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RESEARCH ARTICLE

Chinese Medicine Monomers Inhibit Biofilm Formation in Multidrug-Resistant *P. multocida* Isolated from Cattle Respiratory Infections

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ABSTRACT

Bovine respiratory diseases are a major problem for animals and are caused by *Pasteurella multocida*, commonly found in animals' respiratory tracts. *P. multocida* is considered as a secondary pathogen, and the epidemiology of this bacteria related to the respiratory tract poorly investigated yet. By use of high antimicrobial drugs in the clinics, coupled with the fact that the biofilm formed by *P. multocida* is conducive to increase its resistance to unfavourable environment, the resistance of *P. multocida* in the bovine respiratory tract is becoming increasingly serious, which poses a certain challenge to the treatment of bovine respiratory diseases. In this study, the Kirby Bauer (K.B) disk diffusion method was used to conduct *in vitro* drug susceptibility testing of 20 antimicrobial drugs on 15 isolates of *P. multocida*; PCR detected the carriage of relevant resistance genes of the isolates; the crystal violet staining method was used to determine the ability of the isolates to form biofilm, and the effects of thymol and berberine on the adherence value (B-value) of the biofilm of the isolates and their growth curves; laser confocal microscopy was used to observe the effects of thymol and berberine on the formation of biofilm; the effects of thymol and berberine on the expression of genes *luxS*, *fliA*, and *motA* related to biofilm formation were detected by qRT-PCR. The results showed that all 15 isolates showed 100% sensitivity to tilmicosin, ceftriaxone, ampicillin/sulbactam, and florfenicol, and 53.3% resistance to erythromycin; the resistance rates to lincomycin, clindamycin, and streptomycin were 93.3, 46.7, and 40%, respectively. The aminoglycoside resistance genes *strA* and *strB* had the highest detection rates of 93.3 and 80%, respectively, followed by the macrolide resistance gene *mefE* with a detection rate of 66.7% and the β-lactam resistance gene *blaROB-1* with a detection rate of 60%. There were 13 biofilm-positive isolates, accounting for 86.7% of the total. The Thymol MIC group, berberine MIC group, and 1/2 MIC group could inhibit the formation and growth of the strain's biofilm in a concentration-dependent manner. Thymol MIC, berberine MIC, and 1/2 MIC groups all significantly down-regulated the expression of *luxS*, *fliA*, and *motA*, genes related to biofilm formation. The results of this study provide medication references and theoretical guidance for the prevention and treatment of bovine respiratory diseases caused by biofilm-positive *P. multocida* infections in clinics and reduce the resulting economic losses.

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INTRODUCTION

Bovine respiratory disease is a collective term for diseases such as pneumonia and bronchitis in cattle caused by viral or bacterial infections alone and in combination (Grissett *et al.*, 2015). Their pathogenesis shows complexity and involves several different infectious agents (Zamri-Saad and Annas, 2016). The infection is caused when the animal is stimulated by stressful conditions such as weaning or transportation,

compromising the immune barrier (Kishimoto *et al.*, 2017). Under these stress conditions, bacteria are more likely to invade the animal's respiratory tract and induce severe respiratory disease. *Pasteurella multocida*, *Mannheimia haemolytica*, and *Haemophilus somnolentus* are the three main bacterial pathogens of bovine respiratory diseases. The bacteria have evolved to colonize and persist on respiratory tract mucosa and evade host immunity. A number of adaptive mechanisms are involved, including adhesin expression, the production and secretion of toxins and proteases, and the formation of biofilms (Griffin, 2010). Affected animals clinically exhibit symptoms such as high fever, depression, decreased appetite, respiratory distress, coughing, and salivation, which may even lead to death in severe cases. The disease can occur in animals of all ages, especially young animals are more susceptible. According to statistics, 65% of the diseases in the farming industry are related to respiratory diseases (Ozsvari *et al.*, 2012), the morbidity rate reaches 30 to 80%, and the morbidity and mortality rate can reach 10 to 35%. The annual global economic loss due to bovine respiratory diseases is as high as 30 billion dollars. After the first isolation of *P. multocida* bovis from the lungs of cattle with respiratory diseases in China in 2008, reports of bovine respiratory diseases caused by *P. multocida* have been reported in various provinces of China (Ma WenGe and Yu Li, 2008).

Gram-negative bacterium *P. multocida* produces sticky polysaccharides in its biofilm (Boulianne *et al.*, 2020). Upon successfully colonizing certain parts of the host, the bacteria form a biofilm on the epithelial surface. In the early stages of colonization, the bacteria are encapsulated in a mucopolysaccharide matrix secreted by the bacteria, and these mucopolysaccharides can make the bacteria resistant to the host's defense mechanisms and the action of antimicrobial drugs. Antimicrobial drugs are commonly used to control and treat diseases caused by *P. multocida*, and resistance may develop due to the irrational use of subinhibitory concentrations of antimicrobial drugs. Bacteria in the biofilm are extremely drug-resistant, and once the bacteria at the site of infection have formed a biofilm, their resistance to antimicrobial drugs is more than 500 to 1000 times that of the free state (Prajapati *et al.*, 2020), and they can evade the host's immune response, which has become one of the significant reasons why infections are challenging to solve in the clinics. In order to reduce the residue of antimicrobial drugs in the body and avoid the development of drug-resistant strains, some studies have attempted to use herbal medicines to manage infectious diseases caused by *P. multocida*.

Thymol (5-methyl-2-isopropylphenol) is an aromatic compound derived from the Chinese plant Thymus vulgaris family Thymusaceae and commonly used as a broad-spectrum against the different bacterial and fungal diseases (Hajibonabi *et al.*, 2023). Thymol, also known as muskgrass phenol, is an organic compound, colourless crystals, or colourless crystalline powder at room temperature, with the special smell of thyme or muskgrass can be used as a spice. In addition, thymol is an active phenolic ingredient from the terpenoid group, and it can be found in different parts of plants, such as oregano, thyme sweet basil, black cumin and savoury (Valdivieso-Ugarte *et al.*, 2019). Besides antibiotic effects, thymol can be used as an anti-inflammatory, anticarcinogenic, and antispasmodic drug (Hajibonabi *et al.*, 2023). Berberine is a quaternary ammonium alkaloid naturally occurring in a variety of plants, a yellow needle-like crystal with a bitter taste. In Chinese medicine, berberine is commonly used as a heat-removing and detoxifying drug. The main active ingredient is berberine, which has a wide range of biological activities, including antibacterial, anti-inflammatory, and antioxidant effects. Therefore, it has an important application value in the field of medicine. Berberine is a broad-spectrum antibacterial drug used against different bacteria, viruses, fungi, protozoa and helminths. It is an isoquinoline alkaloid commonly used in bacterial infections and particularly in multi-resistant strains (Sun *et al.*, 2019).

Therefore, in this study, we determined the drug resistance status and resistance gene carriage of *P. multocida* in vitro drug susceptibility testing and resistance gene detection; Screening of biofilm-positive strains, determination of biofilm adhesion values, determination of biofilm growth curves, and observation of biofilm morphology by laser confocal scanning microscopy were used to determine the formation of biofilm in *P. multocida*, and whether thymol and berberine had an effect on the formation of the biofilm in *P. multocida*. The expression of genes related to biofilm formation after the action of thymol and berberine was detected by qRT-PCR to determine the mechanism associated with the inhibition of biofilm formation of *P. multocida* by thymol and berberine.

MATERIALS AND METHODS

Bacterial isolates: The isolates of *Pasteurella multocida* were isolated and identified from large-scale pastures in Inner Mongolia, Gansu, and Anhui, China, and stored in 25% concentration of glycerol at -80°C in a freezer. The standard strain of *Escherichia coli*, ATCC 25922, was provided by the Laboratory of Pharmacology and Toxicology, College of Veterinary Medicine, Inner Mongolia Agricultural University, China.

Drug susceptibility testing: The K-B method was used for the sensitivity test of the isolates of 20 antimicrobial drugs, and the *P. multocida* isolates were inoculated in tryptic soy broth (TSB) medium containing 5% neonatal bovine serum, incubated at 37°C for 12h, and the concentration of the bacterial solution was adjusted to 1.5×10^8 CFU/mL. Aspirate 100μL of bacterial solution was spread evenly on tryptic soy agar (TSA) medium containing 5% neonatal bovine serum, and the drug-sensitive paper sheet was placed after the surface of the medium was dried, and incubated at 37℃ for 24h (Murray *et al.*, 2015). later, we measured the diameter of the circle of inhibition and statistically counted the susceptibility, intermediary, and drug-resistance rates. The results were determined with reference to the determination criteria (Table 1) provided by the American Committee for Clinical Laboratory Standardization, CLSI 2020 edition, Appendix M100-S24 (CLSI, 2020). The quality control organism was the standard strain of *Escherichia coli*, ATCC 25922.

Table 1: Sensitivity criteria for 20 antibiotics

Drug type	Drug name	Drug content	Judgment Criteria (Inhibition Circle Diameter/mm)			*Escherichia coli* ATCC 25922 (QC range/mm)
		(μg/tablet)	Sensitivities	Intermediary	Drug-resistant	
Aminoglycosides	Streptomycin	10	≥15	12～14	≤11	12-20
	Amikacin	30	≥17	15～16	≤14	19-26
	Daecamycin	100	≥14	11～13	≤10	10-16
	Gentamicin	10	≥15	11～14	≤12	19-26
Quinolones	Ciprofloxacin	5	≥21	16～20	≤15	29-37
	Ofloxacin	5	≥17	14～16	≤13	29-33
	Enrofloxacin	5	≥21	17～20	≤16	-
Sulfonamides	Compound sulfamethoxazole	23.75/1.25	≥16	11～15	≤10	23-29
Tetracyclines	Tetracycline	30	≥19	15～18	≤14	18-25
	Doxycycline	30	≥23	16～22	≤15	18-24
Macrolides	Erythromycin	15	≥23	14～22	≤13	-
	Tilmicosin	15	≥14	11～13	≤10	-
β-lactams	Ampicillin	10	≥15	12～14	≤11	15-22
	Cefotaxime	30	≥25	17～24	≤16	29-35
	Ceftriaxone	30	≥21	14～20	≤13	29-35
	Ampicillin / sulbactam	10	≥15	12～14	≤11	19-24
Chloromycetins	Florfenicol	30	≥19	15～18	≤14	21-27
Lincosamides	Lincomycin	2	≥30	25～29	≤24	-
	Clindamycin	2	≥21	15～20	≤14	-
Polypeptides	Polymyxin B	300IU	≥12	9～11	≤8	13-19

Drug resistance Gene testing: After extracting the DNA of the isolated strains, PCR was used to detect the aminoglycoside resistance genes *aadA25*, *aadB*, *strA*, *strB*; quinolone resistance genes *qnrA*, *qnrB*, *qnrD*, *qepA*; sulfonamide resistance genes *Sul1*, *Sul2*, *Sul3*; tetracycline resistance genes *tetA*, *tetB*, *tetG*, *tet(H)*; macrolide resistance genes *erm42*, *ermA*, *mphE*, *mefE*; β-lactam resistance genes *blaTEM*, *blaOXA-1*, *blaROB-1*; lincosamide resistance gene *lnuA* (Table 2). PCR products were detected by agarose gel electrophoresis (Sambrook and Russell, 2001).

Screening of biofilm-positive strains: The diluted bacterial solution 200uL (1:100) was added into the wells of sterile 96 microtiter plates, and the corresponding amount of TSB medium was put into the blank control wells, sealed the plate, and incubated at 37°C for 24h. Discarded the liquid in the wells at the end of the incubation and rinsed it with sterile PBS solution three times; 99% methanol was fixed for 15 min, and the methanol was discarded; the plate was stained with 2.5% crystal violet for 5 min, and rinsed with running tap water until the washings were colourless; the plates were naturally air-dried, and the dye in the binding wells was dissolved with 33% glacial acetic acid, and after dissolution, the OD_{570nm} values of the wells were determined using microplate reader. The test was repeated three times, and the results were averaged (Sun *et al.*, 2019).

Biofilm forming capacity: critical value (ODc) = mean of blank control + (3 x standard deviation of blank control). When 4ODc<OD, *P. multocida* was judged to have strong biofilm formation ability; 2ODc<OD<4ODc, *P. multocida* was judged to have medium biofilm formation ability; ODc<OD<20Dc, *P. multocida* was judged to have weak biofilm formation ability; OD<ODc, *P. multocida* was judged to have no biofilm formation ability (Saxena *et al.*, 2014).

Determination of MIC of thymol and berberine: The MICs of thymol and berberine on one strain of *P. multocida* with multiple compound resistant phenotypes and a strong positive biofilm were determined by the microdilution method (CLSI, 2020) , and the concentrations of thymol and berberine were 5mg/mL and 3mg/mL, respectively, according to the results of the pre-tests, and then prepared for use. Before using the microdilution method to determine the MICs of thymol and berberine on three multicidal *P. multocida* strains, the susceptibility of the isolates to 20 antimicrobial drugs were detected by the K-B method. Based on the results of drug sensitivity of multicidal *P. multocida* (PM) isolates, the resistance phenotypes of them were determined. PM 1 was resistant to ciprofloxacin, ofloxacin, lincomycin, and streptomycin, moderately resistant to amikacin, enrofloxacin, and trimethoprim-sulfamethoxazole; PM 2 was resistant to gentamicin, ofloxacin, lincomycin, trimethoprim-sulfamethoxazole, streptomycin, amikacin, ciprofloxacin and enrofloxacin; PM 3 was resistant to gentamicin, ofloxacin, lincomycin, streptomycin, amikacin, ciprofloxacin, and enrofloxacin. Three parallel samples are set up for each experiment. Then *P. multocida* biofilm positive strains were screened by ELISA method (CLSI, 2020). When 4ODc<OD, critical value (ODc), *P. multocida* was judged to have strong biofilm formation ability, the ODs of the selected strains were all greater than 4ODc, so we selected the above strains for further research.

Determination of biofilm adhesion value: According to the determined MIC of thymol and berberine on the isolated strains, the drug concentration of the two herbal monomers was configured as 2 MIC, MIC, 1/2 MIC, and 1/4 MIC. In 96 microtiter plates, 100μL of diluted drug solution and 100μL of bacterial solution at a concentration of 1.5×10^8CFU/mL were added to each well and then mixed so that the final concentration of the drug in the wells became 1/2 of the original, and three replicates were set up in each well, with drug control, bacterial solution control, and blank control, respectively, and the culture was incubated at 37°C for 24h. At the end of the incubation, the OD_{595nm} value of each well was determined using an enzyme marker and expressed as B1; the liquid in the wells was discarded, and the wells were rinsed with sterile distilled water and then dried at room temperature. After drying, each well was stained by

Table 2: Primer information of related drug resistance genes

Gene	Primer sequence (5′→3′)	Annealing temperature/°C	Product size/bp
aadA25	TATGGAGGCTTCGGCTTT GCTATGTTCTCTTGCTTTTGTC	55	217
aadB	CAGTCGCCCTAAAACAAA CAGTCAAGTAACTCATCCCC	55	247
strA	AAACGAGGCTGGAAAAGG ATCAACTGGCAGGAGGAA	53	681
strB	GTTGCTCCTCTTCTCCATC CACCTTTTCCAGCCTCGT	55	722
qnrA	CAAGAGGATTTCTCACGCCAG AATCCGGCAGCACTATTACTCC	53	628
qnrB	AGCGGCACTGAATTTATCGG CGCAATGTGTGAAGTTTGCT	55	418
qnrD	CACGAGCTCAATTTACGGGGAA CGCCTGCTCTCCATCCAAC	56	572
qepA	CTGCAGGTACTGCGTCATG CGTGTTGCTGGAGTTCTTC	54	403
SuI I	TTCGGCATTGTGAATCTCAC ATGATCTAACCCTCGGTCTC	56	822
SuI2	ATGATCTAACCCTCGGTCTC CGGCATCGTCAACATAACC	49	722
SuI3	CAAGGCATCTGATAAAGACTTA TTAGATACAGATAAGGCAATTG	48	705
tetA	GTGAAACCCAACATACCCC GAAGGCAAGCAGGATGTAG	49	888
tetB	TTGGTTAGGGGCAAGTTTTG GTAATGGGCCAATAACACCG	49	659
tetG	GCTCGGTGGTATCTCTGC AGCAACAGAATCGGGAAC	49	468
tet(H)	AACCAAACTGCCTCAAATAC GCCATAACAGACCATCCC	55	526
erm42	TGCACCATCTTACAAGGAGT CATGCCTGTCTTCAAGGTTT	50	173
ermA	GTTCAAGAACAATCAATACAGAG GGATCAGGAAAAGGACATTTTAC	57	421
mphE	ACAAAGATAGCCCGAAATAC TGACCAATCAATAACGCC	50	314
mefE	GCGTTTAAGATAAGCTGGC CCTGCACCATTTGCTCCTAC	55	1743
blaTEM	GAGTATTCAACATTTTCGT ACCAATGCTTAATCAGTGA	55	857
blaOXA-I	GCAGCGCCAGTGCATCAAC CCGCATCAAATGCCATAAGTG	55	198
blaROB-I	CATTAACGGCTTGTTCGC CTTGCTTTGCTGCATCTTC	50	852
lnuA	GCATGTTATTGATTTTAAATT GCTTAGGAGGGATAAAATGAA	49	500

adding 1% crystal violet (Stepanović *et al.*, 2007) 200μL for 30 min, and rinsed with running water until the washings were colourless, then continued to be dried at room temperature, and after drying, 95% ethanol was added to dissolve the solution in 200μL for 30 min, and the OD_{595nm} values of the wells were determined, which was expressed as B_2. B value = $(B_2-B_{2a})/(B_1-B_{1a})$, where B_{1a} and B_{2a} denote the OD_{595nm} values of the blank wells, respectively (Song, 2023).

Determination of biological periplasmic growth curves: In the 96 microtiter plates, the diluted medicinal and bacterial culture were added to each well. After mixing, the concentration of the drugs of thymol and berberine is MIC, 1/2 MIC, 1/4 MIC, 1/ 8 MIC, there was a solution control, the bacterial solution control, and the blank control, and 3 repeats per well, and 37°C cultivates 0, 2, 4, 6, 8, 10, 12, 16, 24h. At the end of incubation for each time point, rinsed 3 times with sterile PBS, added 20μL of dimethoate yellow (XTT) working solution and 100μL of TSB medium, incubated for 2h at 37°C, protected from light, and measured the OD_{450nm} value using an enzyme marker at the end of incubation (CLSI, 2020).

Observation of biological change in periplasmic space of bacteria after the action of different drug concentrations by laser confocal scanning microscopy: The cells were placed in 24-well cell culture plates, and 1 mL each of diluted bacterial solution and drug solution was added to each well after mixing so that the final concentration of the drugs of thymol and berberine was MIC, 1/2 MIC, 1/4 MIC, and 1/8 MIC, and drug control, bacterial solution control, and blank control were made, respectively, and three replicates were set up for each well, and the cells were incubated at 37℃ for 24h. At the end of the incubation, the cells were removed and rinsed with sterile PBS and fixed with 2.5% glutaraldehyde (40μL) for 1.5h. Subsequently, the cells were rinsed again with sterile PBS, incubated dropwise with 40 μg/mL FITC-conA (40μL) for 30 min at room temperature and protected from light, and then continued to be rinsed with sterile PBS, and incubated dropwise with 33.4μg/mL PI (40μL) for 20min at 37 °C and protected from light. After incubation for 20min at room temperature, the membrane was rinsed with sterile PBS, and 20μL of anti-fluorescence attenuator was added dropwise, and the growth of the bioepithelial membrane was observed under a fluorescence microscope with a filter at an emission wavelength of 519nm and 617nm (Reichhardt and Parsek, 2019).

Effects of different drug concentrations on genes related to bioepidermal membrane formation: The effects of thymol and berberine on the main genes *fliA*, *luxS*, and *motA* (Table 3) related to bioepidermal membrane formation of the isolated strains at different concentrations were detected by qRT-PCR. Based on the determination of bioepidermal membrane B-value, bioepidermal membrane growth curves, and the observation of the bioepidermal membrane growth by laser confocal scanning microscope, the MIC group and 1/2 MIC group of thyme phenol and berberine were chosen as the drug concentration grouping for this part of the experiment.

Table 3: Primer information for genes involved in reference genes and biofilm formation

Gene	Primer sequence (5′→3′)	Annealing temperature/°C	Product size/bp
16S rRNA	AGCGCAACCCTTATCCTTTGT ATCCCCACCTTCCTCCAGTT	60	85
fliA	GCTGGCTGTTATTGGTGTCG CAACTGGAGCAGGAACTTGG	60	112
luxS	TGCCACACTGGTAGACGTTC TGATTGGTACGCCAGATGAG	60	116
motA	CTTCCTCGGTTGTCGTCTGT CTATCGCCGTTGAGTTTGGT	60	120

Total RNA was extracted according to the instructions of RNAiso Plus (Takara) reagent, and the extracted RNA was reverse transcribed according to the instructions of PrimeScript™ RT reagent Kit with gDNA Eraser (Takara) after removing the genomic DNA under the following conditions: 15min at 37°C, 5 s at 85°C. The cDNA conforming to the quality after reverse transcription was stored at -80°C in a freezer for backup. Real-time fluorescence quantitative PCR was

amplified with reference to the TB Green® Premix Ex Taq™ II (Takara) kit instructions, and three replicates were set up for each gene of each sample. A VIIA7 fluorescent quantitative PCR instrument was used for amplification. The following amplification procedure was followed: predenaturation at 95°C for 30 seconds, PCR at 95°C for 5 seconds, 60°C for 30 seconds, 40 cycles, melting at 95°C for 5 seconds, 60°C for 1 minute, 95°C for 1 second, and cooling at 50°C for 30 seconds. To calculate the relative expression of the genes associated with the formation of the biofilm under the effect of different drug concentrations in the isolated strains, the relative quantitative method ($2^{-\Delta\Delta Ct}$) was utilized as the internal reference gene (Shah *et al.*, 2023). The smaller the Ct value, the larger the initial expression of the target gene and the relative expression of the target gene was expressed by ΔΔCt, which was calculated by the following methods: ΔΔCt=Ct value of the target gene - Ct value of the internal reference gene, ΔΔCt = ΔCt (test group) - ΔCt (control group) (Zhao *et al.*, 2024).

RESULTS

Drug susceptibility testing: All of the isolated strains were completely sensitive to tilmicosin, ceftriaxone, ampicillin/sulbactam, and florfenicol, with a sensitivity rate of 100%; most of them were intermediary to erythromycin, with an intermediary rate of 53.3%; they had the highest resistance to lincomycin, 93.3%; and they had a resistance rate to clindamycin and streptomycin, 46.7 and 40%, respectively (Table 4).

Drug resistance gene testing: The detection rate of the *strA* gene was 80%; the detection rate of the *strB* gene was 93.3%; the detection rate of the *qnrA* gene was 13.3%; the detection rate of the *Sul2* gene was 26.7%; the detection rate of the *mefE* gene was 66.7%; the detection rate of the *blaROB-1* gene was 60%; and the detection rates of the *qnrD*, *qepA*, *tetB*, *tet(H)* and *blaTEM* genes were all detected at 6.7%.

Screening of biofilm-positive strains: Among the 15 isolated strains, 5 strains of *P. multocida* had strong biofilm-forming ability (33.3%); 4 strains of *P. multocida* had moderate biofilm-forming ability (26.7%); 4 strains of *P. multocida* had weak biofilm-forming ability (26.7%); and 2 strains of *P. multocida* had no biofilm-forming ability (13.3%).

Determination of MIC of thymol and berberine: The MIC of thymol against the experimental strain was 0.1563mg/mL, and berberine against the experimental strain was 0.0938mg/mL.

Determination of biofilm adhesion value: The B value of experimental strains under different concentrations of thymol and berberine (Table 5). As shown in the table, the thymol MIC group can significantly reduce the B value of the experimental strain, while the 1/2 MIC group can significantly reduce the B value of the experimental strain; Berberine MIC, 1/2 MIC, and 1/4 MIC groups can significantly reduce the B value of the experimental strains.

Determination of biological periplasmic growth curves: Effects of different drug concentrations of thymol and berberine on the growth of biofilm of experimental strains (Fig. 1). As shown in the left figure, the MIC group of thymol can inhibit the growth of the biofilm of the experimental strain, but the 1/2 MIC, 1/4 MIC, and 1/8 MIC groups have no significant effect on the growth of the biofilm of the experimental strain; As shown in the figure on the right, the MIC and 1/2 MIC groups of berberine can inhibit the growth of the biofilm of the experimental strain, but the 1/4 MIC and 1/8 MIC groups have no significant effect on the growth of the biofilm of the experimental strain.

Observation of biological periplasm after the action of different drug concentrations by laser confocal scanning microscopy: From the MIC group to the 1/8 MIC group, it can be observed that as the concentrations of the two drugs decrease, the green fluorescence of FITC-conA labeled biofilms and the red fluorescence of PI labeled bacterial cells gradually increase. The MIC group of thymol and berberine showed no red or green fluorescence, indicating that the drug concentration can significantly inhibit the growth of bacterial cells and biofilms (Fig. 2); There was a small amount of red fluorescence in the 1/2 MIC group of berberine, but no obvious green fluorescence was observed, indicating that the drug concentration can inhibit the growth of biofilms, but cannot completely inhibit bacterial growth (Fig. 3).

Effects of different drug concentrations on genes related to bioepithelial membrane formation: Effects of different concentrations of thymol and berberine on genes related to bioepithelial membrane formation in experimental strains (Fig. 4). As shown in the left figure, it can be seen that the MIC group of thymol could reduce the expression of bioepidermal membrane formation related genes *fliA*, *luxS*, and *motA* very significantly; As shown in the figure on the right, it can be seen that the MIC and 1/2 MIC groups of berberine could reduce the expression of bioepidermal membrane formation related genes *fliA*, *luxS*, and *motA* very significantly.

DISCUSSION

Antimicrobial drugs such as quinolones and macrolides are often used clinically to prevent and control bovine respiratory diseases caused by *Pasteurella multocida*, but due to irrational use of drugs or even drug abuse is widespread, resulting in a decrease in the sensitivity of the pathogenic bacteria to antimicrobial drugs, which in turn brings difficulties to the treatment of the disease. The results of the drug susceptibility test in this study found that 15 isolates of *P. multocida* from three regions showed different degrees of resistance to antimicrobial drugs commonly used in the clinical treatment of bovine respiratory diseases, and the resistance rate to lincomycin, clindamycin, and

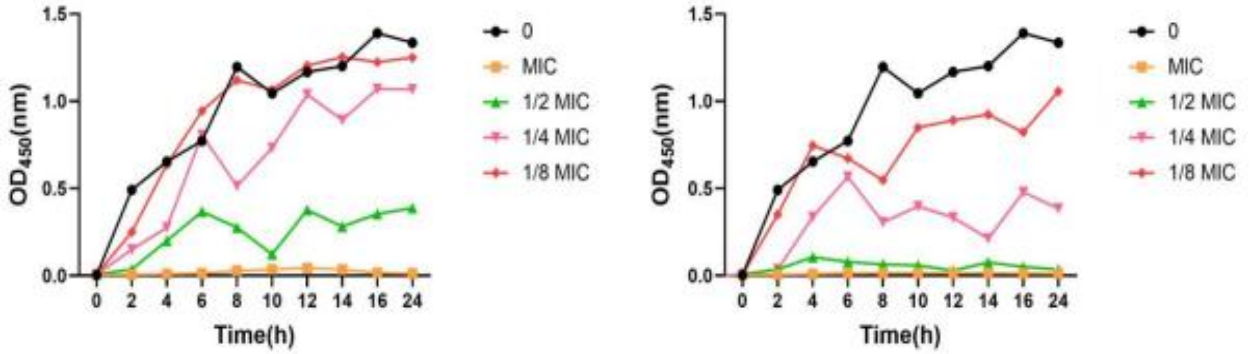

Fig. 1: Effect of thymol and berberine on biofilm growth curves of experimental strain. Note: The left figure shows the growth curve of biofilm under different concentrations of thymol; The figure on the right shows the growth curve of biofilm under different concentrations of berberine.

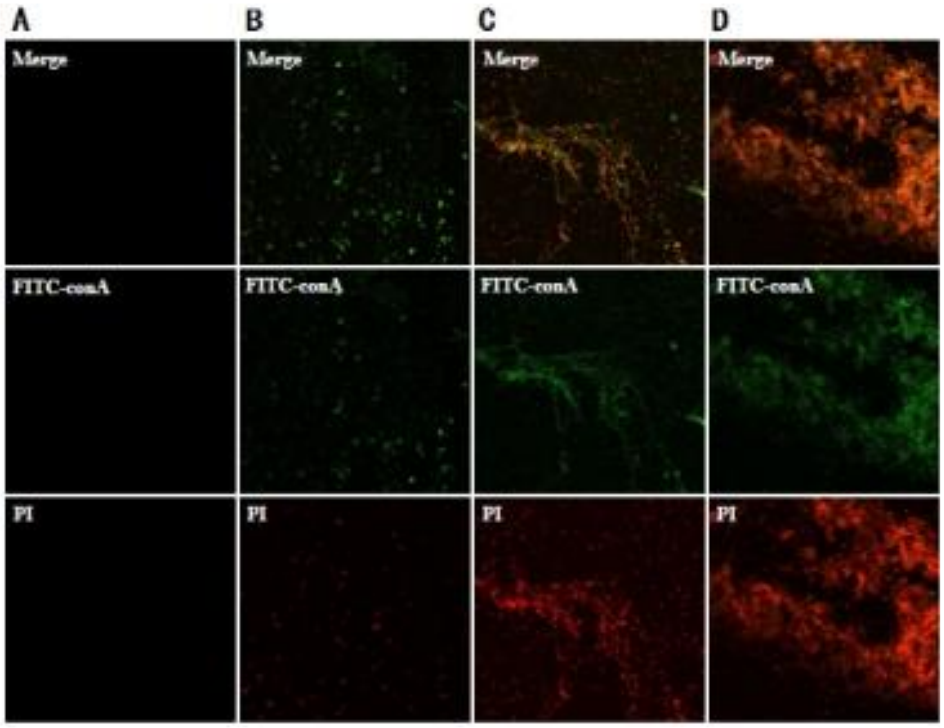

Fig. 2: Laser confocal results of different concentrations of thymol on experimental strain after 24 h incubation (200×). Note: A: MIC group; B: 1/2 MIC group; C: 1/4 MIC group; D: 1/8 MIC group.

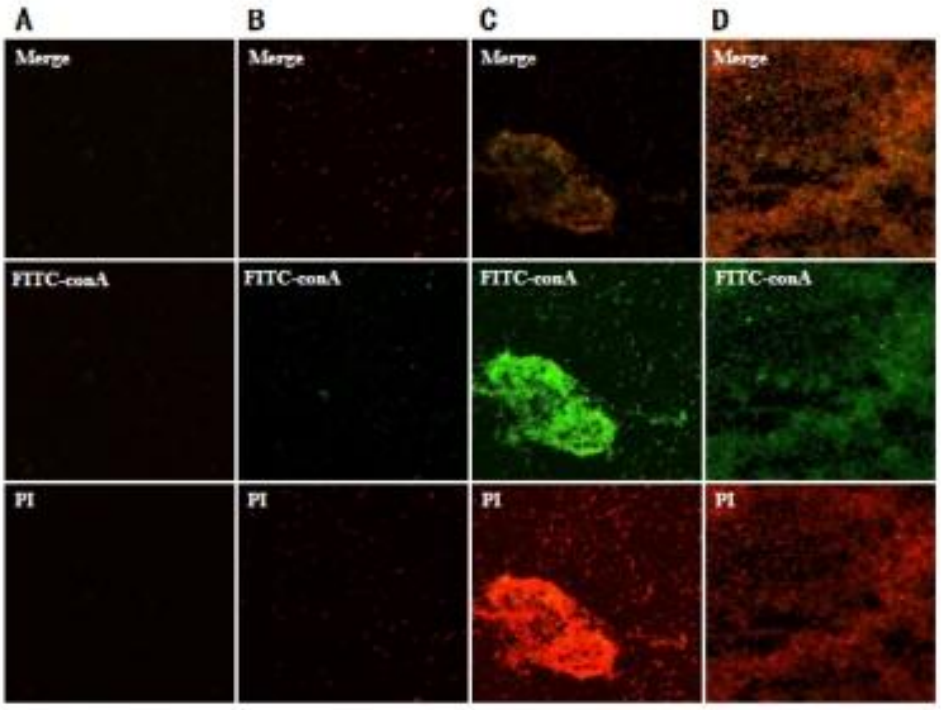

Fig. 3: Laser confocal results of different concentrations of berberine on experimental strain after 24 h incubation (200×). Note: A: MIC group; B: 1/2 MIC group; C: 1/4 MIC group; D: 1/8 MIC group.

Table 4: Results of drug susceptibility testing of isolates

Drug type	Drug name	S (%)	I (%)	R (%)
Aminoglycosides	Streptomycin	40.0 (6/15)	20.0 (3/15)	40.0 (6/15)
	Amikacin	80.0 (12/15)	6.7 (1/15)	13.3 (2/15)
	Daecamycin	93.3 (14/15)	0.0 (0/15)	6.7 (1/15)
	Gentamicin	73.3 (11/15)	0.0(0/15)	26.7 (4/15)
Quinolones	Ciprofloxacin	80.0 (12/15)	13.3 (2/15)	6.7 (1/15)
	Ofloxacin	93.3 (14/15)	0.0 (0/15)	6.7 (1/15)
	Enrofloxacin	93.3 (14/15)	0.0 (0/15)	6.7 (1/15)
Sulfonamides	Compound sulfamethoxazole	93.3 (14/15)	0.0 (0/15)	6.7 (1/15)
Tetracyclines	Tetracycline	86.7 (13/15)	0.0 (0/15)	13.3 (2/15)
	Doxycycline	93.3 (14/15)	6.7 (1/15)	0.0 (0/15)
Macrolides	Erythromycin	40.0 (6/15)	53.3 (8/15)	6.7 (1/15)
	Tilmicosin	100 (15/15)	0.0 (0/15)	0.0 (0/15)
β-lactams	Ampicillin	93.3 (14/15)	6.7 (1/15)	0.0 (0/15)
	Cefotaxime	86.7 (13/15)	0.0 (0/15)	13.3 (2/15)
	Ceftriaxone	100.0 (15/15)	0.0 (0/15)	0.0 (0/15)
	Ampicillin / sulbactam	100.0 (15/15)	0.0 (0/15)	0.0 (0/15)
Chloromycetins	Florfenicol	100.0 (15/15)	0.0 (0/15)	0.0 (0/15)
Lincosamides	Lincomycin	0.0 (0/15)	6.7 (1/15)	93.3 (14/15)
	Clindamycin	33.3 (5/15)	20 (3/15)	46.7 (7/15)
Polypeptides	Polymyxin B	86.7 (13/15)	13.3 (2/15)	0.0 (0/15)

Note: S: sensitivities; I: intermediary; R: drug-resistant.

Table 5: B-values after treatment with different concentrations of thymol and berberine (x±s)

Chinese medicine monomer	0	MIC	1/2 MIC	1/4 MIC	1/8 MIC
thymol	1.354±0.037	0.800±0.048**	1.072±0.041*	1.264±0.078	1.279±0.067
berberine	1.354±0.037	0.565±0.004**	1.072±0.041**	1.148±0.055**	1.328±0.015

streptomycin was higher, with the resistance rate ranging from 40 to 93.3%. It was susceptible to tilmicosin, ceftriaxone, ampicillin/sulbactam, and florfenicol, with a sensitivity rate of 100%. Mao *et al*. (2021) conducted drug resistance testing on three strains of *P. multocida* isolated from fattening cattle with respiratory diseases on a certain cattle farm, the results showed that the isolated strains were all resistant to ciprofloxacin, enrofloxacin, compound sulfamethoxazole, clindamycin, temicoxacin, etc., and were only sensitive to tetracycline and florfenicol. Katsuda *et al.* (2013) conducted susceptibility tests for 9 antimicrobial drugs on 378 strains of *P. multocida* isolated from calves suffering from respiratory disease, and the tests revealed that the isolated strains had varying degrees of susceptibility to florfenicol, kanamycin, ampicillin, ceftolozolin, ceftiofur, erythromycin, enrofloxacin, tetracycline, and ciprofloxacin. The results of the above studies are all somewhat different from the present study, and the analysis may be related to the occurrence of respiratory diseases in different farms in different countries and regions, the different types of antimicrobial drugs used,the different methods of use and dosage standards, but all of them are more resistant to lincosamide, which is not recommended to be used in the clinic, and they are still more sensitive to β-lactam, quinolone and amidol antimicrobial drugs, which is recommended to be used in the clinic for the treatment of respiratory diseases caused by *multicidal Bartonella* species. It is still sensitive to β-lactam, quinolone, and amidol antibacterial drugs and is recommended to treat respiratory diseases caused by *P. multocida* in the clinic, but the emergence of drug resistance should also be considered.

This study conducted resistance gene testing based on the resistance phenotype of *P. multocida* isolates and found that the detection rates of aminoglycoside resistance genes *strA* and *strB* were 80 and 93.3%, respectively, and that the isolated strains were resistant to streptomycin, gentamicin, spectinomycin, and amikacin to varying degrees. Wang (2016) conducted aminoglycoside resistance gene testing on 23 strains of *P. multocida* from 6 provinces in China and found that they all had *strB, aphA*, and *aacA4* genes. Some strains were resistant to streptomycin, amikacin, and kanamycin, but relatively sensitive to spectinomycin and gentamicin, which were similar to the results of this study. The detection rate of the sulfonamide-resistant gene *Sul2* in this study was 26.7%, but all isolated strains were sensitive to compound sulfamethoxazole. The detection rates of β-lactams resistance genes *blaROB-1* and *blaTEM* were 60 and 6.7%, respectively. The strains carrying *blaTEM* resistance genes were sensitive to β-lactams drugs. In contrast, some strains carrying *blaROB-1* resistance genes were resistant to cefotaxime, while the rest were sensitive to β-lactams drugs. Ma (2021) performed resistance gene detection on calf respiratory *P. multocida* isolates from Xinjiang, China, and the results showed that the highest detection rate of resistance genes was 19% for β-lactams *blaTEM*, and 15.5, 13.8, and 6.9% for sul2, sul1, and qnrB, respectively, and no tetracyclines and polypeptides resistant genes were detected, which were similar to the results of this study. The detection rate of macrolide resistance gene *mefE* was 66.7%, all isolates with *mefE* gene detected were erythromycin resistant or mediated, and no methylase genes *ermA*, *ermB*, etc. were detected. Ujvári and Magyar (2022) found that macrolide resistance determining genes *erm42*, *msrE*, and *mphE* were detected in *P. multocida* and mediated erythromycin and tilmicosin resistance, which differs from the results of this study. The detection rate of tetracycline resistance genes *tet(H)* and *tetB* were both 6.7% , which mediated the strain's resistance to tetracycline; Wang *et al.* (2021) conducted resistance gene testing on *P. multocida*, and the results showed that the isolated strains only contained sulfonamide *sul2* genes and tetracycline tetB genes, which were resistant to drugs such as streptomycin and tetracycline, similar to the results of this study.

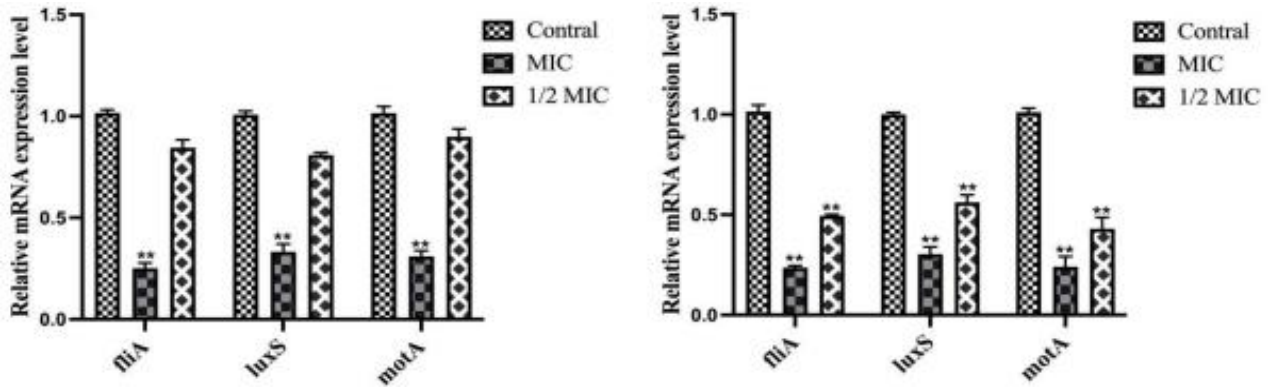

Fig. 4: Effect of thymol and berberine on expression of genes involved in biofilm formation in experimental strain. Note: The left figure shows the effect of thymol MIC and 1/2 MIC concentrations; The figure on the right shows the effect of berberine MIC and 1/2 MIC concentrations; ** *P*<0.01, Compared with the untreated group* 0.01<P<0.05, compared with the untreated group.

The detection rates of quinolone resistance genes *qnrA*, *qnrD* and *qepA* in this study were 13.3, 6.7 and 6.7%, respectively, which mediated the resistance of the strains to ciprofloxacin and enrofloxacin, suggesting that the main reason for the development of resistance to quinolones by *P. multocida* is due to the presence of the *qnr* resistance genes. In this study, the resistance genes carried by *P. multocida* isolates showed some correlation with their resistance phenotypes, but not exactly the same, such as the results of drug sensitivity test of some strains showed that they were resistant to a certain antimicrobial drug, but the related resistance genes were not detected, and the analysis may be related to the existence of a variety of resistance mechanisms in the strains, which still need to be further studied.

The isolation rate of biofilm-positive *P. multocida* strains causing bovine respiratory diseases was relatively high, at 86.7%, which indicates that the prevalence of biofilm is more serious. The resistance rate of *P. multocida* with strong biofilm-forming ability is high. Even the phenomenon of multi-drug resistance occurs, and at the same time, biofilm-positive *P. multocida* can infect the animal organism repeatedly, making it difficult to cure. Song (2023) study found that biofilm-positive *Escherichia coli* were more resistant to antimicrobial drugs, which is consistent with the results of this study. Petruzzi *et al*. (2017) found that the formation of biofilm of the strains could enhance the resistance and virulence of the strains, which is consistent with the results of this study.

More and more traditional Chinese medicines have broad-spectrum antimicrobial activity and can inhibit the growth of many pathogenic bacteria. Compared with antimicrobial drugs, traditional Chinese medicines have a wide range of sources, multiple active ingredients, and various advantages such as minimal toxic side effects and less susceptibility to drug resistance. Among them, thymol and berberine both have sound antibacterial effects. Therefore, this study explores the impact of the two traditional Chinese medicine monomers on the formation of biofilms of *P. multocida*. The initial stage of biofilm formation is the reversible adhesion stage, which is the basis for biofilm formation. Therefore, reducing the B value of the strain indicates that it can effectively inhibit biofilm formation in the strain. This study found that the MIC and 1/2 MIC groups of thymol and berberine reduced the B-value of the strong biofilm-forming ability of multicidal *P. multocida* in a concentration-dependent manner in highly significant and significant ways. Dong *et al.*(2023) found that when the adhesion factors related to bacterial biofilm formation are lacking, the ability of bacterial biofilm formation is significantly reduced, which is consistent with the results of this study. The results of biofilm growth curves in this study showed that the MIC group of thymol and the MIC and 1/2 MIC groups of berberine could inhibit the growth of biofilm of *P. multocida* strains, and the other drug concentrations had no significant effect on the growth of biofilm of *P. multocida* strains. The results of laser confocal observation of biofilm formation showed that the MIC group of thymol and berberine could inhibit the growth of the bacterium and biofilm, and the 1/2 MIC group of berberine could inhibit the growth of the biofilm but could not inhibit the growth of the bacterium completely. Penesyan *et al.* (2020) research found that sub-inhibitory concentrations of cefoxitin can inhibit the growth rate of *Escherichia coli* and the formation of biofilms, similar to this study's results. Combined with the B-value, biofilm growth curve, and laser confocal observation results, it can be shown that the MIC group of thymol and the MIC and 1/2 MIC groups of berberine can inhibit the initial reversible adhesion stage of biofilm formation of *P. multocida* strains, as well as the subsequent colonization and proliferation stages, jointly inhibiting the formation and growth of *P. multocida* biofilm, and thereby reducing the drug resistance of the strains caused by biofilm formation.

The process of biofilm formation in *P. multocida* is regulated by the expression of a variety of related genes, such as the *luxS* gene, which regulates the synthesis of LuxS (AI-2 synthase) proteins, and the *fliA* and *motA* genes, which regulate flagellar synthesis and motility (Ferrières and Clarke, 2003). Studies have shown that the LuxS/AI-2 QS system regulates the biological behavior of various bacteria, mainly involved in the formation of biofilms (Zhou, 2023). In the process of biofilm formation, as the moving organs on the surface of bacteria, flagella can drive the bacterial body to move towards a surface conducive to growth and fix it on the surface of the organism, playing an important role in biofilm formation. The LuxS/A1-2 QS system can regulate the expression of flagella synthesis gene *fliA* and

flagella movement gene *motA*, and when the extracellular signal molecule AI-2 reaches a certain concentration, it is transported to the bacterial body, upregulating the expression of *fliA* and *motA*, thereby promoting the formation of biofilms (Moreira *et al.*, 2006). This study found that the MIC group of thymol and the MIC and 1/2 MIC groups of berberine could both significantly reduce the expression of *luxS*, *fliA*, and *motA* genes of strongly biofilm-positive *P. multocida* strains and also significantly inhibit the formation and growth of biofilm of *P. multocida* strains. The above research results indicate that thymol and berberine may inhibit the expression of the *LuxS* gene in the *P. multocida* strain, reduce the synthesis of LuxS protein in the LuxS/AI-2 QS system, and thereby decrease the synthesis of the signal molecule AI-2, preventing its concentration from reaching the threshold and inhibiting the formation and growth of biofilms. Due to the decrease in the synthesis of the signal molecule AI-2, the expression levels of *fliA* and *motA* genes in the bacterial body are reduced, which inhibits the synthesis and movement of flagella and weakens the initial reversible adhesion of the *P. multocida* strain itself, leading to the inhibition of biofilm formation and growth, and ultimately weakening the drug resistance of the *P. multocida* strain itself.

Conclusions: In summary, the strains of *P. multocida* isolated from the bovine respiratory tract are severely resistant to lincomycin, clindamycin, and streptomycin. They are more sensitive to temicoxacin, ceftriaxone, and fluphenicol. The prevalence of biofilm-positive strains was high, and thymol and berberine can regulate the expression of genes related to biofilm formation in strongly positive strains, inhibiting the formation of biofilms and thereby inhibiting the development of resistance mediated by biofilms. It provides drug reference and a theoretical basis for the clinical prevention and treatment of bovine respiratory diseases caused by *P. multocida*, and the traditional Chinese medicine monomer thymol and berberine can be used as a potential biofilm inhibitor to treat infectious diseases caused by multi-drug resistant strains of *P. multocida*, which needs further research.

Animal Ethical approval: Current research was conducted according to the rules and regulations of the College of Veterinary Medicine (2022-2414), Inner Mongolia Agricultural University, Huhhot 010010, Inner Mongolia, China.

Authors contribution: Data curation, Puguo Hao; Formal analysis, Yue Sun, Wei Mao and Kerui Gua; Funding acquisition, Hongxia Zhao; Investigation, Kaiwen Yin; Methodology, Yue Sun, Puguo Hao, Su Jian Guo and Kaiwen Yin; Project administration, Hongxia Zhao; Software, Jinshan Cao and Puguo Hao; Validation, Wei Mao, Su Jian Guo and Hongxia Zhao; Visualization, Jinshan Cao and Puguo Hao; Writing – original draft, Yue Sun; Writing – review & editing, Yue Sun, Jinshan Cao, Kerui Gua and Hongxia Zhao.

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