INTRODUCTION

The weaning period is one of the most stressful phases for pigs during production. During this time, young pigs must rapidly adapt to a multitude of psychosocial (maternal and littermate separation, and mixing stress) and environmental (transport, abrupt changes in diet and increased pathogen exposure) stressors (Moeser, Ryan, Nighot, & Blikslager, 2007). Influenced by environmental changes or changes in food composition, weaning piglets often experience gastrointestinal dysfunctions such as villous atrophy, increased crypt depth and intestinal permeability (Paßlack, Vahjen, & Zentek, 2015), and decrease in the activity of lactase and maltase (Boudry, Péron, Le

Effects of Cortex Phellodendri extract on post-weaning piglets diarrhoea

Xiaofan Xu¹ | Yunxin Pan¹ | Baoyang Xu¹ | Yiqin Yan¹ | Boqi Yin¹ | Yanqing Wang¹ | Shuxin Hu² | Libao Ma¹

¹College of Animal Science and Technology, Huazhong Agricultural University, Wuhan, China
²Hubei New Agricultural Technology Company, Wuhan, China

Correspondence
Libao Ma, College of Animal Science and Technology, Huazhong Agricultural University, Wuhan 430070, China. Email: mailbao@mail.hzau.edu.cn

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Abstract
The diarrhoea incidence rate is often high among weaning piglets. In light of the fact that Cortex phellodendri has long been used to treat diarrhoea in China, this study aimed to evaluate the effects of Cortex Phellodendri Extract (CPE) on diarrhoea in weaning piglets and the mechanism behind such effects. In the first trial, 36 diarrhoeal weaning piglets were randomly divided into three groups. The control group was injected with 20 mg oxytetracycline/kg BW, while the two treatment groups were orally administered with 10 mg and 20 mg CPE/kg BW respectively. In the second trial, 96 weaning piglets were randomly divided into two groups. The control group was fed basal diet, while 300 mg CPE/kg BW was added to the diet of the treatment group. The pathogenic bacteria were then isolated and identified from the diarrhoeal faecal samples. Cell adhesion and RT-PCR tests were used to investigate the effect of CPE on the adhesion of pathogenic bacteria to IPEC-J2 cells. 16S rDNA-based high-throughput sequencing was used to analyse faecal microflora. The results showed that CPE reduced the diarrhoea incidence rate \((p < 0.05)\) and diarrhoea index \((p < 0.05)\) compared to control group, and increased the richness and evenness of weaning piglets’ gut microbiota. Escherichia coli \((E. coli)\) was identified as the causative organism. Cell adhesion and RT-PCR tests suggested that CPE reduced the adhesion of \(E. coli\) to IPEC-J2 cells \((p < 0.05)\) and the expression of \(fae\) and \(faeG\) gene \((p < 0.05)\) responsible for encoding \(E. coli\) fimbriae protein.

KEYWORDS
cell adhesion, cortex phellodendri extract, diarrhoea, Escherichia coli, weaning piglets
Huërou-Luron, Lalès, & Sève, 2004). Accordingly, the diarrhoea incidence rate of weaning piglets is often very high (Smith et al., 2010).

Diarrhoea in weaning piglets could decrease their survival rate and slow down their growth (Moeser, Ryan, et al., 2007). The causes of diarrhoea are complicated, but bacterial infection has been identified as a major risk factor. Particularly, enterotoxigenic Escherichia coli (ETEC) is thought to be a major cause of diarrhoea in weaning piglets (Zhang et al., 2018). The gastrointestinal system of weaning piglets is very vulnerable and susceptible to ETEC infection. The pathogenicity of ETEC depends on its colonization and production of enterotoxin in small intestinal epithelial cells (Wu, Wang, & Liu, 2010). Bacteria use their Fimbriae to recognize the receptors of intestinal epithelial cells. Based on the type of its fimbriae, E. coli strains are classified as F4 (K88), F5 (K99), F6 (987P), F18, F17, F41 and F42, among which F4 is the most prevalent one that causes piglet diarrhoea during the post-weaning period. (Van Breda, Dhungyel, & Ward, 2018).

Traditional Chinese herbal medicines can serve as potential alternatives to antibiotics in treatment of diarrhoea owing to their relatively low toxicity and reliable anti-diarrhoea efficacy (Prasad, Parmar, Danta, Laloo, & Hemalatha, 2017; Xu, Chen, & Li, 2017). Cortex phellodendri extract (CPE), whose major bioactive component is berberine (84.25%), is derived from a traditional Chinese herb Cortex phellodendri. Cortex phellodendri has been clinically used for thousands of years in China to treat gastrointestinal pathogenic bacterial infection, especially bacteria-induced diarrhoea (Li et al., 2017). In earlier studies, CPE has been shown to ameliorate the symptoms of gastrointestinal disorders through regulating the anti-inflammation cytokine signalling pathways, and modulating the ubiquitin proteasome system or protein synthesis (Park, You, Cho, Choi, & Lee, 2019). CPE could treat gastrointestinal disorders, suggesting a strong correlation between gut microbiota, host metabolism and health (Freile et al., 2003).

Previously studies suggested that CPE could inhibit bacteria reproduction as well as virus replication (Cecil, Davis, & Cech, 2011; Cernáková & Kostálová, 2002). However, our in vitro bacteriostatic experiment found that the minimum inhibitory concentration (MIC) of CPE is higher than antibiotics, which indicates that CPE’s bacteriostatic effect is weak. Therefore, we hypothesize that there is another explanation to the mechanism of CPE’s action, for example, bacterial adherence or bacterial toxins secretion. Our study attempts to evaluate the effects of CPE, whose major component is berberine, on post-weaning diarrhoea, and further reveal the mechanism behind such effects.

2  |  MATERIALS AND METHODS

2.1  |  Plant materials and chemicals

The following are the plant materials and chemicals used in our study:

- Cortex phellodendri extract (Phellodendron chinense Schneid) (containing 84.25% berberine, judged by chromatography; Hengruitong Company, Sichuan, China)
- Faecal genomic DNA extraction kit, Bacterial genomic DNA extraction kit, and Bacterial total RNA extraction kit (Tiangen Biotech Company, Beijing, China)
- Porcine small intestinal epithelial cells IPEC-J2 (National Key Laboratory of Agricultural Microbiology, Wuhan, China)
- SYBR green supermix (Bio-Rad, California, US)
- 10 × Easy taq buffer, Taq DNA polymerase (Promega, Madison, US)
- DNA ladder DL2000, and Restriction enzyme ecor I and hinc II (Takara Bio (Dalian))
- Reverse transcription kit (Thermo, Waltham, US)
- Standard strain of E. coli K88ab (C83901), K88ac (C83715), and K88ad (C83923) (China Institute of Veterinary Drug Control, Beijing, China)

2.2  |  Experimental animals

(1) Thirty-six 28-day-old Duroc × Landrace×Yorkshire weaning piglets with diarrhoea, each weighing about 7.5 kg, were randomly divided into three groups (12 in each) with male and female halves. Each group lived in a separate pen. The experiment lasted 4 days (from d28 to d31). The piglets in the control group were injected with oxytetracycline (20 mg/kg BW/day) for three days. The piglets in the two experimental groups were orally administered with CPE (10 mg/kg BW/day and 20 mg/kg BW/day, respectively) for three days. Faecal samples were collected in the mornings from d29 to d31, and faecal scores were recorded according to a 4-grade faecal scoring system (Table 1) by a person who did not know about the experimental scheme. Then diarrhoea indices and diarrhoea incidence rates were calculated and recorded (Figure 1).

(2) Ninety-six 28-day-old Duroc × Landrace×Yorkshire non-diarrhoeal weaning piglets, each weighing about 7.5 kg, were randomly divided into two groups with male and female halves. Each group had six replicates with eight piglets in each replicate, and each replicate lived in a separate pen. The experiment lasted 11 days (from d28 to d38). The piglets in the control group were fed the basal diet. The experimental group was fed the basal diet supplemented with CPE 300 mg/kg for 10 days. The weaning piglets’ diarrhoea incidence rates were recorded on d28, d31 and d38 using the same method adopted in the first trial.

**TABLE 1**  Faecal scoring standard

| Score | Evaluation     | Faecal appearance     |
|-------|----------------|-----------------------|
| 0     | Normal         | Solid and cloddy      |
| 1     | Light diarrhoea| Soft with shape       |
| 2     | Mild diarrhoea | Very soft or viscous liquid |
| 3     | Diarrhoea      | Watery or with blood  |
All piglets were raised indoors, and each pen was 24 square metres with cement floor. The piglets could obtain water freely by biting drinkers. The pigpens were ventilated to maintain the temperature at about 25°C.

All diets were formulated to meet or exceed the NRC (2012) nutrient requirements (Supporting Information 1). The study was approved by the Institutional Review Board for Clinical Research at Huazhong Agricultural University (HZAUSW2013-0006). Written informed consent was also obtained from all participants before the study protocol began.

2.3 | Isolation and identification of pathogenic bacteria

Under sterile conditions, faecal samples from the diarrhoeal pigs were inoculated on the Agar plate at 37°C for 24 hr. Suspected colonies were picked and inoculated respectively on MacConkey plate at 37°C for 24 hr. Lastly, the purified bacteria were inoculated in the microbiochemical reaction tube under sterile conditions.

2.4 | Determination of the minimum inhibitory concentration (MIC) of CPE

A single colony of fresh K88ac was picked out of the purified bacteria and inoculated in Tryptic Soy Broth (TSB) medium. It was then placed in a biochemical incubator at 37°C for 16–18 hr. The bacterial content was counted with the surface plate method, and diluted to 10^8 CFU/ml with TSB medium before use. The 96-well plate microdilution method was used to determine the MIC value of CPE, ceftiofur sodium, colistin sulphate, amoxycillin, enrofloxacin and ciprofloxacin. The results were recorded after 18 hr of culture. The MIC value was determined by the drug concentration in the lowest dilution well with no bacterial growth at the bottom.

2.5 | Cell adhesion test

IPEC-J2 cells were cultured in 12 orifices for 20 hr until they grew into monolayer cells. The cell concentration was adjusted to around 10^6 for each hole. Pathogenic bacteria were inoculated in the TSB culture medium.
medium at 37°C for 10–16 hr. The concentration of bacterial solution was adjusted to about 10^8 CFU/ml. Four millilitre of pathogen suspension was added into each of four centrifugal tubes for centrifugation at 4,000 r/min for 15 min. The supernatant in each tube was discarded, and 4 ml of PBS, 100 μg/ml CPE, 200 μg/ml CPE and 300 μg/ml CPE were respectively added into each of the four tubes, which were then cultured at 37°C for 15 min. One millilitre of the new suspensions from the four tubes was added into the cell culture holes, respectively, which was repeated three times. After 2 hr of incubation, each of the 12 holes was washed with sterile PBS buffer three times to remove the bacteria that had not adhered to the cells. Then 0.5% of Triton X-100 was added into each hole to crack the cells so that the adhering bacteria fell off. After 15–20 min, a pipette was used to blow the bacterial suspensions gently and the bacteria were collected. Finally, the bacteria were inoculated on the MacConkey tablet for incubation at 37°C for 12 hr, and then counted. (Guo et al., 2016).

2.6 | Real-time fluorescence quantitative PCR

Pathogenic bacteria were cultured in TSB medium at 37°C for 10–16 hr, and the concentration of bacteria solution was adjusted to about 10^8 CFU/ml. Four microliter of pathogen suspension was added into each of three centrifugal tubes for centrifugation at 4,000 r/min for 15 min. The supernatant in each tube was discarded, and 4 ml of PBS, 150 μg/ml CPE and 300 μg/ml CPE were respectively added into the three tubes for incubation at 37°C for 15 min. One millilitre of the pathogen suspensions from each tube was added into another three centrifugal tubes for centrifugation at 12,000 r/min for 2 min. Then the precipitate in each centrifugal tube was collected. Bacterial total RNA extraction kit was used to extract the total RNA of the precipitates, and reverse transcription kit was used to reverse transcribe the extracted total RNA of the precipitates (Luo et al., 2020).

The primers of each gene are listed below (Table 2). The thermal cycle conditions were initial heating at 50°C for 2 min and then at 95°C for 10 min, followed by multiple cycles of 95°C for 15 s and 60°C for 1 min.

2.7 | Faecal microflora analysis

Fresh faeces were individually collected from the rectums of slaughtered piglets, immediately snap-frozen in liquid nitrogen and then stored at –80°C before faecal microbial genomic DNA extraction. Total DNA was extracted using bacterial total DNA extraction kit. The DNA concentration was then measured with micro UV spectrophotometer. The quality of DNA was measured with agarose gel electrophoresis. The genomic DNA was used as a template for PCR amplification. Universal primers 338F and 806R were used for PCR amplification of the V3–V4 hypervariable regions of 16S rRNA genes (338F, 50-ACTCTACGGGAGGCAGCAGGA-30; 806R, 50-GGACTACHVGGGTWTCTAAT-30). Sequencing was performed with an Illumina MiSeq PE300. The forward and reverse reads were joined and assigned to samples based on barcode and truncated by cutting off the barcode and primer sequence. Quality filtering on joined sequences was performed and sequence which did not fulfil the following criteria were discarded: sequence length < 200bp, no ambiguous bases, mean quality score>= 20. Then the sequences were compared with the reference database (RDG Gold database) using UCHIME algorithm to detect chimeric sequence, and then the chimeric sequences were removed.

The effective sequences were used in the final analysis. Sequences were grouped into operational taxonomic units (OTUs) using the clustering program VSEARCH (1.9.6) against the Silva 119 database pre-clustered at 97% sequence identity. The Ribosomal Database Program (RDP) classifier was used to assign taxonomic category to all OTUs at confidence threshold of 0.8. The RDP classifier uses the Silva 123 database which has taxonomic categories predicted to the species level.

Sequences were rarefied prior to calculation of alpha and beta diversity statistics. Alpha diversity indexes were calculated in QIIME from rarefied samples, using the Shannon index for diversity and the Chao1 index for richness. Beta diversity was calculated using weighted and unweighted UniFrac and principal coordinate analysis (PCoA). Unweighted Pair Group Method with Arithmetic mean (UPGMA) tree from beta diversity distance matrix was built. Statistical comparisons were performed using an unpaired two-tailed Student t test, paired Student t test or one-way analysis of variance (O’ Donovan, Connor, Madigan, Cotter, & O’ Sullivan, 2020).

2.8 | Statistical analysis

The results were presented as mean ± SEM. Experimental data were analysed by one-way ANOVA and the Duncan multiple comparison test with GraphPad 8.0 software. Significance was presented as *p < 0.05, and **p < 0.01.

3 | RESULTS

3.1 | Effect of CPE on treatment of post-weaning diarrhoea

In the first trial, with the increase in treatment time, diarrhoea indices decreased in all the groups. No significant difference was found
among the three groups (p > 0.05) after the first treatment. After the third treatment, the diarrhoea indices and the diarrhoea incidence rates of the treatment groups all indicated significant decrease though higher than those of the control group. While no significant difference was found in their diarrhoea incidence rate, the diarrhoea index of the 20 mg/kg BW group was significantly lower than that of the 10 mg/kg BW group (p < 0.05) (Figure 1a).

In the second trial, the diarrhoea incidence rate of the treatment group reduced from 22.2% to 3.7% (p < 0.05) following the addition of 300 mg/kg extract to the weaning piglets’ diet. The decrease was significantly greater than that of the control group (p < 0.05) (Figure 1c). During the experiment, no piglet died or showed symptoms of other diseases.

3.2 | Isolation and identification of pathogenic bacteria

We isolated 24 pathogenic strains from diarrhoeal piglets and selected all the Gram-negative colonies for biochemical identification. Seven of the Gram-negative colonies were positive for glucose, mannitol, indole, arabinose and lactose, but negative for citrate, urea and hydrothion. These biochemical characteristics conform to those of E. coli designated in Berger’s manual of systematic bacteriology (Table 3). Thus, we concluded that the seven colonies were E. coli.

Afterward, we used the gene specific primers of K88ab (A), K88ac (B) and K88ad (C) for PCR analysis. The result indicated that all the standard strains could be amplified to the fragment size of about 500 bp as expected (Figure 2). PCR analysis showed that the seven colonies carried K88ac flagellum whereas neither K88ab nor K88ad flagellum was detected.

3.3 | CPE’s ability to reduce E. coli adhesion to IPEC-J2

Previous researchers generally believe that berberine exerts its effect through direct bacteriostatic action, but our records of MIC values do not seem to support their opinion. The MIC value of each drug for E. coli was recorded as follows: CPE, 1,000 μg/ml; Ceftiofur sodium, 0.195 μg/ml; Colistin sulphate, <0.097 μg/ml; Amoxycillin, 1.562 μg/ml; Enrofloxacin, 0.097 μg/ml; Ciprofloxacin, <0.097 μg/ml (Figure 3a). Apparently, the bacteriostatic effect of CPE was not significant, compared with the other drugs. In the cell adhesion test, the 100 μg/ml group was not significantly different from the PBS group (p > 0.05). However, the 200 μg/ml (p < 0.05) and 300 μg/ml (p < 0.01) groups exhibited significantly greater ability to reduce the adhesion of E. coli to IPEC-J2, compared with the PBS group (Figure 3b).

3.4 | CPE’s ability to reduce Fae and FaeG gene expression of ETEC

In the RT-PCR test, compared with the PBS group, the expression of fae gene (p < 0.01) and faeG gene (p < 0.05) were both significantly reduced in the 300 μg/ml CPE group, though no significant difference was found between the PBS and 150μg/ml CPE groups (Figure 4a and b).

3.5 | CPE’s influence on the gut microbiota composition of diarrhoeal piglets

According to operational taxonomic unit (OTU) analysis, there were 125 OTUs in the diarrhoeal piglets’ guts after treatment with CPE, 4 OTUs more than before treatment (Figure 5a). Also, the OUT-rank graph became wider and flatter after treatment, indicating that the richness and evenness of the species had both increased (Figure 5b and c). Based on Non-metric multidimensional scaling (NMDS) analysis, we found a significant difference between the compositions of the gut microbiota before and after treatment (Figure 5d).

3.6 | CPE’s inhibition of the growth of harmful intestinal bacteria

We randomly selected four piglets’ faeces to analyse faecal microflora before and after treatment. OTU analysis showed that the abundance distribution of the 29 genus changed after treatment, and as nine genus abundances decreased, the other 20 increased (Figure 6a). After treatment with CPE, the abundances of Escherichia and Shigella reduced significantly (p < 0.05). These bacteria are often the cause of inflammatory bowel disease (IBD) and are regarded as harmful. On the other hand, the abundances of Bacteroides, Phascolarctobacterium and Faecalibacterium, bacteria that can maintain the balance of digestive and immune system, increased significantly (p < 0.05) (Figure 6b).

4 | DISCUSSION

When piglets are weaned, their feed is changed from milk to a weaner diet, and they are separated from their mothers and often

| TABLE 3 | Results of biochemical experiment |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Gram stain      | Glucose | Citrate | Mannitol | Indole | Urea | Hydrothion | Arabinose | Lactose |
| Pathogenic bacteria | G-      | +      | -      | +      | +    | -        | -        | +        |

Note: G-... gram-negative; +... 90% to 100% positive; –... 90% to 100% negative.
moved from the farrowing pens to other pens where they are mixed with unfamiliar pigs. Moreover they can no longer receive antibodies through sows’ milk, thus losing passive intestinal immunity. Also, there are changes in the intestinal \textit{E. coli} flora of piglets (Muns & Magowan, 2018). Impairment of immune functions in early-weaning piglets has been documented (Levast et al., 2010). During the first two weeks after weaning, pathogenic \textit{E. coli} is a key factor in the aetiology of bowel disease though infection with pathogenic \textit{E. coli} does not unequivocally lead to the development of diarrhoea in weaned pigs (Melin, Katouli, & Lindberg, 2000).

ETEC is the main intestinal pathogenic bacteria and its pathogenicity depends on its ability to colonize and to produce enterotoxin in small intestinal epithelial cells. Moreover \textit{E. coli}’s adhesion to intestinal epithelial cells is an essential step for its colonization (Li et al., 2010). Our results indicated that 300 μg/ml CPE reduced the adhesion of \textit{E. coli} to IPEC-J2 cells significantly. In light of the fact that \textit{E. coli}’s adhesion depends on its fimbriae, we further carried out a RT-PCR test, whose result showed that 300 μg/ml CPE reduced the expression of \textit{fae} and \textit{faeG} gene significantly. Therefore, it can be inferred that CPE is able to reduce \textit{E. coli}’s adhesion to IPEC-J2 cells by reducing the expression of \textit{E. coli}’s fimbiae gene, thus successfully preventing \textit{E. coli}’s colonization in the weaning piglets’ small intestinal epithelial cells to achieve its positive effects on post-weaning diarrhoea. Although such explanation has not been proposed by previous researchers, some researchers did show that berberine can directly interfere with the adhesion of \textit{Streptococcus} to host
cells through two different mechanisms: interfering with adhesion by inhibiting the release of an adhesive from the Streptococcus’ cell surface, or directly blocking or dissolving a fibronectin complex (Shi, Wu, Liu, & Xie, 2013). Therefore, it is probable that CPE’s reduction of E. coli’s fimbrae gene is the mechanism behind its ability to treat and prevent post-weaning diarrhoea.

The weaning piglet’s intestinal microbiota plays a critical role in health and disease, including the pathogenesis of IBD (Wang et al., 2019). Numerous studies have demonstrated that altered bacterial diversity and abundance at varying taxonomic levels in IBD mouse can improve disease symptoms (Jostins et al., 2012; Neish, 2009). Intestinal dysbiosis (specifically, a compositional imbalance of commensal bacteria) is the central characteristic of the gut microbiota in IBD and has been considerably documented. A common feature of the intestinal microbiota in IBD is reduction in the abundance of several types of bacteria, particularly members of Firmicutes and Bacteroidetes phyla (Frank et al., 2007).

According to the results of OTU abundance analysis, we found that after treatment with CPE, the abundances of Escherichia, Shigella, Sutterella, Bacteroides, Phascolarctobacterium and Faecalibacterium changed significantly. Previous studies have reported that the abundances of Faecalibacterium and Phascolarctobacterium usually decreased and the abundances of Escherichia and Shigella increased in Crohn’s disease and ulcerative colitis patients (Imanshahidi & Hosseinzadeh, 2008). Faecalibacterium and Phascolarctobacterium are associated with the production of short-chain fatty acids (SCFAs) (e.g. acetate, propionate and butyrate). Bacteroides can provide antigens that induce regulatory T cells to generate anti-inflammatory cytokines to protect commensal gut microbiota (Ramakrishna et al., 2019). That explains why CPE has positive effect on intestinal microbiota health, and can thus be used to treat diarrhoea.

However, in this experiment, the number of samples analysed by faecal 16s was not large, which may have a negative impact on the reliability of the results. Moreover the concentration of CPE in

**FIGURE 5** OUT Venn graph of gut microbiota (Figure 5a), OTUs rarefaction curves of gut microbiota (Figure 5b), Rank-abundance graph of gut microbiota (Figure 5c), and NMDS plot analyses of gut microbiota (Figure 5d). Note: Group A refers to piglets after treatment; Group B refers to piglets before treatment.
the second trial was not set gradient, but only according to previous experience. In addition, there were no statistics of some important performances of piglets like weight and food intake.

5 | CONCLUSIONS

In summary, CPE has positive effect on diarrhoea in weaning piglets. The mechanism behind such effects is that CPE can reduce the adhesion of ETEC to intestinal epithelial cells by inhibiting the expression of bacterial flagellum gene. Furthermore, CPE can improve the composition of gut microbiota, and enhance the diversity and evenness of gut microflora. It can also inhibit the growth of harmful intestinal bacteria such as Escherichia and Shigella. Our results showed that adding CPE to piglet diet can be an effectively method to relieve weaned diarrhoea.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS’ CONTRIBUTION

xiaofan Xu: Conceptualization; Formal analysis; Methodology; Resources; Software; Supervision; Validation; Visualization; Writing-original draft; Writing-review & editing. yunxin Pan: Funding acquisition; Methodology; Writing-original draft. baoyang Xu: Data curation; Formal analysis; Funding acquisition. yiqin Yan: Resources; Software; Validation. boqi Yin: Conceptualization; Data curation; Investigation. yanqing Wang: Conceptualization; Data curation; Formal analysis; Project administration. libao Ma: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Resources; Supervision; Validation; Visualization. XFX and YXP participated in all portions of the project including experimental design, the conduct of the experiments, interpretation and analysis of the results, and the writing and revision of the manuscript. BYX, YQY, BQY, YQW and SXH participated in the conduct of the experiments, including sample collection, animal care, and the conduct of the assay. All authors have read and approved this copy.

ORCID

Xiaofan Xu  https://orcid.org/0000-0002-8454-6996

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