glutamic acid; GLN, glutamine; LEU, leucine. C, molecular docking of 5-FU and ThyA. HIS, glutamine; ALA, glutamic acid; TYR, tyrosine.

down-regulation effect on the expression of adhesion and virulence related genes in Δ*thyA* strain (Fig. 2-G). Virulence experiments demonstrated similar outcomes, with the survival rate of *G mellonella* larvae infected with the Δ*thyA* strain reaching 80%, indicating significantly lower toxicity of the Δ*thyA* strain (Fig. 1-A and B). Additionally, adhesion and phagocytosis results indicated weaker capabilities in the Δ*thyA* strain, and the impact of 5-FU on adhesion and phagocytosis in the Δ*thyA* strain was not significant (Fig. 1-C and D). By comparing the effects of 5-FU on the wild-type strain and the Δ*thyA* strain, we demonstrated that 5-FU can affect the biofilm and virulence of *S. suis* by regulating *thyA*.

Since 5-FU is an inhibitor of thymidylate synthase and thymidylate synthase is located upstream of the methyl cycle, we speculate that 5-FU can regulate the methyl cycle through the regulation *thyA* gene in the folate cycle and then affect the LuxS/AI-2 QS system (Fig. 5-A). To confirm whether 5-FU can affect the LuxS/AI-2 QS system of *S. suis* through *thyA*, we used the Δ*thyA* strain to quantitatively measure the expression levels of the *luxS* gene, LuxS protein, and AI-2 signal molecule in the presence or absence of 5-FU. The results showed that compared with the wild-type strain, the expression of *luxS* gene and LuxS protein in Δ*thyA* strain were significantly down-regulated, and the expression level of AI-2 signaling molecules was also significantly reduced at different time points, and was not regulated by 5-FU (Fig. 5-B and D). In addition, we also detected the expression levels of key genes in the folate and methionine cycles in *S. suis* WT and Δ*thyA* strain by using qPCR and WB to measure mRNA and protein expression levels, respectively. The results showed that the deletion of *thyA* gene significantly downregulated the expression of *glyA*, *folA*, *metE*, *metK*, *trmL*, *pfS*, and *luxS* genes (Fig. 5-E), but did not reach the level of Δ*luxS* strain. Western blotting showed that the expression of GlyA, FolA, MetE, MetK, TrmL, PfS and LuxS in *S. suis* decreased significantly in the absence of *thyA* (Fig. 5-F).

3.4. Therapeutic effect of 5-FU on *S. suis* infection in mice

To evaluate the potential therapeutic effect of 5-FU as a LuxS/AI-2 QS inhibitor, we used a mouse infection model. BALB/c mice were inoculated with 5×10^6 CFU/wild-type strain or the Δ*luxS* strain, respectively. The experimental design is depicted in Fig. 5-A, with subsequent dissection of mouse organs illustrated in Fig. 5-B. Remarkably, administration of 5-FU exhibited a notable reduction in brain edema, with relatively mild damage observed in the lungs, liver, and spleen. Two days post-infection, evaluation of bacterial loads in tissues and organs was conducted (Fig. 5-C). The 5-FU treatment of wild-type strain demonstrated a significant decrease in bacterial load in the brain, lungs, liver, and spleen compared to the untreated wild-type strain, while no significant difference was observed in the Δ*luxS* strain. Histopathological examination *via* Hematoxylin and Eosin (H&E) staining of tissue sections (Fig. 5-D) revealed distinct pathological features in the untreated wild-type strain, including submeningeal congestion, inflammatory cell infiltration, and signs of shedding. Additionally, lung tissues exhibited loss of structural integrity and alveolar congestion, the central vein of liver cells displayed congestion, and the

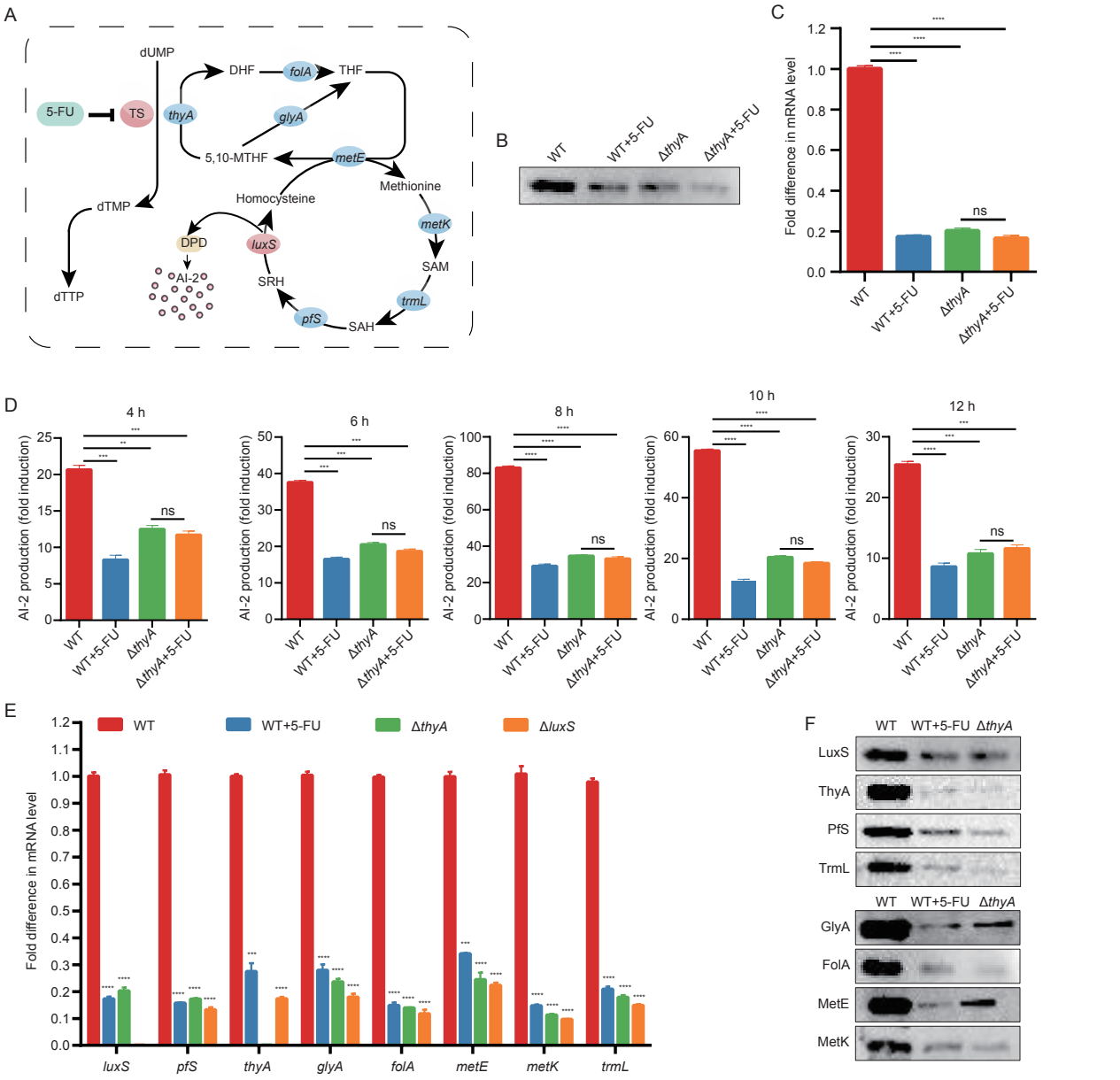


Fig. 5 5-fluorouracil (5-FU) affects the LuxS/AI-2 quorum sensing (QS) system of *Streptococcus suis* through *thyA*. A, proposed model for the effect of 5-FU on folic acid cycle and methyl cycle in *S. suis*. DHF, dihydrofolate; THF, tetrahydrofolate; 5,10-MTHF, 5,10-methylenetetrahydrofolate; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; SRH, *S*-ribosylhomocysteine; DPD, 4,5-dihydroxy-2,3-pentanedione; *thyA*, thymidylate synthase gene; *glyA*, serine hydroxymethyltransferase gene; *folA*, dihydrofolate reductase gene; *metE*, methionine synthase gene; *metK*, *S*-adenosylmethionine synthetase gene; *pfS*, *S*-adenosylhomocysteine nucleosidase gene and *luxS*, *S*-ribosylhomocysteinase gene. B, production of LuxS protein in WT and Δ*thyA* strains in the presence or absence of 5-FU. C, production of *luxS* gene in WT and Δ*thyA* strains in the presence or absence of 5-FU. D, production of AI-2 signaling molecules in WT and Δ*thyA* strains in the presence or absence of 5-FU. E, expression of *thyA*, *glyA*, *folA*, *metE*, *metK*, *trmL*, *pfS*, and *luxS* genes in *S. suis*. F, Western blot analysis of LuxS, ThyA, GlyA, PfS, FolA, MetE, MetK, and TrmL expression in *S. suis*. Data are mean±SD of three biological replicates. **, ***, and **** indicate significant differences at $P<0.01$, $P<0.001$ and $P<0.0001$ (one-way ANOVA test), respectively. ns, did not show a significant difference.

portal vein showcased inflammatory infiltration. The spleen displayed moderate congestion with an unclear boundary between red and white marrow, accompanied by inflammatory cell infiltration. Notably, 5-FU treatment resulted in significant amelioration, presenting only mild lesions compared to the Δ*luxS* strain.

4. Discussion

Streptococcus suis is an important pathogen of zoonosis, which can cause a variety of diseases in humans and pigs (Gottschalk *et al.* 2010). In severe cases, it can lead to fatal outcomes, causing substantial economic losses in the pig industry (Wang *et al.* 2022). A variety of virulence factors synthesized and secreted by *S. suis* increases its pathogenic chances. Once it forms biofilm, its pathogenicity is further enhanced, and it has become a bottleneck in the treatment of related infectious diseases (Kerdsin *et al.* 2018). At present, controlling the formation of biofilm or reducing the virulence

of bacteria to prevent infection has become a new research hotspot (Kerdsin *et al.* 2011; Takeuchi *et al.* 2012; Huong *et al.* 2014). Our previous investigations have demonstrated that *S. suis* orchestrates biofilm formation and virulence factor production through the LuxS/AI-2 QS system (Wang *et al.* 2019). This regulatory mechanism results in increased pathogenicity, complicating treatment strategies. Therefore, inhibiting the formation of biofilm, reducing the production of virulence factors and increasing the sensitivity of antibiotics by intervening quorum sensing system is a promising anti-infective therapy. We previously screened an FDA-approved drug, 5-FU, as an inhibitor of *S. suis* quorum sensing to control biofilm formation and reduce virulence. Many studies have shown that 5-FU also shows effective anti-biofilm or anti-quorum sensing activity against a variety of gram-positive and gram-negative bacteria. It was found that 5-FU is an effective population quencher, which can inhibit the production and release of AI-2 by MRSA, *S. epidermidis*, *E. coli* and *V. harveyi*, and effectively reduce the adhesion of MRSA and block the formation of biofilm *in vitro* (Sedlmayer *et al.* 2021). Another study showed that 5-fluorouracil inhibits the formation of *P. aeruginosa* biofilms, eliminates quorum sensing phenotypes, and reduces virulence (Garcia-Contreras *et al.* 2013).

We first identified the effect of 5-FU on reducing the virulence of *S. suis* and inhibiting biofilm formation. It was then demonstrated that 5-FU inhibited biofilm and virulence by regulating LuxS/AI-2 QS system. Quantitative and observation of the biofilm formation of wild-type strain of *S. suis* and Δ*luxS* strain under the effect of 5-FU showed that 5-FU still had a significant inhibitory effect on the biofilm formation of *S. suis* at a concentration that did not inhibit bacterial growth (0.625 μg mL^{-1}, 1/8 MIC) (Fig. 2-A). In addition, 5-FU significantly reduced the formation and aggregation of *S. suis* biofilm, and its biofilm formation capacity was similar to that of the Δ*luxS* strain. The biofilm formation capacity of the Δ*luxS* strain supplemented with AI-2 increased to the level of the wild-type strain and was not affected by 5-FU (Fig. 2-B and C). The disintegration effect of 5-FU on mature biofilm was also significant (Fig. 2-D). This suggests that 5-FU can influence the biofilm formation capacity of *S. suis* through the LuxS/AI-2 QS system. Biofilms are defined as microbial aggregates encapsulated in secreted polysaccharides. It has been reported that bacterial polysaccharides serve as the substrate for bacterial biofilms. The primary constituents of bacterial polysaccharides include CPS and EPS. CPS functions as the structural cell surface component of bacteria. Previous studies have indicated that clinical pneumococci coated with CPS exhibit a diminished ability to form biofilms. Exopolycans secreted in mucus can promote intercellular adhesion and thus promote biofilm formation. Previous studies have shown that pomegranate extract reduces the formation of *Streptococcus mutans* biofilm and insoluble extracellular polysaccharides in biofilm (Gulube and Patel 2016). Therefore, we further measured the production of *S. suis* capsular polysaccharide and extrinsic polysaccharide. The results showed that 5-FU significantly reduced the content of CPS and EPS (Fig. 2-E and F), suggesting that the biofilm may be affected by the weakening of CPS and EPS. This may be a mechanism by which 5-FU leads to the weakening of *S. suis* biofilm. Previous studies in our laboratory have shown that overexpression of AI-2 and deletion of *luxS* gene can regulate some virulence factors of *S. suis*. In *S. suis*, the expression levels of *ef*, *sly*, and *mrp* genes are down-regulated, indicating reduced virulence and pathogenicity of *S. suis*. The *fbps* and *gapdh* genes have previously been shown to mediate cell adhesion, and reduced expression of these two genes may lead to reduced biofilm formation and adhesion. Therefore, we selected adhesion and virulence related genes regulated by *luxS* gene for quantitative experiments. The results showed that 5-FU significantly down-regulated the expression of *gapdh*, *ef*, *sly*, *fbps*, *mrp*, *gor*, and *ccpa* genes in *S. suis* (Fig. 2-G).

Bacterial virulence factors can also promote adherence, invasion of host tissues, and enable them to escape immune defenses. Watcharapong Mitsuwan *et al.* (2020)'s study found that treatment with subinhibitory concentrations of rhodomyrtus tomentosa leaf extract and rhodomyrtone could significantly reduce the invasion of pneumococcal lung epithelial cells. Similarly, *S. aureus* treated with rhodomyrtus tomentosa leaf extract showed decreased adhesion to human cheek cells and decreased invasiveness to bovine mammary epidermal tissue (Limsuwan *et al.* 2014). Our results are consistent with previous studies. The results showed that 5-FU could significantly reduce *S. suis* adhesion to HEp-2 cells and phagocytose to RAW264.7 cells (Fig. 1-C and D). Caterpillar larvae have been successfully used to study the virulence of some important pathogens, including *S. suis* (Velikova *et al.* 2016), so we used a caterpillar infection model to assess the effects of 5-FU on *S. suis* virulence. Our preliminary findings suggest that 5-FU can significantly reduce the virulence of *S. suis* (Fig 1-A and B).

As commonly acknowledged, the central component of the LuxS/AI-2 QS system is the AI-2 signaling molecule synthetase, LuxS. In this study, it was observed that 5-FU exerted an influence on the expression of AI-2 signaling molecules in *S. suis*. Given that LuxS serves as a pivotal enzyme in the control of AI-2 signaling molecule synthesis in *S. suis*, we posited its potentially significant role. Consequently, we explored the interaction between 5-FU and LuxS through virtual molecular docking analysis. The results showed that the docking effect of 5-FU with LuxS protein was not significant (Fig. 4-B). Since 5-FU is an inhibitor of thymidylate synthase, and thymidylate synthase is located upstream of the methyl cycle, we speculated that 5-FU could regulate the methyl cycle through *thyA*, the thymidylate synthase regulatory gene in the folate cycle, and thus affect the LuxS/AI-2 QS system (Fig. 5-A). Virtual molecular docking results demonstrated that 5-FU may bind to ThyA protein (Fig. 4-C). Zhao *et al.* (2010) demonstrated that the LuxS/AI-2 QS system can regulate the production of capsulated polysaccharide through the two-component system kdpDE by constructing the deletion strain of the two-component system KdpDE. Jakobsen *et al.* (2017) demonstrated by deletion of *rsmY* and *rsmZ* genes that ajoene's inhibition of quorum sensing is achieved by inhibiting the small regulatory RNA (sRNA) of *P. aerosa* and *S. aureus*. Based on the above findings, to further determine

whether the inhibition of 5-FU on LuxS/AI-2 QS is affected by thymidylate synthase, we knocked out the *thyA* gene for verification. The detection of biofilm formation ability of Δ*thyA* strain showed that the amount of biofilm formation of Δ*thyA* strain was significantly lower than that of wild-type strain, and 5-FU had no inhibitory effect on biofilm formation ability of Δ*thyA* strain (Fig. 2). Virulence tests showed similar results, with Δ*thyA* strains being extremely virulent and unaffected by 5-FU (Fig. 1). By comparing the effects of 5-FU on wild-type strain and Δ*thyA* strains, we determined that 5-FU can affect the biofilm and virulence of *S. suis* by regulating *thyA*. Peng *et al.* (2019) illustrated that baicalin inhibits the release of inflammatory factors by reducing the activation of the NF-κB signaling pathway induced by APEC-O78. Additionally, it interferes with the QS regulation of biofilm formation and the expression of virulence genes. Folate metabolism is involved in the remethylation of homocysteine to methionine (Lan *et al.* 2018). Trimethoprim-sulfamethoxazole (TMP/SMX), as a folate inhibitor, interferes with the folate mechanism process, resulting in the production of thymine-dependent small colony variants (Chatterjee *et al.* 2008). The results from Yu *et al.* (2018) showed that exogenous AI-2 down-regulates transcription of folate synthase coding genes *folA* and *folC*, and folate synthesis-related genes *luxS*, *metE*, and *metH*. To determine whether 5-FU can affect the LuxS/AI-2 QS system of *S. suis via thyA*, we quantified the *luxS* gene, LuxS protein, and AI-2 signaling molecules here. In addition, the expression levels of key genes in folate cycle and methyl cycle in wild-type strain and Δ*thyA* strains were also detected, and mRNA expression levels and protein expression levels were detected by qPCR and WB, respectively. The results showed that the deletion of *thyA* gene inhibited folate cycling and methyl cycling, thereby affecting the LuxS/AI-2 QS system (Fig. 5). It can be seen that the regulation of 5-FU on LuxS/AI-2 QS system is influenced by *thyA*, but not completely regulated by *thyA*. We know that this study has some limitations, and further research is needed to fully understand other mechanisms of 5-FU regulation on LuxS/AI-2 QS system. Finally, we explored the therapeutic effect of 5-FU *in vivo* through a mouse infection model, and the results showed that 5-FU significantly reduced the damage of tissues and organs in mice and reduced the bacterial load in the mouse infection model (Fig. 6).

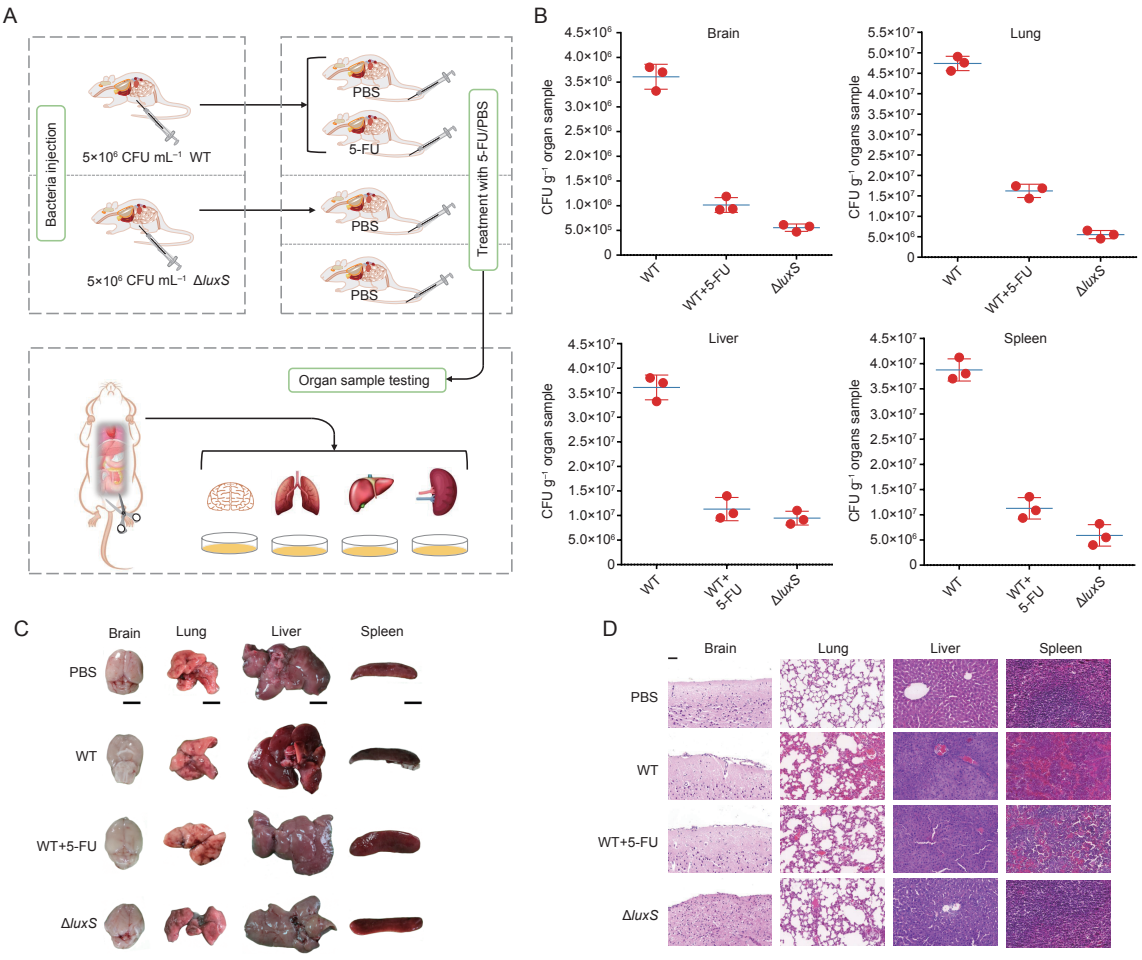


Fig. 6 *In vivo* therapeutic effect of 5-fluorouracil (5-FU) in a mouse model of *Streptococcus suis* infection. A, schematic diagram of mouse infection model. B, bacterial load in the brain, lung, liver and spleen of *S. suis* wild type (WT)-infected or the Δ*luxS*-infected BALB/c mice treated with 5-FU (20 μg g^{-1}). C, the morphological changes of brain, lung, liver and spleen, scale bar: 0.5 cm. D, histopathologic changes of *S. suis* infections caused, magnification: 200×, scale bar: 50 μm.

5. Conclusion

Taken together, our results suggest that 5-FU affects the LuxS/AI-2 QS system of *S. suis via thyA*, thereby reducing biofilm production and virulence, ultimately leading to reduced pathogenicity. Therefore, 5-FU can be used to guide the development of novel anti-biofilm drugs to control *S. suis* infections.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

Ethical approval

The animal research protocol received approval from the Animal Care and Use Committee of Henan University of Science and Technology (approval number: HAUST-023-M0913011).

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