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Effects of Paeonol Sub-inhibitory Concentration on *Streptococcus suis* Biofilm and Expression of Virulence Genes

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ABSTRACT

Background: *Streptococcus suis* is a zoonotic pathogen closely related to the respiratory tract of swine. Paeonol is a traditional Chinese medicine with antibacterial effect. This study demonstrated the effect of paeonol on biofilm formation and the expression of virulence factors of *Streptococcus suis*.

Methods: The minimum inhibitory concentration (MIC) of paeonol on *Streptococcus suis* was determined by double dilution method. Crystal violet staining method was used to determine the effect of paeonol on the biofilm formation. The effects of paeonol on the morphology and structure of *Streptococcus suis* biofilm were observed by field emission scanning electron microscopy (FE-SEM). Real-time quantitative PCR (qRT-PCR) was used to detect the expression levels of *Streptococcus suis* related virulence factors under sub-MIC (1/4 MIC, 1/8 MIC) paeonol.

Result: The results showed the effect of 1/4 MIC and 1/8 MIC paeonol on biofilm formation and mature biofilm was significant (*P<0.05). Under the action of paeonol, the biofilm structure becomes sparse. qRT-PCR results showed that 1/8 MIC paeonol inhibits bacterial adhesion by up-regulating the expression of *mrp* virulence genes, thereby inhibiting the formation of *Streptococcus suis* biofilm.

Key words: Biofilm, Inhibition, Paeonol, *Streptococcus suis*, Virulence genes.

INTRODUCTION

Streptococcus suis (*S. suis*) is a vital gram-positive zoonotic pathogen, that can stick to each other, adhere to the host cell to form a biofilm shape and cause persistent infection, which is related to arthritis, septicaemia, endocarditis, bronchopneumonia, meningitis and other diseases in swine (Tang *et al*., 2006). The bacterial carrying rate of swines is close to 100% and the mortality rate is close to 20% (Cloutier *et al*., 2003), which cause huge property damage to the global swine industry (Guo *et al*., 2009).

Bacterial Biofilm (BF) is a membrane-like structure formed by bacteria that combine together by secreting polysaccharide complex and polysacchari, de matrix in order to adapt to harsh environment (Fuente *et al*., 2013; Wang *et al*., 2014). The biological activities of bacteria under the biofilm are changed (Wang *et al*., 2011; Wang *et al*., 2018; Li *et al*., 2021). In addition, studies have shown that biofilms are related to the production of bacterial resistance and the expression of virulence factors (Penesyan *et al*., 2015; Qi *et al*., 2016).

Antibiotics are commonly used to prevent and treat infections caused by *S. suis*, but the overuse of antibiotics has led to strains showing multiple resistance (Devi *et al*., 2017; Liu *et al*., 2020). Chinese herbal medicine has become a hot spot in the research and development of new bacteriostatic agents because of its green, natural and non-toxic advantages (Abdallah *et al*., 2018; Raju *et al*., 2018; Millar *et al*., 2021). Many studies have shown that Chinese herbal medicine has an inhibitory effect on bacterial (Szliszka *et al*., 2009; Chen *et al*., 2016; Ding *et al*., 2017; Acharya *et al*., 2020). Paeonol, also known as paonol or paeoniflorin, is an active ingredient extracted from the dry root bark of the belladonna vitex (Xu *et al*., 2005). Studies have shown that paeonol has such effects as sedation, cooling blood and clearing heat, bacteriostasis and anti-tumor (Li *et al*., 2010; Zhang *et al*., 2019). Recent studies have also shown that paeonol can inhibit bacterial adhesion and thus affect their biofilms (Qian *et al*., 2021). Based on this, paeonol was selected in this study to explore its effects on *S. suis* biofilm and the expression of virulence genes.

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MATERIALS AND METHODS

This experiment was conducted in Luoyang Key Laboratory of Animal Molecular Pathogens and Immunology, Henan University of Science and Technology from November 2020

to August 2021 to study the effects of paeonol on *S. suis* biofilm and the expression of related virulence factors.

Bacterial strains and paeonol susceptibility testing

HA9801 (HA) was isolated from swine infected with *S. suis* in Jiangsu Province which identified as a virulent strain (Wang *et al*., 2014). Add 100 mg paeonol to 10 ml sterile distilled water which containing 10% Tween-80 (Papandreou *et al*., 2002), the final concentration of paeonol mother liquor was 10 mg/ml. HA strains were grown in Tryptone soya broth (TSB) (Thermo Fisher Scientific, Shanghai, China). The MIC of Paeonol to HA was determined according to the double dilution method stipulated by CLSI.

Effects of Paeonol on biofilm formation under sub-inhibitory concentration

Crystal violet staining method was used to determine the effect of paeonol on the biofilm formation. A single colony was placed into 3 ml of TSB and incubated density to 10^6 CFU/mL (Li *et al*., 2021). HA suspension was added to the 96-well plate (Costar, Corning, Shanghai Aiyan Biological Technology Co., LTD, Shanghai, china), followed by adding 1/2 MIC, 1/4 MIC, 1/8 MIC and 1/16 MIC paeonol solutions respectively. After 48 h of culture, the sample wells were washed lightly twice with 200 µl Phosphate buffered saline (PBS) and immobilized with 95% methanol (w/w) at 37°C for 20 min. Then stained the biofilm with 0.1% crystal violet for 15 min and dissolved with 95% ethanol for 30 min, the OD value was measured at OD_{595} nm using Automatic microplate reader (Infinite M Nano, Tecan Trading Co., Ltd., Shanghai, China). All assays were set 6 replicate controls and repeated 3 times.

Field emission scanning electron microscopy (FE-SEM)

The structure of *Streptococcus suis* biofilm were observed by Field emission scanning electron microscopy (FE-SEM). 1 ml of HA with the final concentration of 10^6 CFU/ml was added to the 24-well plate of cell culture plate (Corning, Beijing Yaanda Biotechnology Co., Ltd. Beijing, China) and 1 ml of TSB containing 1/2 MIC and 1/4 MIC paeonol were added respectively and cultured at 37°C for 48 h. The slides were washed with PBS lightly, adding 2.5% pentaglycol and immobilized at 4°C overnight and washed with PBS for 3 times, the slides were dehydrated in gradients of 30%, 50%, 70%, 80% and 90% ethanol (w/w). After being added for 15 min each time, the slides were finally washed twice with 100% ethanol (Yi *et al*., 2020). After drying, the gold-plated film is sputtered and the air is pumped, then the scanning electron microscope is used to observe the biofilm structure at 5.0 KV (JSM-7800F; Japan Electronics Co. LTD, Tokyo, Japan).

Effects of paeonol on mature biofilm under Sub-inhibitory concentration

A single colony was placed into 3 ml of TSB and incubated density to 10^6 CFU/mL After multiple dilution, HA was added to 96-well plates with 6 replicates in each group. Culture 48 h, suspension was absorbed, 1/2 MIC, 1/4 MIC, 1/8 MIC and 1/16 MIC paeonol solutions were added respectively. Incubate for another 8 h, the detection method was carried out in accordance with the method for the effect of paeonol on the formation of biofilm.

Quantitative RT-PCR

Detection of HA virulence genes expression level by qRT-PCR. In brief, cultivate a single colony in TSB overnight, transfer to TSB containing 1/4 MIC or 1/8 MIC paeonol according to 1:100 and cultivate for 8 h. The total RNA of HA was extracted by TRIzon (Shenggong Biological Engineering Co., Ltd, Shanghai, china) method and the cDNA obtained by reverse-transcription was used PrimeScript™ reagent kit (CoWin Biosciences Co., Ltd, Biejing, china). qRT-PCR was used for detecting the transcription levels of virulence genes *ef*, *gapdh*, *srtA*, *fbps*, *gdh*, *mrp* and 16S rRNA were calculated by using the $2^{-\Delta\Delta CT}$ method, the 16S rRNA gene as an internal control. Primer information is shown in Table 1.

Data analysis

Statistical analysis was performed using SPSS 24.0. Biofilm formation and mature biofilm results are based on one-way analysis of variance (ANOVA), qRT-PCR results are based on two-way analysis of variance. The data presented represent three independent experiments and are represented as mean±SE. Control group without paeonol. *p<0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Paeonol susceptibility testing

The results showed the MIC of paeonol to HA was 640 µg/mL. The tween-80 which used to dissolve paeonol had no significant effect on the life activities of HA, so we excluded the interference caused by tween-80 to the experiment.

Sub-inhibitory concentration paeonol inhibited biofilm formation

Sub-MIC drug was selected to rule out the possibility that paeonol could weaken the biofilm by inhibiting the growth

Table 1: Primers needed for the real-time quantitative PCR analysis.

Genes	Primer sequence
ef-1	TCCAA TCACA GATCC AGATA GCG
ef-2	CTGAC CCATT TGGAC CATCT AAG
gapdh-1	CTTGG TAATC CCAGA ATTGA ACGG
gapdh-2	TCATA GCAGC GTTTA CTTCT TCAGC
SrtA-1	GAACC GCAAT CCCAC CAAT
SrtA-2	AAAAG AATAA ACAGG CTGAG ACAAC A
fbps-1	AACCA TCTTG CCAGG CTCCA C
fbps-2	CAGTT CAGAA GCCGT ATCCC GAC
gdh-1	CACCT TTACC ACCGC CGATT G
gdh-2	GGAAA TGTTC AAGTC AACCG TGG
mrp-1	CAGGT AACAT CAGAA TCACC ACTTT T
mrp-2	AAGTT TTGTT TGAGC ATCCT CTATA GC

of HA. The results (Fig 1) showed that in the range of 1/2 MIC to 1/8 MIC, the number of biofilms showed a downward trend as the concentration of paeonol changed. 1/4 MIC and 1/8 MIC Paeonol significantly affected the ability of HA biofilm formation. Then as the concentration of paeonol decreased, the ability of HA to form biofilm gradually recovered.

Morphology of biofilm under field emission scanning electron microscope (FE-SEM)

The structural variation difference of HA biofilm was observed under scanning electron microscope after paeonol treatment in control group (A), 1/4 MIC paeonol (B) and 1/8 MIC paeonol (C). The FE-SEM image shows a three-dimensional structure by the adhesion of HA on the cell slipper. The magnification of electron microscope is 5000 ×. From the electron microscope (Fig 2), It was observed that the biofilm of the control group was compact and HA was tightly folded. After the sub-MIC (1/4 MIC, 1/8 MIC) paeonol action, the HA quantity was reduced and the membrane structure became dispersed and fragmental. In addition, the biofilm structure under the effect of 1/8 MIC paeonol was looser than that under the effect of 1/4 MIC paeonol, indicating that the effect of 1/8 MIC paeonol on the biofilm formation was greater than that under the effect of 1/4 MIC paeonol. This is also consistent with our previous conclusion that paeonol has a significant effect on the biofilm formation at 1/4 MIC and 1/8 MIC.

Sub-inhibitory concentration paeonol inhibited mature biofilm

The biofilm was placed in automatic microplate reader to determine the value of OD_{595} nm. Compared with the control group (Fig 3), the paeonol in the experimental concentration range has a certain degree of influence on the biofilm of HA. But the effect of paeonol on the formation of biofilms is much greater than that on mature biofilms.

Regulation of paeonol on virulence factors

1/4 MIC and 1/8 MIC paeonol had the most significant effect on the biofilm. Therefore, the 1/4 MIC and 1/8 MIC paeonol were selected in this experiment to explore the effects on the expression levels of HA-related virulence genes. The results (Fig 4) showed that 1/4 MIC paeonol down-regulated the transcription levels of *ef*, *gapdh* and *srtA* virulence genes in HA, while 1/8 MIC paeonol up-regulated the expression levels of *ef*, *gapdh*, *srtA*, *fbps*, *gdh* and *mrp* virulence genes in HA, with the most significant effect on *mrp*. Up-regulation of *mrp* virulence gene expression can inhibit bacterial adhesion, so as to inhibit the formation of HA biofilm (Wang *et al*., 2011). Studies have also shown that *mrp* is related to bacterial biofilm and the expression of virulence and the up-regulation of *mrp* is also inextricably linked to the decrease of biofilm (Wang *et al*., 2011; Wang *et al*., 2014).

In the presence of sub-MIC tetracycline, the biofilm formation ability and the number of viable bacteria under

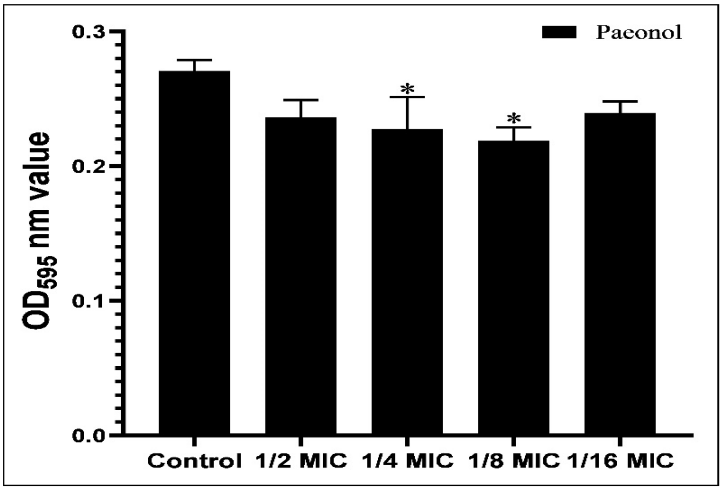

Fig 1: Sub-MIC paeonol effect the formation of HA biofilm.

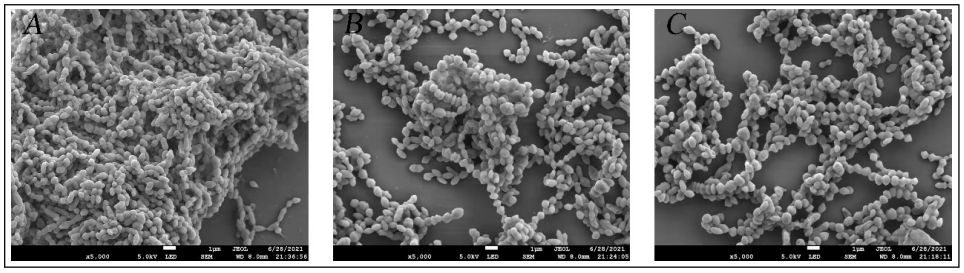

Fig 2: Field emission scanning electron microscopy image of HA biofilms (WT, 1/4 MIC paeonol and 1/8 MIC paeonol).

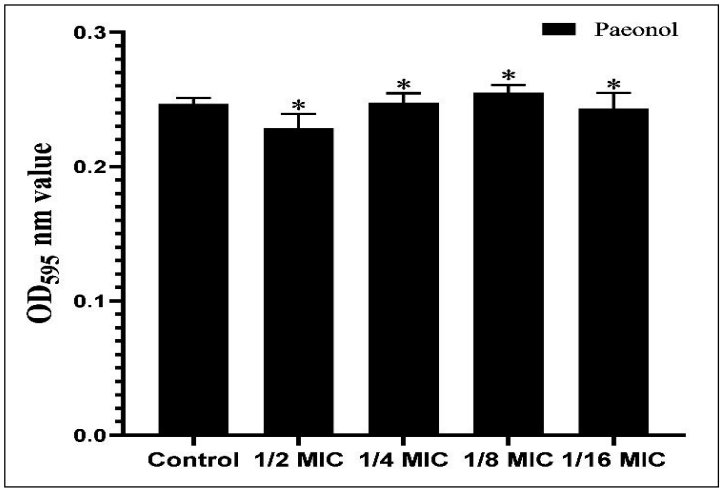

Fig 3: Effects of sub-MIC paeonol on HA mature biofilm.

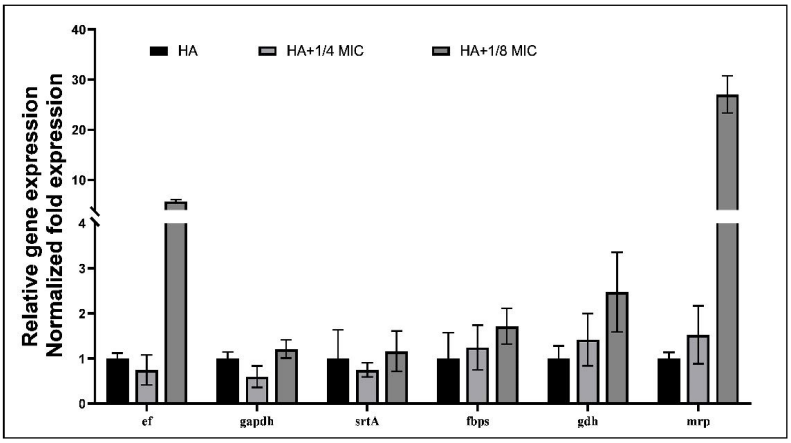

Fig 4: Effects of paeonol on the relative expression of virulence genes in HA.

the envelope increase in *Streptococcus pneumoniae,* which may be regulated by the LuxS/AI-2 density sensing system (Ahmed *et al*., 2009). Sub-MIC amoxicillin can induce the production of extracellular genes of *Staphylococcus aureus*, thereby stimulating the formation of biofilms (Mlynek *et al*., 2020), Azithromycin with sub-MIC could weaken the formation of biofilm and increase the content of capsular polysaccharide (Yang *et al*., 2016), Paeoniflorin inhibits the AI-2 signaling molecules in *Streptococcus suis* through mass effect, thereby attenuating the formation of *Streptococcus suis* biofilm (Li *et al*., 2021), Paeonol down-regulated genes related to density sensing in *Pseudomonas aeruginosa* and attenuated the virulence of the pathogen (Yang *et al*., 2021). Therefore, the effect of paeonol with low inhibitory concentration on HA biofilm may also be realized by affecting the density sensing system or the synthesis and secretion of exopolysaccharide and the specific regulatory mechanism needs to be further studied.

CONCLUSION

Paeonol sub-MICs affects biofilm formation and virulence gene expression in *S. suis*. These findings suggested that investigating the effect of Chinese herbal medicine paeonol on bacterial biofilms may lead to the development of natural substance treatments.

Data availability

The datasets generated during and analyzed during the study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

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Declaration of competing interest

The author declares that there is no conflict of interest between the publication of this paper and other persons or organizations.

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Author contributions

JG contributed to the execution of the trial and the writing of the initial draft. PL and QX performed the design of the experiment. Material preparation, data collection and analysis were conducted by YF and LZ. The funds were collected by YW and YW. All authors read and approved the final manuscript.

Conflict of interest : None.

REFERENCES

Abdallah, A., Zhang, P., Zhong Q., Sun, Z. (2018). Application of Traditional chinese herbal medicine by-products as dietary feed supplements and antibiotic replacements in animal production. Current Drug Metabolism. 20: 54-64.

Acharya, P., Lathwal, S.S., Baithalu, R., Patnaik, N., Thul, M.R., Moharana, B. (2020). Supplementing rumen protected choline with green tea extract improves reproductive performances in transition karan fries cows. Indian Journal of Animal Research. 54: 452-455.

Ahmed, N.A., Petersen, F.C., Scheie, A.A. (2009). AI-2/LuxS Is Involved in increased biofilm formation by *Streptococcus intermedius* in the presence of antibiotics. Antimicrobial Agents and Chemotherapy. 53: 4258-4263.

Chen, J., Zhou, Y., Yang, Y., Bai, J., Li, H. (2016). Screening of Chinese herbal extracts against *Streptococcus suis* biofilm formation. Chinese Journal of Veterinary Medicine. 52: 14-19.

Cloutier, G., Allaire, S.D., Martinez, G., Surprenant, C., Lacouture, S., Gottschalk, M. (2003). Epidemiology of *Streptococcus suis* serotype 5 infection in a pig herd with and without clinical disease. Veterinary Microbiology. 97: 135-151.

Devi, M., Dutta, J.B., Rajkhowa, S., Kalita, D., Saikia, G.K., Das, B.C., Hazarika, R.A., Mahato, G. (2017). Prevalence of multiple drug resistant *Streptococcus suis* in and around Guwahati, India. Veterinary World. 10: 556-561.

Ding, W., Li, Y., Lian, H., Ai, X., Zhao, Y., Yang, Y., Han, Q., Liu, X., Chen, X., He, Z. (2017). Sub-Minimum Inhibitory Concentrations of Rhubarb Water Extracts Inhibit *Streptococcus suis* Biofilm Formation. Frontiers in Pharmacology. 8: 425.

Fuente-Núñez, C., Reffuveille, F., Fernández, L., Hancock, R.E. (2013). Bacterial biofilm development as a multicellular adaptation: Antibiotic resistance and new therapeutic strategies. Current Opinion in Microbiology. 16: 580-589.

Guo, Y., Ryan, U., Feng, Y., Xiao, L. (2009). *Streptococcus suis*: An Emerging Human Pathogen. Clinical Infectious Diseases. 48: 617-625.

Li, J., Fan, Q., Jin, M., Mao, C., Zhang, H., Zhang, X., Sun, L., Grenier, D., Yi, L., Hou, X., Wang, Y. (2021). Paeoniflorin reduce *luxS*/AI-2 system-controlled biofilm formation and virulence in *Streptococcus suis*. Virulence. 12: 3062-3073.

Li, J., Fan, Q., Mao, C., Jin, M., Yi, L., Wang, Y. (2021). Effect of biofilm formation on the *Escherichia coli* drug resistance of isolates from pigs in Central China. Indian Journal of Animal Research. 55: 697-703.

Li, N., Fan, L.L., Sun, G.P., Wan, X.A., Wang, Z.G., Wu, Q., Wang, H. (2010). Paeonol inhibits tumor growth in gastric cancer *in vitro* and *in vivo*. World J Gastroenterol. 16: 4483-4490.

Liu, B., Yi, L., Li, J., Gong, S., Dong, X., Wang, C., Wang, Y. (2020) Norfloxacin sub-inhibitory concentration affects *Streptococcus suis* biofilm formation and virulence gene expression. Indian Journal of Animal Research. 54: 342-348.

Millar, B.C., Rao, J.R., Moore, J.E. (2021). Fighting antimicrobial resistance (AMR): Chinese herbal medicine as a source of novel antimicrobials - an update. Letters in Applied Microbiology. 73: 400-407.

Mlynek, K.D., Bulock, L.L., Stone, C.J., Curran, L.J., Sadykov, M.R., Bayles, K.W., Brinsmade, S.R. (2020). Genetic and biochemical analysis of codY-mediated cell aggregation in *Staphylococcus aureus* reveals an interaction between extracellular DNA and polysaccharide in the extracellular matrix. Journal of Bacteriology. 202: e00593-19.

Papandreou, V., Magiatis, P., Chinou, L., Kalpoutzakis, E., Skaltsounis, A.L., Tsarbopoulos, A. (2002). Volatiles with antimicrobial activity from the roots of Greek Paeonia taxa. Journal of Ethnopharmacology. 81: 101-104.

Penesyan, A., Gillings, M., Paulsen, I.T. (2015). Antibiotic discovery: Combatting bacterial resistance in cells and in biofilm communities. Molecules. 20: 5286-5298.

Qi, L., Li, H., Zhang, C., Liang, B., Li, J., Wang, L., Du, X., Liu, X., Qiu, S., Song, H. (2016). Relationship between antibiotic resistance, biofilm formation and biofilm-specific resistance in *Acinetobacter baumannii*. Frontiers in Microbiology. 7: 483.

Qian, W., Li, X., Yang, M., Mao, G. (2021). Antibacterial and anti-biofilm activities of paeonol against *Klebsiella pneumoniae* and *Enterobacter cloacae*. Biofouling. 37: 1-14.

Raju, A.B., Reddy, N.S. (2018). Antiarthritic and antioxidant activities of gossypium herbaceum plant (cottonplant) leaves. Agricultural Science Digest - A Research Journal. 38: 88-94.

Szliszka, E., Czuba, Z.P., Domino, M., Mazur, B., Zydowicz, G., Krol, W. (2009). Ethanolic Extract of Propolis (EEP) Enhances the Apoptosis- Inducing Potential of TRAIL in Cancer Cells. Molecules. 14: 738-754.

Tang, J., Wang, C., Feng, Y., Yang, W., Song, H., Chen, Z., Yu, H., Pan, X., Zhou, X., Wang, H. (2006). *Streptococcal* toxic shock syndrome caused by *Streptococcus suis* serotype 2. PLoS Medicine. 3: 668-676.

Wang, Y., Wang, Y., Sun, L., Grenier, D., Yi, L. (2018). *Streptococcus suis* biofilm: Regulation, drug-resistance mechanisms and disinfection strategies. Applied Microbiology and Biotechnology. 102: 9121-9129.

Wang, Y., Yi, L., Zhang, Z., Fan, H., Cheng, X., Lu, C. (2014). Biofilm formation, host-cell adherence and virulence genes regulation of *Streptococcus suis* in response to autoinducer-2 signaling. Current Microbiology. 68: 575-580.

Wang, Y., Zhang, W., Wu, Z., Lu, C. (2011). Reduced virulence is an important characteristic of biofilm infection of *Streptococcus suis*. FEMS Microbiology Letters. 316: 36-43.

Wang, Y., Zhang, W., Wu, Z., Zhu, X., Lu, C. (2011). Functional analysis of *luxS* in *Streptococcus suis* reveals a key role in biofilm formation and virulence. Veterinary Microbiology. 152: 151-160.

Xu, X., Gao, J., Chen, J., Li, S., Yang, X., Song. H. (2005). Preparation and Crystal Structure of Paeonol. JIEGOU HUAXUE. 24: 436-438.

Yang, D., Hao, S., Zhao, L., Shi, F., Ye, G., Zou, Y., Song, X., Li, L., Yin, Z., He, X., Feng, S., Chen, H., Zhang, Y., Gao, Y., Li, Y., Tang, H. (2021). Paeonol Attenuates Quorum-Sensing Regulated Virulence and Biofilm Formation in *Pseudomonas aeruginosaz*. Frontiers in Microbiology. 12: 692474.

Yang, Y., Chen, J., Zhao, Y., Bai, J., Ding, W., Zhou, Y., Chen, X., Liu, D., Li, Y. (2016). Sub-MICs of Azithromycin Decrease Biofilm Formation of *Streptococcus suis* and Increase Capsular Polysaccharide Content of *S. suis*. Frontiers in Microbiology. 7:1659.

Yi, L., Li, J., Fan, Q., Mao, C., Jin, M., Liu, Y., Sun, L., Grenier, D., Wang, Y. (2020). The otc gene of *Streptococcus suis* plays an important role in biofilm formation, adhesion and virulence in a murine model. Veterinary Microbiology. 251. 108925

Zhang, L., Li, D., Liu, L. (2019). Paeonol: Pharmacological effects and mechanisms of action. International Immuno Pharmacology. 72: 413-421.