Tea polyphenols suppress growth and virulence-related factors of *Haemophilus parasuis*

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**ABSTRACT.** The bacterium *Haemophilus parasuis* (*H. parasuis*) is the primary cause of Glässer's disease. Currently, there are no effective vaccines that can confer protection against all *H. parasuis* serovars. Therefore, the present study aimed to investigate the effect of tea polyphenols on growth, expression of virulence-related factors, and biofilm formation of *H. parasuis*, as well as to evaluate their protective effects against *H. parasuis* challenge. Our findings demonstrated that tea polyphenols can inhibit *H. parasuis* growth in a dose-dependent manner and attenuate the biofilm formation of *H. parasuis*. In addition, tea polyphenols exerted inhibitory effects on the expression of *H. parasuis* virulence-related factors. Moreover, tea polyphenols could confer protection against a lethal dose of *H. parasuis* and can reduce pathological tissue damage induced by *H. parasuis*. In summary, our findings demonstrated the promising use of tea polyphenols as a novel treatment for *H. parasuis* infection in pigs.

**KEY WORDS:** biofilm, *Haemophilus parasuis*, protection, tea polyphenols, virulence-related factor

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*Haemophilus parasuis* (*H. parasuis*) is a bacterial pathogen that colonizes the upper respiratory tract of pigs, thereby causing Glässer's disease [15]. Glässer’s disease is characterized by fibrinous polyserositis, polyarthritis, and meningitis, which can lead to sudden death [4, 19]. In total, 15 *H. parasuis* serovars have been identified to date, with serovars 4 and 5 being the most prevalent worldwide, although more than 20% of the isolates remain to be serotyped [3, 7]. The pathogenesis of *H. parasuis* infection remains poorly understood and represents a challenge for the development of preventative and control measures, making Glässer’s disease a significant problem in the pig industry.

Tea is the most popular beverage in the world and comprises an aqueous infusion of dried leaves from the plant *Camellia sinensis* [13]. In particular, green tea is rich in polyphenols, which have been shown to have numerous important biological functions [29]. Previous research has shown that oral administration of green tea polyphenols can prevent the progression of abdominal aortic aneurysm mediated by its anti-inflammatory effects [21] and they can inhibit colorectal tumorigenesis in azoxymethane-treated F344 rats [8]. *H. parasuis* has been demonstrated to be capable of biofilm formation [11]. In addition, tea polyphenols can inhibit the growth of *Escherichia coli* by promoting endogenous oxidative stress [24], attenuating the expression of virulence factors, and preventing biofilm formation of *Fusobacterium nucleatum* [2]. Tea polyphenols can reduce disease pathogenesis and suppress inflammatory responses in interleukin-2-deficient mouse models for chronic inflammatory disease [23]. In addition, tea polyphenols were demonstrated to attenuate the pathogenicity of *Pseudomonas aeruginosa* [26]. However, the effects of tea polyphenols on *H. parasuis* infection remain to be fully understood.

The present study aimed to evaluate the influence of tea polyphenols on *H. parasuis*, including their effects on cell integrity, virulence-related factors, and biofilm formation. Our findings demonstrated that tea polyphenols could inhibit growth, reduce biofilm formation, and downregulate the expression of virulence-related factors in *H. parasuis* SH0165. In addition, tea polyphenols were demonstrated to confer protection against a lethal dose of *H. parasuis* and reduce pathological tissue damage induced by *H. parasuis*. Therefore, the use of tea polyphenols could serve as a novel strategy to control and treat pig infections caused by *H. parasuis*.
Biofilm susceptibility assay

The kinetics of the bactericidal effects of tea polyphenols against H. parasuis were determined by performing a quantitative assay [25]. Briefly, H. parasuis (1 \times 10^{6} \text{ CFU/ml}) and tea polyphenols (0, 40, 80, 160 and 320 \mu g/ml) were co-cultured in tubes at 37°C. Bacterial samples were collected at 0, 1, 2, 3, 4 and 5 hr. Samples were diluted in sterile phosphate buffered saline (PBS) and subsequently plated onto TSA plates. All plates were incubated for 24 hr at 37°C. The number of colonies was counted, and kinetic curves were constructed by plotting the log_{10} CFU/ml vs. time over a 5-hr period.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC of tea polyphenols against H. parasuis were determined as previously described [9, 20]. The MIC of tea polyphenols was defined as the minimum concentration at which no bacterial growth can be observed. To determine the MBC, 10-\mu l aliquots from each well of the plates with no visible growth were spread on TSA plates for 48 hr at 37°C. The MBC of the tea polyphenols were determined by measuring the lowest concentration at which no colony formation of H. parasuis was detected.

Analysis of H. parasuis cellular integrity by transmission electron microscopy (TEM)

The cellular integrity of H. parasuis was examined by transmission electron microscopy as previously described with minor modifications [17]. Briefly, H. parasuis was grown in TSB and harvested by centrifugation at 12,000 rpm for 10 min and subsequently washed with sterile PBS. Bacteria were then suspended in sterile PBS to obtain an OD of 0.6 and incubated with 10 \mu l of TSB containing 10 \mu g/ml NAD, 10% newborn calf serum, and 1% inoculum. The contents of each tube were removed, and the tubes were stained with 1% Hucker crystal violet solution at 37°C for 15 min. Then, the dye solution was removed from the glass tubes, and the glass tubes were washed with distilled water for 10 min until no color was observed in the flowing water. Excess water was removed from the glass tubes with tissue paper. In addition, the effects of tea polyphenols on biofilm formation were examined by performing a quantitative assay [17]. Briefly, we evaluated the effects of tea polyphenols on biofilm formation by treatment of H. parasuis in the wells of a 96-well microplate with tea polyphenols (80, 160 or 320 \mu g/ml) for 2 hr at 37°C. Wells treated with PBS were used as controls. Following treatment with tea polyphenols, H. parasuis was cultured for 16 hr, and the resulting culture was diluted with TSB to obtain an OD660 of 0.1. The samples (200 \mu l) were added to treated wells of the 96-well microplate. After incubation for 16 hr at 37°C, free-floating bacteria and spent media were removed using a 26-g needle. The wells were washed thrice with PBS, after which the H. parasuis biofilms were stained with 0.05% crystal violet (100 \mu l) for 30 min. Afterwards, wells were washed five times with PBS to remove unbound crystal violet dye and subsequently dried for 3 hr at 37°C. Afterwards, 100 \mu l of 95% (v/v) ethanol was added to each well, and the plate was shaken for 30 min. The absorbance at 550 nm was measured.

Determination of virulence related factor expression by quantitative RT-PCR

To explore the effects of tea polyphenols on the expression of virulence-related factors, H. parasuis was grown to the early-log phase. Then, tea polyphenols were added at 1/2 MIC (160 \mu g/ml) and co-cultured at 37°C for 5 hr. Bacterial cells were collected, and total RNA was extracted using the GenElute Total RNA Purification Kit (Sigma) according to the manufacturer’s instructions. Total RNA from H. parasuis was reverse-transcribed into cDNA using reverse transcriptase (TaKaRa). Expression levels were quantified using the SYBR Green PCR Kit (TaKaRa) according to the manufacturer’s instructions. The 16S rRNA gene was used as the internal control. Primer sequences used for quantitative RT-PCR are listed in Table 1.

Determination of the protective effects of tea polyphenols against H. parasuis challenge

The present study was carried out in strict accordance with the recommendations by China’s Regulations for the Administration.
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TEA POLYPHENOLS SUPPRESS H. PARASUIS

Table 1. Primers used for qRT-PCR

|       | Nucleotide sequence (5ʹ–3ʹ) Tm (°C) |       |
|-------|-------------------------------------|-------|
| 16S RNA | Forward TGAAGTCCGGAATCGCTAGTA | 53.4  |
|       | Reverse CCTACGGTTCACCTTTGATTCG    | 55.4  |
| ArcA  | Forward GGAGGGAGGAGGAAAGAGGAGATA | 54.0  |
|       | Reverse AAGACCTGAGAAGGAGCCACTAC    | 57.7  |
| ClpP  | Forward ACAACAAACACGACTGCAC        | 52.1  |
|       | Reverse CTGCCGTCATGCTGCTCCTTCCC   | 55.8  |
| RfaE  | Forward GAGGAGGAAGGAGGACTGTTT     | 55.8  |
|       | Reverse AGATTGGAACCTGTTGAGGAGT     | 54.0  |
| RfaD  | Forward TCTGCATGAGCTTTGCAAG        | 54.0  |
|       | Reverse ACAAGGGAGACTCGAATTCAC      | 54.0  |
| OmpP2 | Forward AGTAACCATCTCTGTGCGATT      | 59.5  |
|       | Reverse TCTTATCATGATGTCAGAAC       | 50.2  |
| OmpP5 | Forward CGCTCTTCTGCTACTGCACTTC     | 55.8  |
|       | Reverse CTGCCGTCAGCCTGAGCATT       | 52.1  |
| GalU  | Forward CCAAGGAACCAGCTATGAA        | 57.7  |
|       | Reverse CTGCACAGCGCTGACATT         | 52.1  |
| GalE  | Forward CAGAGCCAGGAGAGACT          | 52.1  |
|       | Reverse GACCAGCACAGGAATGAG         | 57.7  |

Table 2. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values of tea polyphenols for H. parasuis

| Medium | Compound       | MIC (µg/ml) | MBC (µg/ml) |
|--------|----------------|-------------|-------------|
| TSB    | Tea polyphenols | 160         | 320         |

of Affairs Concerning Experimental Animals 1988, and Hubei Regulations for the Administration of Affairs Concerning Experimental Animals 2005. The protocol was approved by China Hubei Province Science and Technology Department [permit number SYXK (ER) 2010-0029]. All experimental animals were euthanized at the end of the experiments.

Female BALB/c mice (five to six weeks old) were purchased from the Laboratory Animal Center of the Center for Disease Control of Hubei Province (Wuhan, China). The mice were divided randomly into four treatment groups (12 mice per group). Three groups received intragastrically administered with tea polyphenols at concentrations of 80, 160 and 320 µg/ml. The fourth group served as the negative control group and received PBS alone. After 30 min, all mice were challenged intraperitoneally with a lethal dose of 1.0 × 10⁹ CFU of the H. parasuis SH0165 strain. All mice were monitored for 7 days after the challenge. The morbidity and mortality rates were recorded.

Histopathology

The lungs and brains of all mice that survived the H. parasuis SH0165 challenge were collected and fixed by immersion in 10% neutral-buffered formalin and embedded in paraffin. The 4-µm-wide tissue sections were stained with hematoxylin-eosin (H&E) based on a standard protocol and examined using light microscopy.

Statistical analysis

Experimental data were expressed as mean ± SD. Differences between two groups was analyzed using the two-tailed Student’s t-test. Survival analysis was performed using the log-rank test. P<0.05 was considered statistically significant. *, P<0.05 and **, P<0.01.

RESULTS

Tea polyphenols inhibit the growth of H. parasuis in vitro

The results demonstrated that the tea polyphenols exhibited antibacterial activity against H. parasuis SH0165 (Table 2). The tea polyphenols had MIC and MBC values of 160 µg/ml and 320 µg/ml, respectively (Table 2). The kinetics of the antimicrobial effects of tea polyphenols against H. parasuis SH0165 indicated that tea polyphenols exerted a strong dose-dependent bactericidal effect against H. parasuis SH0165. In particular, H. parasuis SH0165 growth was significantly inhibited at a concentration of 160 µg/ml tea polyphenols over the 5-hr incubation period relative to the original inoculum (P<0.05) (Fig. 1).
Tea polyphenols inhibit the formation of *H. parasuis* biofilms

The effects of tea polyphenols on *H. parasuis* biofilm formation were evaluated by performing glass tube biofilm assay and the quantitative assay. Results indicated that tea polyphenols significantly reduced the formation of *H. parasuis* biofilms at concentrations of 80, 160 and 320 µg/ml relative to the control (0 µg/ml) (*P*<0.05) in a dose-dependent manner (Fig. 2).

**Tea polyphenols affect the cellular integrity of *H. parasuis***

Cell ultrastructure was analyzed by transmission electron microscopy to determine the effect of tea polyphenols on the cellular integrity of *H. parasuis*. *H. parasuis* cells treated with tea polyphenols at a concentration of 160 µg/ml showed evident damage to the cell ultrastructure relative to those of negative control cells (Fig. 3). Treated cells displayed severe cellular damage, which led to disruption of the cell wall and cytoplasmic membrane, accompanied by the leakage of cytoplasmic inclusions (Fig. 3B and 3C).

**Tea polyphenols inhibit the expression of virulence-related factors of *H. parasuis* in vitro***

To determine the effect of tea polyphenols on virulence-related factors, we evaluated the expression of the virulence-related factors ArcA, ClpP, RfaE, RfaD, OmpP2, OmpP5, GalU and GalE in *H. parasuis* SH0165. The results showed that tea polyphenols at 80 µg/ml downregulated the expression of ArcA, ClpP, RfaE, OmpP2, OmpP5 and GalE by greater than 40% relative to the untreated control in TSB medium (*P*<0.05) (Fig. 4). Furthermore, tea polyphenols significantly inhibited the expression of GalU by around 16% (Fig. 4). However, tea polyphenols significantly upregulated RfaD expression relative to the untreated control (*P*<0.05) (Fig. 4).

**Tea polyphenols exert protective effects against *H. parasuis* challenge***

The protective effects of tea polyphenols were assessed by evaluating survival in a mouse model following lethal dose challenge with *H. parasuis*. After intragastrical administration of tea polyphenols for 30 min, mice were challenged with a lethal dose of 1.0 × 10^9 CFU *H. parasuis* SH0165. Results showed that mice administered with tea polyphenols had higher survival rates compared to mice in the negative control group (*P*<0.05) (Fig. 5). No protective effects were observed in the negative control group (Fig. 5), and all mice died within 48 hr of the challenge. Mice administered with 80 or 160 µg/ml tea polyphenols presented mild clinical symptoms associated with *H. parasuis* infection. However, the mice administered with 320 µg/ml tea polyphenols displayed no clinical symptoms (data not shown).

**Histopathology analysis***

To examine the protective effects of tea polyphenols against the development of disease, we analyzed tissue pathologies of the lung and brain samples. Results showed that lung tissues from the negative control group presented extensive edema with fibroblast proliferation and connective tissue formation (Fig. 6A). Brain tissues from the negative control group exhibited meningitis with meningeal edema and detachment, as well as inflammatory cell exudation (Fig. 6E). However, only minor pathological changes were detected in the lung and brain tissues of animals treated with tea polyphenols at the concentrations of 80, 160 and 320 µg/ml (Fig. 6B, 6C, 6D, 6F, 6G and 6H).

**DISCUSSION***

In recent years, *H. parasuis* has emerged as one of the most serious respiratory bacterial pathogens that infect livestock worldwide, and *H. parasuis* infections have caused substantial economic loss [7]. In the present study, tea polyphenols were demonstrated to confer protective effects against a lethal dose of *H. parasuis*, thereby suggesting that administration with tea...
polyphenols could serve as a novel strategy for disease control and treatment of *H. parasuis* infections.

In the present study, tea polyphenols were dissolved in ethanol, thereby raising concern on the potential effects of ethanol in the experiments. Consistent with previous reports [14, 22], the working concentration of tea polyphenols was obtained by diluting the stock solution with very high dilutions using the culture medium. Therefore, the effects of ethanol on the bacterial were considered negligible.

Biofilms are sessile communities of bacterial cells enclosed in a self-generated extracellular polysaccharide matrix, which subsequently conglutinates to a biotic or abiotic surface [11]. In recent years, bacterial biofilms produced by microbial pathogens have become an important focus of scientific research [18]. Previous studies showed that *Staphylococcus aureus* causes chronic infections through biofilm formation [16]. *Candida albicans* causes catheter-related bloodstream infections via the formation of biofilms, which exhibit increased tolerance to antimicrobials [1]. *H. parasuis* serovar 5 has been reported to exhibit a high degree of biofilm formation [11] and are considered highly virulent [12]. The *H. parasuis* SH0165 strain is classified under serovar 5 [3]. In a recent study, we used *H. parasuis* SH0165 as the model organism because of its ability to form biofilms [11]. Our findings showed that tea polyphenols can inhibit *H. parasuis* biofilm formation and can thus be effective for the treatment of chronic...
infections caused by *H. parasuis*.

Virulence-related factors play important roles in bacterial pathogenesis. Previous studies showed that deletion of the *ArcA* gene increased bacterial sensitivity to porcine serum and produced lower biofilm mass [5]. The *ClpP* gene is essential for stress tolerance and acts as a negative regulator of biofilm formation in *H. parasuis* [10]. Deletion of the *RfaE* gene can attenuate serum resistance, adherence, and invasion of porcine kidney epithelial cells [28]. *RfaD* overexpression in the ΔompP2 mutant of *H. parasuis* could enhance adherence and invasion to PUVEC and PK-15 cells [27]. Deletion of *GalU* resulted in impaired biofilm formation, whereas *GalE* mutants produced more biofilm mass compared with wild-type *H. parasuis* [30]. Therefore, we investigated the mRNA expression of these important virulence-related factors (*ArcA*, *ClpP*, *RfaE*, OmpP2, OmpP5, *GalU* and *GalE*) following treatment with tea polyphenols. Our results showed that tea polyphenols could downregulate the mRNA expression of *ArcA*, *ClpP*, *RfaE*, OmpP2, OmpP5, *GalU* and *GalE* to different degrees. Notably, *RfaD* mRNA levels were significantly upregulated by treatment with tea polyphenols. The mechanism by which tea polyphenols induce *RfaD* upregulation warrants further study.

In this study, mice were administered with tea polyphenols via intragastrical administration for 30 min, after which increased plasma concentrations of the tea polyphenols were detected. Therefore, we selected 30 min as the treatment period for *H. parasuis* SH0165 challenge. Piglets were used as the infection model and were challenged by intranasal inoculation to mimic the natural infection route [6]. Mice were selected as the infection model for evaluating the protective effects of tea polyphenols. Consistent with the methods of our previous research, mice were challenged via intraperitoneal injection [7].

Our results demonstrated that tea polyphenols can inhibit growth, attenuate biofilm formation, and downregulate the expression of virulence-related factors of *H. parasuis* SH0165. In addition, tea polyphenols can confer protection against *H. parasuis* challenge and reduce infection-associated tissue damage. Our findings suggested that tea polyphenols exhibit potential as a novel anti-bacterial compounds for the prevention and control of *H. parasuis* infection.

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