Inhibition of *Streptococcus suis* Adhesion and Biofilm Formation in Vitro by Water Extracts of *Rhizoma Coptidis*

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**INTRODUCTION**

*Streptococcus suis* is a pathogen causing huge economic and financial losses in the pork industry and an emerging threat to human health (Staats et al., 1997; Hill et al., 2005; Lun et al., 2007). *S. suis* can form biofilms, trapping nutrients, and shielding the pathogen from antagonistic effects (Brady et al., 2008; Wang et al., 2011). Biofilms are consortia of microorganisms attached to biotic or abiotic surfaces. Generally, the initial step in biofilm formation is a non-specific, reversible attachment of bacteria to substrate surfaces. Once permanently attached, the bacteria start to synthesize insoluble exopolysaccharides that encase the adherent bacteria in a three-dimensional...
matrix (Costerton et al., 1987). Therefore, reducing S. suis adhesion to surfaces may be an effective way to mitigate biofilm formation.

Studies have suggested that some genes and proteins play crucial roles in a series of complex molecular processes leading to biofilm formation (Sauer, 2003; Latasa et al., 2006; Beloin et al., 2008; Gaddy and Actis, 2009). A previous study reported deletion of the atl gene from S. suis type 2 strain HA9801, which encodes an autolysin, reduced adhesion to HEP-2 cells by 50% compared with wild-type S. suis, suggesting a role for Alt in biofilm formation and cell adhesion (Ju et al., 2012). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), an S. suis protein, has been identified as an adhesin. GAPDH mediates cell adhesion, encouraging biofilm production (Brassard et al., 2004; Wang and Lu, 2007). Muramidase-released protein (MRP) is a cell wall protein allowing bacteria to resist phagocytosis by macrophages and aids adhesion to epithelial cells (Liang et al., 2011). MRP induces expression of the cell surface protein BapA1 in Streptococcus pneumonia. Deletion of mrp reduces the bacterium’s ability to aggregate and form biofilms (Liang et al., 2011).

Rhizoma Coptidis (RC), used for over 2000 years in Traditional Chinese Medicine, has been studied for its antibacterial, antiviral, anti-inflammatory, anti-hyperglycemic, and hypolipidemic effects (Ye et al., 2009; Wu et al., 2014). In recent years, there has been a surge in the study of plants rich in bioactive components. These components have been shown to possess various beneficial properties including anti-adhesive effects (Dixon, 2001). It is reported that Rhizoma Coptidis can inhibit biofilm formation by Staphylococcus epidermidis (Wang et al., 2009). Previous studies on the anti-pathogenic effects of Rhizoma Coptidis have focused on its anti-biofilm activity (Zhu and Li, 2006; Hayashi et al., 2007; Yu et al., 2007). Our previous study indicated that a water extract from Rhizoma Coptidis (C. deltoidea C.Y.Cheng & P.K.Hsiao, obtained from Sichuan Province), berberine hydrochloride and coptisine can all inhibit S. suis biofilm formation in a tissue culture plate (TCP) assay (Liu et al., 2015), though the mechanisms involved are poorly understood.

Since in the previous study we only found that water extract from Rhizoma Coptidis could interfere with the formation of S. suis biofilms, but we did not know the mechanism involved. So the aim of this study was therefore to investigate the mechanisms by which Rhizoma Coptidis (C. deltoidea C.Y.Cheng & P.K.Hsiao, obtained from Sichuan Province) extracts disrupt S. suis biofilm formation and bacterial adherence, and to guide strategies to prevent S. suis biofilm infection.

MATERIALS AND METHODS

Preparation of Extract

Rhizoma Coptidis (C. deltoidea C.Y.Cheng & P.K.Hsiao, obtained from Sichuan Province) was purchased as a crude drug from the Beijing Tong Ren Tang Pharmacy. Its identity was authenticated by Professor Mingxia Bai at the Horticulture Branch of the Heilongjiang Academy of Agricultural Sciences. A berberine standard (877-200001) was purchased from the Ministry of Health of Drug Products. To generate water extracts of Rhizoma Coptidis, 50 g Rhizoma Coptidis powder were boiled in 500 mL distilled water for 60 min at 100°C before decanting and filtration. The filtrate was collected and added to 300 mL of distilled water and boiled for 60 min at 100°C. The final filtrate mass was lyophilized and concentrated into a dried powder with a yield of 0.25 g mL⁻¹ and stored at 4°C. The amount of berberine, the major active ingredient, in Rhizoma Coptidis water extracts was measured by high-performance liquid chromatography (HPLC) on a Waters Alliance HPLC system (Waters e2695, United States) consisting of a binary pump and a UV/Vis detector. Separation was carried out using a 5 µM DL-Cl8 column (4.6 mm × 150 mm, Japan) at 37°C. Acetonitrile (solvent A) and 0.05 M potassium dihydrogen phosphate (solvent B) were used as the mobile phase at a ratio of 40:60 (solvent A:solvent B), supplemented with 0.015 M sodium dodecyl sulfate. The flow rate was set at 1.2 mL min⁻¹. A detection wavelength of 345 nm and an injection volume of 5 µL were used in the study. The amount of berberine in the Rhizoma Coptidis aqueous extract was determined by comparing the HPLC retention time to the authentic standard. Quantification of berberine in the aqueous extract was done using a linear calibration plot of the peak area in HPLC at 345 nm against concentration using the external standard method. The calibration curve was calculated by plotting peak areas against six different concentrations of the standard solutions (0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mg mL⁻¹).

Minimum Inhibitory Concentrations

The MIC (minimal inhibitory concentration) was determined by the microtiter broth dilution method, as recommended by the Clinical and Laboratory Standards Institute [CLSI] (2016). Dilutions were performed in Todd–Hewitt Broth (THB) medium using 1 × 10⁶ colony-forming units (CFU) of bacteria per milliliter. Cell suspensions (100 µL) were inoculated into 96-well microtiter plates in the presence of Rhizoma Coptidis water extracts with different final concentrations (0, 1.625, 3.125, 6.25, 12.5, 25, 50, 100, or 200 µg mL⁻¹). Azithromycin was used as a positive control, with the susceptibility (MIC) of S. suis ATCC 700794 to azithromycin found to be 32 µg mL⁻¹ (Yang et al., 2016). Inoculated microplates were incubated at 37°C for 24 h before examination. Susceptibility (MIC) of S. suis ATCC700794 to Rhizoma Coptidis water extracts was 100 µg mL⁻¹ (Berberine, the active ingredient, in Rhizoma Coptidis water extracts was 36.3 µg mL⁻¹).

Growth Conditions of S. suis Biofilms

Streptococcus suis ATCC700794 was grown overnight in THB (Sigma-Aldrich) at 37°C with constant shaking. Biofilm culture production was described previously (Wang et al., 2011). Briefly, S. suis grown in THB medium at 37°C was added to a 1% fibrinogen solution in 100 mm polystyrene dishes and grown for 24 h. After decanting the growth medium, plates were thoroughly rinsed twice with 50 mM Tris–HCl (pH 7.5). Biofilms were then harvested by scraping. Cells were sonicated for 5 min and centrifuged at 12,000 × g for 10 min at 4°C. Supernatants were then removed and cell pellets were washed twice with 50 mM Tris–HCl (pH 7.5).
Determination of the Effect of *Rhizoma Coptidis* Water Extracts on Biofilm Formation by TCP Assay

*Streptococcus suis* cultures in mid-exponential growth phase with an optical density of 0.2 at 600 nm (OD_{600}) were used for TCP assays. In each well of a 96-well plate, 100 µL of *S. suis* culture and 100 µL of *Rhizoma Coptidis* water extract were combined. The final tested concentrations were 6.25, 12.5, 25, or 50 µg mL^{-1}. Wells filled with sterile growth medium were included as blank controls, azithromycin (1/2MIC) as a positive control. Wells containing 200 µL culture without extract served as negative controls. After incubation at 37°C for 24 h, all wells were washed with sterile phosphate-buffered saline (PBS) and stained with crystal violet.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) was performed as described previously (Zhao et al., 2015). Briefly, cultures were diluted to an OD_{600} of 0.1 before adding 2 mL to wells of a six-well microplate containing a 10 mm × 10 mm sterilized rough organic membrane (Mosutech Co., Ltd., Shanghai, China). After incubation without shaking for 24 h at 37°C, planktonic cells were decanted. Attached cells were removed by addition of 0.5 M sodium hydroxide. Adherence was quantified by OD_{600}. Percentage adherence = [OD_{600} of adhered cells/(OD_{600} of adhered cells + OD_{600} of planktonic cells)].

Anti-adherence Activity of Extract Against *S. suis*

*Anti-adherence to organic membranes*. Assays were prepared as previously described (Hamada et al., 1981). Briefly, *S. suis* ATCC700794 cultures at mid-exponential growth phase were diluted to an OD_{600} of 0.1 before combining with 2 mL of THB or sub-MICs of *Rhizoma Coptidis* water extract in a six-well microplate containing a 10 mm × 10 mm sterilized rough organic membrane (Mosutech Co., Ltd., Shanghai, China). After incubation without shaking for 24 h at 37°C, planktonic cells were decanted. Attached cells were removed by addition of 0.5 M sodium hydroxide. Adherence was quantified by OD_{600}. Percentage adherence = [OD_{600} of adhered cells/(OD_{600} of adhered cells + OD_{600} of planktonic cells)].

*Anti-adherence to cells*. Assays were prepared as described previously (Lalonde et al., 2000) with slight modifications. Briefly, PK-15 cells were cultured in DMEM (Hyclone) and grown in 75 cm × 75 cm flasks at 37°C with 5% CO₂. Confluent monolayers of PK-15 cells (1.0 × 10⁵ cells per well) were cultured in 96-well plates (Corning, NY, United States). *S. suis* cells, either supplemented with sub-MICs of *Rhizoma Coptidis* water extracts or untreated, were added to each well at an MOI of 100:1 and incubated at 37°C to allow cells to attach. After 4 h, plates were washed twice with PBS and cells were lysed with sterile distilled water on ice. Both adherent and intracellular bacteria were counted on THB agar. Both assays were repeated three times.

RNA Isolation and Real-Time PCR

Real-time PCR was performed as described previously (Yang et al., 2015). The primer sequences used in the experiment were shown in Table 1. To investigate the effect of *Rhizoma Coptidis* water extracts on gene expression, mid-log growth phase cultures of *S. suis* were supplemented with 50 µg mL^{-1} extract and incubated at 37°C for 24 h. Cells without extract served as control. Cultures were centrifuged at 10,000 × g for 5 min before treatment with an RNase Remover I (Huayueyang Ltd., Beijing, China). Total RNA levels were determined using the E.Z.N.A.™ Bacterial RNA isolation kit. Real-time PCR for each
sample was performed as previously described (Yang et al., 2015).

**iTRAQ Analysis**

Protein was extracted from *S. suis* cells either treated with 50 µg mL⁻¹ *Rhizoma Coptidis* water extract or left untreated (Wang et al., 2011). iTRAQ analysis was performed at Shanghai Applied Protein Technology Co., Ltd. (APT, Shanghai, China). Three biological replicates were evaluated to minimize the influence of less reliable quantitative information. iTRAQ analysis was performed as previously described (Zhao et al., 2015).

**Statistical Analysis**

Values were calculated as the mean of individual experiments in triplicate and compared to those of the control groups. Differences between two mean values were calculated by Student’s t-test using SPSS 11.0.0 statistical software, with *p*-values below 5% designated as statistically significant.

### RESULTS

**Amounts of the Active Ingredient Berberine in *Rhizoma Coptidis* Water Extracts**

High-performance liquid chromatography chromatograms of a *Rhizoma Coptidis* water extract and a standard solution of berberine are shown in Figure 1. The retention time of berberine agreed well with the authentic compound (15.43 min). The calibration curve equation was $y = 7E + 06x + 28,724, R^2 = 0.999$. Using the calibration curve, the portion of berberine, the active ingredient, in *Rhizoma Coptidis* water extracts was calculated to be 36.30%.

**Effect of *Rhizoma Coptidis* Water Extracts Against Biofilm Formation in Vitro**

The TCP method allows quantitative detection of *S. suis* biofilm formation at 24 h. Four different doses of *Rhizoma Coptidis* water extract were tested against *S. suis* biofilms (Figure 2). At 12.5 and 6.25 µg mL⁻¹, the OD₆₀₀ of *S. suis* ATCC700794 were lower than the negative control. At 25 and 50 µg mL⁻¹, there was significant inhibition (*p* < 0.05) of *S. suis* biofilm formation, suggesting that these concentrations were more effective than 6.25 or 12.5 µg mL⁻¹ at inhibiting biofilm formation.

**Scanning Electron Microscopy**

Scanning electron microscopy was performed to examine the effects of 50 µg mL⁻¹ *Rhizoma Coptidis* water extract on *S. suis* biofilm formation. In the absence of extract, the surface of the rough organic membrane was observed to be almost entirely covered by aggregates and micro-colonies of *S. suis* (Figure 3A). However, when 50 µg mL⁻¹ extract was added, most of the cell aggregates were dispersed (Figure 3B), suggesting that *S. suis* biofilm formation was inhibited by the extract in vitro.

![FIGURE 3](image-url) Scanning electron microscopy of biofilm of *S. suis* grown in THB broth. (A) With 0 MIC (0 µg mL⁻¹) concentration of aqueous extracts of *Rhizoma Coptidis*; (B) with 1/2 × MIC (50 µg mL⁻¹) concentration of aqueous extracts of *Rhizoma Coptidis*.

![FIGURE 4](image-url) Effect of *Rhizoma Coptidis* at different concentrations on *S. suis* ATCC700794 adhesion to glass (A), or PK-15 cells (B). Data are expressed as means ± standard. Significant decrease (*p* < 0.05) compared to control in vitro.
more than 1.5-fold and others by less than 0.67-fold ($p < 0.05$). Of the 26 proteins tested using iTRAQ after treatment with 50 µg mL$^{-1}$ extract, expression of 15 proteins increased and 11 were suppressed (Table 2). Among the suppressed proteins were antigen-like protein (D5AGH9), hydrolase (R4NST6), methyltransferase H (G7SM56), glycosyltransferase (M1VJJ3), and helicase (G7S7E3). These proteins had fold-change values of 0.49576886, 0.311630845, 0.644879525, 0.574502756, and 0.57612248, respectively.

**DISCUSSION**

We investigated the relationship between *Rhizoma Coptidis* water extracts and *S. suis* biofilm formation. Previous studies have suggested that there is a relationship between some antimicrobial agents and biofilm formation (Majtan et al., 2008; Nucleo et al., 2009; Mishra et al., 2014; Zhao et al., 2015; Wang et al., 2016). In our study, sub-MICs of *Rhizoma Coptidis* water extracts could inhibit biofilm formation of *S. suis in vitro*, as observed in a TCP assay. Most studies on the anti-infective activities of *Rhizoma Coptidis* have focused on its anti-biofilm effects, with little or no

**TABLE 2** | iTRAQ identification of differentially expressed proteins.

| Accession | Proteins                                                                 | Fold change  |
|-----------|--------------------------------------------------------------------------|--------------|
| R4NST6    | Hydrolase (HAD superfamily)                                              | 0.311630845  |
| G7SEP0    | Putative uncharacterized protein                                         | 0.389353378  |
| G7SM20    | DNA gyrase subunit B                                                    | 0.427259889  |
| D5A9H9    | Antigen-like protein                                                     | 0.49576886   |
| G7SHZ3    | Bacteriophage protein, putative                                          | 0.520900282  |
| F3D5P5    | Putative uncharacterized protein                                         | 0.534269172  |
| G5K2N4    | DNA polymerase IV                                                        | 0.564882743  |
| G7RZW0    | Sugar ABC transporter permease                                           | 0.566616133  |
| A4W5Y3    | Response regulator                                                       | 0.569410189  |
| M1VJJ3    | Glycosyltransferase                                                      | 0.574502756  |
| G7S7E3    | Helicase                                                                | 0.57612248   |
| G7SM66    | Methyltransferase H                                                      | 0.644879625  |
| R4NVK5    | DNA gyrase subunit B                                                    | 1.50073608   |
| G7SN2N    | ABC transporter ATP-binding protein                                      | 1.537200996  |
| KOFG35    | CpsR                                                                    | 1.542930057  |
| R4NW55    | AAA-class ATPase domain protein                                          | 1.55109354   |
| G7S3Q5    | Putative uncharacterized protein                                         | 1.56827867   |
| F4E0C5    | Putative uncharacterized protein                                         | 1.724654275  |
| E9NC29    | CPS16V                                                                  | 1.764888931  |
| B9WUV5    | Transcriptional regulator, DeoR family                                   | 1.913005357  |
| B0FY8     | Neprylsin (Fragment)                                                    | 2.203412347  |
| G7SDS2    | ABC superfamily ATP binding cassette transporter, membrane protein       | 2.332354978  |
| R4NST6    | Hydrolase (HAD superfamily)                                              | 2.805220661  |
| G7SM99    | Type I site-specific restriction-modification system, R (Restriction) subunit and related helicase | 3.177157457  |
| G7SM99    | ABC-type transport system involved in Fe-S cluster assembly, permease component | 3.464547909  |
| G7SM99    | Chlorophenolic acetyltransferase                                         | 3.474123973  |

**Anti-adherence Activity of Extract Against S. suis**

The inhibitory effects of *Rhizoma Coptidis* water extract on adherence of *S. suis* to glass were tested at several concentrations (Figure 4). The extract inhibited adherence to organic membranes (Figure 4A) and PK-15 cells (Figure 4B).

**The Effect of Rhizoma Coptidis Water Extracts on Gene Expression**

The expression profiles of gapdh, sly, and mrp in *S. suis* were determined 24 h post-treatment with 50 µg mL$^{-1}$ *Rhizoma Coptidis* water extract. In treated cultures, gapdh, sly, and mrp gene expression levels were suppressed compared to untreated samples (Figure 5).

**Rhizoma Coptidis Water Extracts Inhibit Biofilm Formation and Modulate Protein Expression by iTRAQ**

*Streptococcus suis* cultures were incubated with extract for 24 h before measurement using iTRAQ. Changes in protein expression levels were observed, with some proteins changing by

**TABLE 1** | Primers used for the quantitative RT-PCR analysis.

| Genes      | Primer sequence                      |
|------------|--------------------------------------|
| 16S rRNA   | Forward: 5′-TGCTAGTACCTAGGCTAAAGGCTAA-3′ Reverse: 5′-GGCTGGAGATTCTTCTGAT-3′ |
| gapdh      | Forward: 5′-GCTGGAAGAAGTAAACCGCTGCT-3′ Reverse: 5′-GTGCGACATCAAATACGAACC-3′ |
| sly        | Forward: 5′-AGTCATGGTTGGACTGCTAGG-3′ Reverse: 5′-TTGTCGTCGAACTGACCGC-3′ |
| mrp        | Forward: 5′-TGGCACAGTATTACGAAGACCC-3′ Reverse: 5′-TACCGTACACGAAAACAT-3′ |

**Figure 5** | Effect of 1/2MIC of aqueous extracts of Rhizoma Coptidis on mRNA decreased expression of genes in *S. suis* ATCC700794. Data are expressed as means ± standard deviations. The expression was normalized to 16S rRNA. Controls refer to the absence of aqueous extracts of Rhizoma Coptidis. Significantly different ($p < 0.05$) compared to untreated control bacteria.
Furthermore, a recent study showed that mutation of the hrpB gene, which encodes RNA helicase, can reduce surface adhesion and inhibit disease spread in citrus leaves (Granato et al., 2016). We thank Shanghai Applied Protein Technology Co., Ltd. for the help with iTRAQ.

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In this study, we identified *S. suis* genes gapdh, sly, and mrp as potential targets of *Rhizoma Coptidis* water extracts. These genes are thought to play key roles in infection and invasion (Liang et al., 2011; Ju et al., 2012) and have been shown to be important in biofilm formation and adhesion. Treatment with 50 µg mL⁻¹ of extract suppressed gapdh, sly, and mrp gene expression. We speculate that this downregulation may be the cause of a reduction in *S. suis* adhesion and therefore biofilm formation. However, the detailed molecular mechanisms behind this reduction are still unknown and should be addressed in further studies.

Using iTRAQ, we found that 26 proteins were differentially expressed upon treatment of *S. suis* with 50 µg mL⁻¹ *Rhizoma Coptidis* water extract compared to untreated cells. Of these proteins, 11 proteins, implicated in surface adhesion and biofilm formation, were significantly suppressed. These include an antigen-like protein (D5AGH9), hydrolase (R4NST6), methyltransferase H (G7SM56), glycosyltransferase (M1VJ3), and helicase (G7S7E3) (Table 2). Antigen-like protein (D5AGH9) has been identified as a novel matricellular protein that promotes cell adhesion and spreading (Tajiri et al., 2010). Hydrolase, from the haloacid dehydrogenase superfamily (R4NST6), plays an important role in *Paracoccidioides brasiliensis* adherence to host cells (Hernandez et al., 2010). A previous study showed that deletion of an orphan C⁵ -cytosine methyltransferase, similar to methyltransferase H (G7SM56), has a significant effect on the expression of genes responsible for pathogenic growth (Kumar et al., 2012). Over-expression of the putative *Brucella* glycosyltransferase can lead to development of clumping and increased adhesion to polystyrene plates (Dabral et al., 2015).

Furthermore, a recent study showed that mutation of the hrpB gene, which encodes RNA helicase, can reduce surface adhesion and inhibit disease spread in citrus leaves (Granato et al., 2016). Biofilm formation and adhesion were reduced by treatment with 50 µg mL⁻¹ of *Rhizoma Coptidis* water extract, likely by down-regulation of expression of the proteins discussed above. In contrast, loss of capsular polysaccharides has previously been described to facilitate and speed up biofilm formation (Qin et al., 2013). Our results suggest that treatment of *S. suis* cells by the extract might cause upregulation of CpsR (K0FG35) and CPS16V (E9NQ29), proteins involved capsular polysaccharide formation, and reduced biofilm formation and adhesion.

Our results show that sub-MICs of *Rhizoma Coptidis* water extracts could inhibit biofilm formation, though the mechanism of action is unclear. We observed anti-adherence activity of the extract on *S. suis*. We also found that expression levels of genes and proteins involved in adhesion were significantly altered in cells treated with sub-MICs of *Rhizoma Coptidis* water extracts compared to untreated cells. Our results indicate that *Rhizoma Coptidis* water extracts inhibit *S. suis* biofilm formation by limiting adhesion.

**AUTHOR CONTRIBUTIONS**

Y-HL designed the whole experiment. The other authors are responsible for completing the experiment.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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