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Efficacy of Ban-Qin-Fei-Re-Qing oral liquid on avian infectious bronchitis

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Abstract

Infectious bronchitis virus (IBV) can cause respiratory infections in animals that often lead to heavy losses for breeding industry. Ban-Qin-Fei-Re-Qing oral liquid (BQ), a Chinese herbal compound, has been used to treat infectious bronchitis (IB). This research aimed to assess the antiviral effect of BQ against IBV and elucidate the underlying mechanisms through bioinformatics analysis. The experiments designed in this study investigated how BQ inhibits IBV propagation in chicken embryos and enhances protective effects on chicken embryos. The findings indicated that, in comparison to the model group (untreated), the BQ-treated groups exhibited a significant protective effect on IBV-infected chicken embryos. Moreover, the groups administered medium or high doses of BQ demonstrated a superior protective effect compared to the group treated with a lower dose. In addition, even at a low dose (2.5 mL/L), BQ successfully treated IB in chickens. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses revealed that the differentially expressed genes were enriched in antiviral pathways, such as the JAK/STAT and type I interferon signaling pathways. In conclusion, the current study demonstrated that BQ has antiviral activity and plays an antiviral role through the combined action of multiple antiviral pathways. These findings could lead to future research on identifying drugs to prevent and treat IB.

Keywords Ban-Qin-Fei-Re-Qing oral liquid, Infectious bronchitis, Gene expression profiling, JAK/STAT signaling pathways, Type I interferon signaling pathway

Introduction

Avian infectious bronchitis (IB) is characterized by acute and high contact and often manifesting clinical signs across multiple organ systems, including the respiratory tract, digestive system, and urogenital system [1]. IB not only has major clinical symptoms, such as sneezing, tracheal rales, and coughing [2], but also affects the quality of laying hens and the performance of broilers, which causes large economic losses in the poultry breeding industry.

The treatment for IB is vaccination, but existing vaccination approaches focus on particular virus serotypes. Nevertheless, vaccines have not proven to be entirely effective in preventing new infections owing to the remarkable recombination-prone characteristics of these viruses [3, 4]. There is a demand for more effective approaches to prevent or treat IBV. Plant extracts could serve as promising resources. Traditional Chinese Medicine (TCM) has been utilized across Asian nations for millennia to address various ailments. Herbal medicine

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is a major branch of TCM and many herbal compounds have been demonstrated to have anti-infectious bronchitis virus (IBV) effects [5, 6]. For example, forsythoside A has been shown to have preventative and therapeutic effects on against IBV infection in chickens [5]. A study also showed that Houttuynia cordata exhibited inhibitory effects against IBV infection in both *Vero* cells and chicken embryo kidney cells [6].

Numerous studies have been conducted to identify effective Chinese Herbal Medicines (CHMs) for the treatment of respiratory diseases caused by IBV. Ban-Qin-Fei-Re-Qing oral liquid (BQ) contains 8 ingredients: Banlangen (Isatidis Radix, *Isatis indigotica* Fort., Cruciferae), Huangqin (Scutellariae Radix, *Scutellaria baicalensis* Georgi, Labiatae), Shigao (Gypsum Fibrosum), Beidougen (Menispermi Rhizoma, *Menispermum dauricum* DC., Menispermaceae), Jiegeng (Platycodonis Radix, *Platycodon grandiflorum* (Jacq.) A.DC., Campanulaceae), Ziwan (Asteris Radix Et Rhizoma, *Aster tataricus L.f.*, Asteraceae), Pengsha (Borax) and Bingpian (Borneolum, *Cinnamomum camphora* (L.) Presl, Lauraceae). Banlangen, a monarch medicine of BQ, has been shown to have a wide range of antiviral activities [7], such as against influenza virus [8], varicella-zoster virus [9], and respiratory syncytial virus [10]. Huangqin has also been reported to have antiviral activity, exemplified by the anti-H1N1 virus [11], coxsackievirus [12], and respiratory syncytial virus [13]. Despite the knowledge of the actions of its key medicines, the effect and mechanism of BQ on treating IB are still poorly understood.

Microarray technology and data mining tools for extracting crucial information from biological data provide opportunities for exploring many genes/targets that could help explain the efficacy of CHMs [14]. Gene Ontology (GO), which functions as a bioinformatics resource rooted in the community, offers ontological representation for biological knowledge, encompassing information related to the functionality of gene products [15]. Kyoto Encyclopedia of Genes and Genomes (KEGG) has been used to further illustrate the biological significance of the genes [16]. Using these tools, the differential gene expression of profiles can be used to analyze the possible targets and mechanisms of many CHMs.

The objective of this study was to determine the effect of BQ on IBV and to explore the underlying mechanisms through bioinformatics. The findings could identify effective drugs for IBV and offer novel ideas for developing new treatments for coronavirus.

Results

Preparation of BQ and its quality control

In this study, BQ, which is composed of Banlangen, Huangqin, Beidougen, Jiegen, Ziwan, Shigao, Pengsha and Bingpian, was prepared by extraction and concentration. We used the HPLC method to determine the content of baicalin and dauricine in BQ to control the quality of BQ (Fig. S1).

Antiviral effect of BQ on chicken embryos

To determine the antiviral effect of BQ in chicken embryos, the chicken embryos were observed twice a day for 8 days, and those that died within 24 h were discarded, and then the protection rates were compared among the six groups (Control, Model, BM (Bo-Ma oral liquid), BQ low dose (BQL), BQ medium dose (BQM), and BQ high dose (BQH) groups).

Based on the clinical observations, the number of abnormal chicken embryos was counted, and the protection rate for each group was calculated as:

$$\text{The protection rate} = \frac{\text{Number of normal chicken embryos}}{\text{Total number of chicken embryos}} \times 100\%$$

After 24 h of observation, one chicken embryo each died in the Model, BM, and BQL groups ($n=15$). The protection rate is shown in Fig. 1A. As expected, the protection rate in the Control group was 100% and that in the Model (no treatment) group was 0%. For the infected and treated groups, the protection rate in the BM group was 53.3% (8/15), in the BQL group was 60% (9/15), and was higher in the BQM group (13/16, 81.3%) and BQH group (12/16, 75%). Clearly, all treated groups had significantly greater did than the infected-only group.

On the virus replication detected by the qRT–PCR method, as shown in Fig. 1B, compared to the Model group (19,318 ± 3,077 copies/μL), treated groups had clear reduction of virus replication: BQL (6,423 ± 1,146 copies/μL; $p<0.05$), BQM (4,095 ± 589 copies/μL; $p<0.01$), BQH (3,849 ± 489 copies/μL; $p<0.01$) and BM (7,101 ± 542 copies/μL; $p<0.05$). This suggested that the treatments, especially with medium or high doses of BQ, could effectively inhibit virus replication after the chicken embryos were infected with the virus.

To compare the effect of different administration times on BQ antiviral therapy, two additional trials (one was BQ administered 2 hours before infection, and the other was BQ and IBV simultaneously) were conducted (details are shown in Fig. S2A-B and Fig. S3A-B). The results were

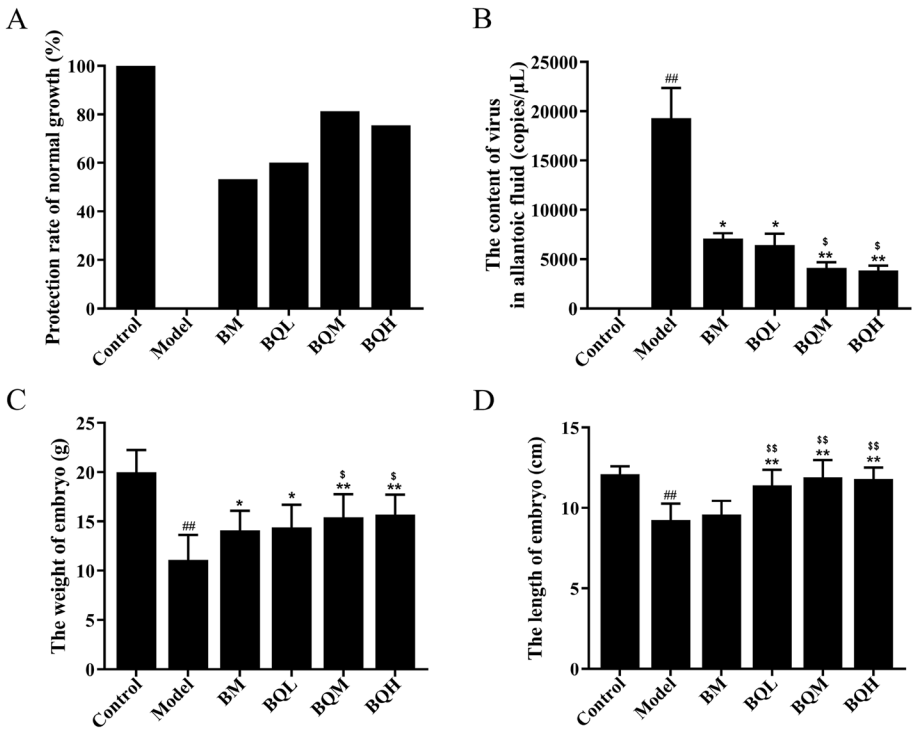

Fig. 1 Effect of BQ on antiviral activity and growing development of chicken embryos after administration of BQ 2 h after infection. The antiviral effect was evaluated by the protection rate of embryonic normal growth (**A**) and the content of the virus in allantoic fluid (**B**). Growing development was demonstrated by the weight (**C**) and length (**D**) of chicken embryos. Except for the protection rate of normal growth, other data are shown as the mean ± SD. $^{\#\#}P < 0.01$, Model group vs. Control group. $^{**}P < 0.01$ and $^{*}P < 0.05$, BM or BQ group vs. Model group. $^{\$\$}P < 0.01$ and $^{\$}P < 0.05$, BQ group vs. BM group. BQ: Ban-Qin-Fei-Re-Qing oral liquid; BM: Bo-Ma oral liquid; BQL: BQ low dose group; BQM: BQ medium dose group; BQH: BQ high dose group

similar to the above results (giving BQ after the infection)—BQ could improve the protection rate of chicken embryos, where BQM and BQH were more effective than BQL and BM (Fig. S2A, Fig. S3A). In addition, BQM and BQH significantly reduced the replication of the virus in the allantoic cavity of chicken embryos compared with that in infected-only group (Fig. S2B, Fig. S3B). Therefore, BQ can inhibit viral replication.

Effect of BQ on the growth performance of chicken embryo

To investigate the effect of BQ on the growth performance of chicken embryos, the embryo length and weight of chicken embryos were measured in each group. The results of embryo weight showed that when BQ was given 2 h after virus injection, all treated groups had significantly greater mean weight than the Model group ($P < 0.01$ for BQM and BQH groups; $P < 0.05$ for BQL and BM groups) (Fig. 1C). Besides, mean weights of BQM and BQH groups were also more than that in the BM group ($P < 0.05$). When administering the BQ 2 h before or at the same time as the time of virus infection, the results also showed that the BQ group had significantly greater mean weight than the Model group (Fig. S2C when given before infection, $P < 0.05$ for BQ. Fig. S3C when given simultaneously with infection, $P < 0.05$ for BQL and BQM, $P < 0.01$ for BQH).

For embryo's length, when given the treatment 2 h after virus injection, Model group had a significantly smaller mean length ($P < 0.01$) compared with Control group, and the BQ groups had a significantly greater mean length than the Model group (Fig. 1D, $P < 0.01$), whereas the mean length in the BM group was not significantly different from that in the Model group ($P > 0.05$). When BQ was given 2 h before virus injection and BQ was administered simultaneously with the virus infection, similar conclusions were reached (Fig. S2D, $P < 0.01$ for BQM groups, $P < 0.05$ for BQL and BQH groups. Fig. S3D, $P < 0.01$ for BQM and BQH groups, $P < 0.05$ for BQL groups).

Safety evaluation of BQ

To gauge the clinical safety of BQ administration, we divided chicks into four groups, including receiving no medicine, those receiving the recommended dose of BQ, those receiving 3 times the recommended dose of BQ, and those receiving 5 times the recommended dose of BQ for examining hematological procedures, blood

Table 1 Routine blood indicators of the experimental chickens in each group

Indicators	Control	BQ-1	BQ-3	BQ-5
Red blood cell (10^{12}/L)	3.24 ± 0.61	3.32 ± 0.72	3.01 ± 0.81	3.36 ± 0.58
White blood cell (10^{9}/L)	20.40 ± 2.05	21.90 ± 1.37	21.30 ± 1.21	22.10 ± 2.16
Lymphocytes (%)	55.50 ± 1.09	55.60 ± 1.96	55.90 ± 1.26	55.80 ± 1.38
Heterophile granulocytes (%)	25.20 ± 1.62	25.40 ± 1.42	25.90 ± 1.54	25.40 ± 1.87
Acidophilic leukocytes (%)	6.10 ± 1.31	5.90 ± 1.25	5.80 ± 1.24	6.10 ± 1.51
Basophils (%)	1.60 ± 0.81	1.60 ± 0.88	1.70 ± 0.72	1.70 ± 0.81
Monocytes (%)	6.50 ± 0.63	6.20 ± 0.57	6.40 ± 0.82	6.30 ± 0.92
Hemoglobin concentration (g/L)	121.00 ± 2.13	124.00 ± 2.49	122.00 ± 2.17	125.00 ± 1.99
The average hemoglobin content (pg)	45.16 ± 0.81	43.18 ± 1.12	44.37 ± 1.31	46.52 ± 1.21
Blood coagulation count(10^{9}/L)	1.09 ± 1.31	0.98 ± 1.45	0.87 ± 1.82	0.91 ± 1.34

Table 2 Blood biochemical parameters of the experimental chickens in each group

Indicators	Control	BQ-1	BQ-3	BQ-5
Cereal third transaminase (U/L)	3.48 ± 0.58	3.02 ± 1.00	3.04 ± 0.72	3.50 ± 1.19
Aspartate aminotransferase (U/L)	217.02 ± 34.17	211.08 ± 35.56	200.86 ± 17.86	219.15 ± 30.59
Alkaline phosphatase (U/L)	2228.80 ± 506.47	2065.20 ± 1394.51	1746.20 ± 1233.74	1935.00 ± 372.57
Serum total protein (g/L)	38.16 ± 4.62	34.94 ± 2.79	40.34 ± 8.91	37.03 ± 5.75
Serum albumin (g/L)	15.98 ± 1.30	15.50 ± 0.35	15.04 ± 0.61	15.52 ± 0.57
Serum globulin (g/L)	22.18 ± 3.88	19.44 ± 2.50	25.30 ± 9.01	21.52 ± 5.85
White ball ratio	0.72 ± 0.13	0.78 ± 0.08	0.66 ± 0.15	0.75 ± 0.21
Creatinine (mmol/L)	0.55 ± 0.10	0.23 ± 0.37	0.55 ± 0.19	0.46 ± 0.22
Urea nitrogen (μmol/L)	2.50 ± 2.93	2.06 ± 2.48	3.30 ± 2.64	2.10 ± 2.26
Blood glucose (mmol/L)	11.96 ± 0.31	12.06 ± 0.29	12.10 ± 0.77	12.52 ± 0.91

Table 3 Organ indices of the experimental chickens in each group

Indicators	Control	BQ-1	BQ-3	BQ-5
Cardiac index	0.86 ± 0.12	0.83 ± 0.09	0.86 ± 0.12	0.88 ± 0.11
liver index	3.12 ± 0.26	3.26 ± 0.31	3.25 ± 0.47	3.20 ± 0.38
Spleen index	0.16 ± 0.04	0.19 ± 0.04	0.18 ± 0.05	0.18 ± 0.02
Gastric index	0.75 ± 0.05	0.74 ± 0.09	0.78 ± 0.06	0.83 ± 0.08
Intestinal index	6.80 ± 0.97	7.06 ± 0.90	7.53 ± 1.24	6.80 ± 1.02
bursa of Fabricius index	0.49 ± 0.15	0.54 ± 0.14	0.58 ± 0.15	0.54 ± 0.13

biochemistry, and organ indices. The results of the routine blood analysis are shown in Table 1, and there were no significant differences ($P > 0.05$) between each administration group and the Control group, demonstrating that BQ had no impact on the target chickens' routine blood parameters.

According to the findings of the chickens' blood biochemistry, there were no significant differences ($P > 0.05$) between each administration group and the Control group, which implied that BQ had no negative effects on the liver, kidney, lipid, protein, or glucose metabolism (Table 2).

To investigate the effect of BQ on the heart, liver, spleen, stomach, intestine, and bursa of Fabricius, organ indices were examined (Table 3). The results showed that no macroscopic pathological changes were found in the internal organs of each group (pictures not shown in the article). Compared with the control group, there were no significant differences in the heart, liver, spleen, stomach, intestine, and bursa of Fabricius in each dose group ($P > 0.05$).

Antiviral ability of BQ in chicks

To explore the clinical effect of BQ, we selected a dose of 2.5 mL/L for mixed drink administration. In addition, because most of the chickens with naturally occurring IB were mixed infections and the medicinal effects cannot be accurately evaluated, artificial modeling was planned to test the effect of BQ. The results of this experiment showed that morbidity was improved in the BQ and BM groups compared with the Model group (Fig. 2A-B). The results in Fig. 2C

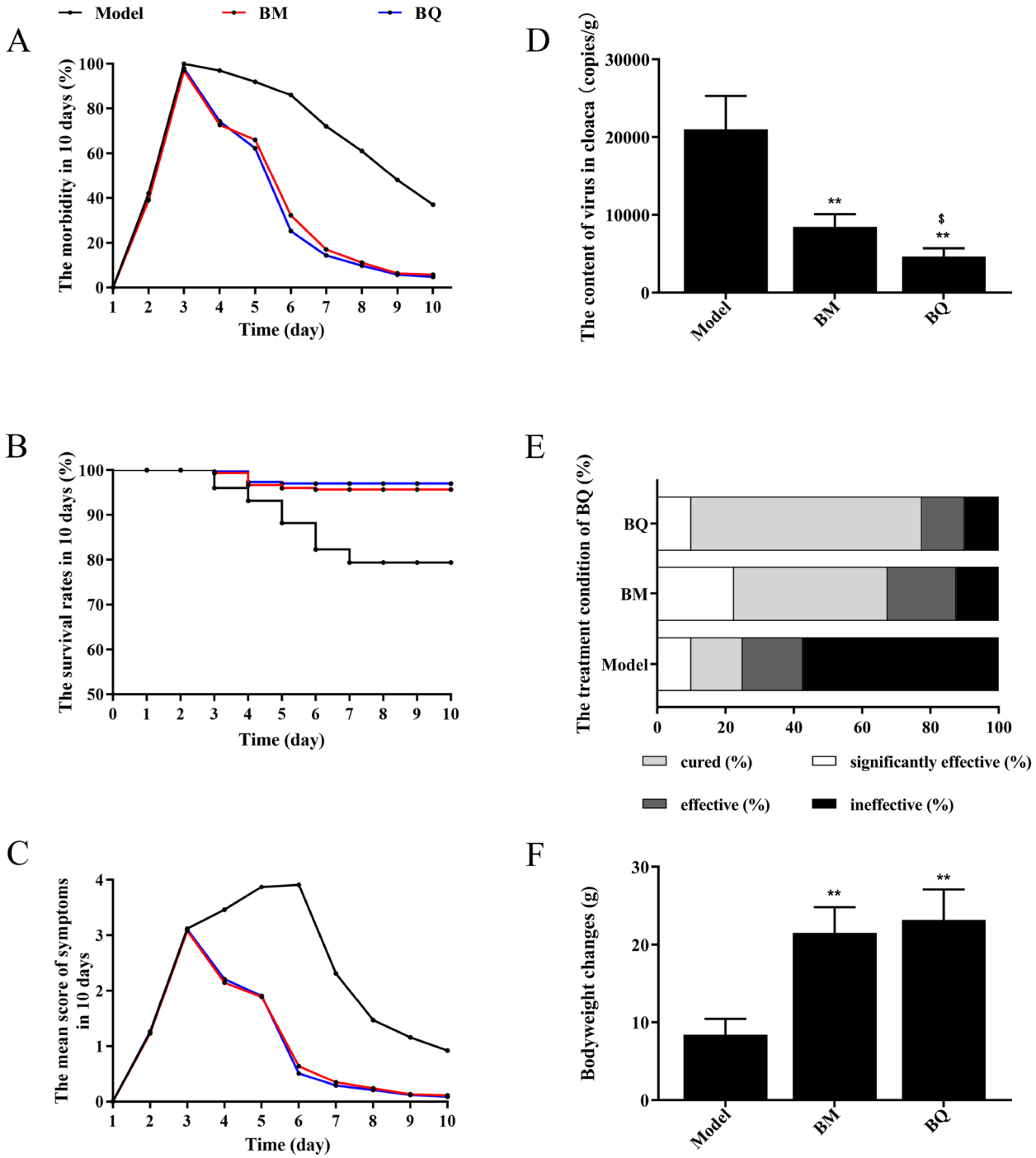

Fig. 2 Effect on the antiviral activity and growing development of Partridge shank chickens. Morbidity (**A**), survival (**B**), and the mean score of symptoms (**C**) are shown for each group for a 10 days of observation period. On the sixth day of the observation period, the virus content of chickens in each group was measured (**D**). The therapeutic effect of BQ was evaluated by the ineffective rate, the effective rate, the significant effective rate, and the cure rate of each group of chickens (**E**). Body weight change was used to evaluate the growth performance of chickens in each group (**F**). $^{**}P < 0.01$, BM or BQ group vs. Model group. $^{\$}P < 0.05$, BQ group vs. BM group

showed that the BQ and BM groups had a great improvement in symptom scores compared to the Model group.

Six days after the chickens were given IBV, cloacal cotton swabs were randomly collected from 20 chickens in each group and viral shedding was determined by qPCR.

Compared with that in the Model group, viral shedding was significantly lower in the BM and BQ groups ($P < 0.01$), and the effect of BQ feeding (4,604.1 ± 1,116.13 copies/g) had a greater effect on viral shedding than BM feeding (8,460.1 ± 1,621.33 copies/g) (Fig. 2D, $P < 0.05$).

After 10 days of observation, 40 chickens from each group were randomly selected for pathology scoring statistics. The statistics showed that the total effective rates (total effective rates = (cured number + significantly effective number + effective number)/40) of BQ and BM treatments were 90.0% and 87.5%, respectively, which were greater than that of the infected group (42.5%) (Fig. 2E). By recording and calculating the changes in daily weight gain of each chicken, it was also found that both BQ and BM were effective at improving body weight ($P < 0.01$ compared to the Model control group), and that BQ was slightly more effective than BM (Fig. 2F).

Microarray gene expression profiling

The potential mechanism of BQ antiviral activity was studied after confirming its antiviral activity. After treatment with BQ, the MCF-7 cells were collected and analyzed with Affymetrix Human Genome U133 Plus 2.0. The differentially expressed genes were shown in volcano plots (Fig. 3A), and genes with a fold change ≥ 2.0 and $P \leq 0.05$ were considered to be differentially expressed genes. The results showed that 304 genes, consisting of 166 downregulated genes and 138 upregulated genes, were differentially expressed in MCF-7 cells treated with BQ (Supplementary File 1). Good repeatability was obtained in each group via cluster analysis (Fig. 3B).

To analyze the functions of these differentially expressed genes, GO and KEGG analyses were performed with DAVID (https://david.ncifcrf.gov/), with detailed results are shown in Supplementary Material Table S1. In addition, the GO and KEGG items with significant differences ($P < 0.05$) were screened according to their biological significance (Fig. 3C-D). It was found that BQ primarily acted through type I interferons and the JAK-STAT signaling pathways.

Discussion

The pandemic caused by coronavirus infection is a major threat to vertebrates worldwide [17]. For instance, the outbreak of coronavirus disease 2019 (COVID-19) has caused major injuries and challenges in more than 200 countries and regions [18]. In addition, the severe acute respiratory syndrome (SARS) that appeared in 2003 seriously threatened human respiratory health [19, 20]. IB is a highly contagions poultry respiratory disease caused by IBV [21]. IBV infection primarily results in respiratory tract infection, nephritis, and decreased productivity. In laying hens, infection not only causes noticeable clinical manifestations, but also causes permanent and irreversible damage to the reproductive system, manifested as diminished egg production and compromised egg quality [22]. Therefore, it is urgent to develop new anti-infectious bronchitis or anti-coronavirus drugs.

In this study, we first prepared a TCM compound, BQ. According to TCM theories, Banlangen and Huangqin are considered principal medicines, which have the functions of heat-clearing, detoxification, dampness elimination and sore throat relief. The use of Shigao and Beidougen, which are ministry medicines, is intended to extend the duration of the effects of Banlangen and Huangqin. Jiegeng, Ziwan, Pengsha and Bingpian, as assistant medicines and guide medicines, aim to relieve cough and asthma, and can resolve phlegm in conjunction with Banlangen, Huangqin, Shigao and Beidougen. Subsequently, we assessed the efficacy of BQ and demonstrated its anti-IBV activity in chicken embryos and chicken. The experiment using IBV-infected chicken embryos revealed that BQ could alleviate the adverse effects induced by IBV, inhibit the replication of IBV, protect the normal development of chicken embryos, and have an antiviral effects. With the use of three different doses (2.5 mL/L, 7.5 mL/L and 12.5 mL/L), the results showed that, even at high doses, BQ was safe for clinical use in chicks. At a BQ concentration of 2.5 mL/L (BQ-1), there were no significant differences between each administration group and the Control group. Meanwhile, there was no significant difference between the groups in routine blood tests, blood biochemistry or organ indices. As a result, BQ-1 (2.5 mL/L) is intended to be used as the therapeutic dosage in official clinical usage, considering both cost and efficacy.

The effects of different administration times on chicken embryos were compared in additional trials using IBV-infected chicken embryos [5]. The findings indicated that the antiviral efficacy of BQ after virus infection is superior to that of BQ after early and simultaneous administration. These findings led to the choice in the subsequent experiments in chickens to test the BQ's antiviral efficacy when it was administered after infection. The results of the chick experiments suggested that BQ can prevent viral replication in chickens compared to self-healing (infected but notreated). In this experiment, 93% to 100% of the chicks were infected after the challenge, suggesting that the artificial challenge model was valid and that BQ and BM could effectively lower the burden of IBV in chicks, increase survival rates, and restore body weight.

The type I interferon signaling pathway is crucial for the defense against viral infections [23]. Type I interferon has been shown to reduce the replication of IBV [24]. However, IBV exhibits a strong inhibitory effect on

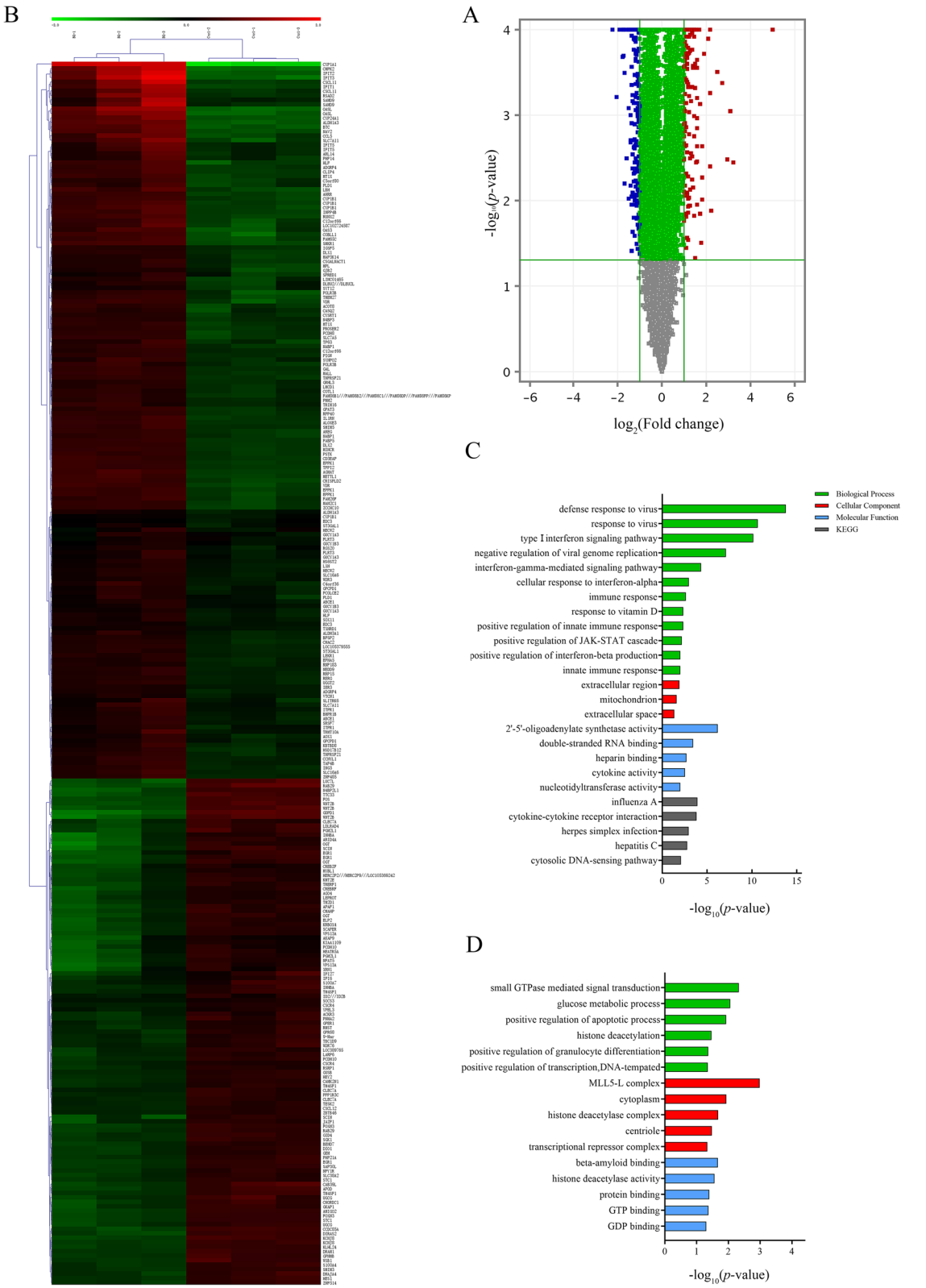

Fig. 3 GO and KEGG analyses of the differentially expressed genes. Volcano map (**A**) of differentially expressed genes. Red dots represent significantly upregulated genes, and blue dots represent significantly downregulated genes. Heatmap (**B**) of differentially expressed genes. GO annotation and KEGG pathway enrichment analyses of the differentially expressed genes revealed upregulated (**C**) and downregulated (**D**) genes

interferon and can actively antagonize the type I interferon response [25]. The finding in this study that up-regulated differentially expressed genes in BQ were enriched in the type I interferon receptor implies a significant role for this receptor. Baicalin, the main active ingredient of Huangqin and found in BQ, can upregulate the expression of type I interferon signals caused by viruses [26]. Therefore, it can be postulated that the antiviral effect of BQ is due to the upregulation of the type I interferon signaling pathway by BQ.

Type I interferon can bind to type I interferon receptor (IFNAR), and the signals can be sent through members of the Janus kinase (JAK) signal transduction pathway and transcriptional activator (STAT) pathway to induce the expression of hundreds of gene transcripts, resulting in the production of antiviral and immunoregulatory proteins [27]. Therefore, the immune response to viruses is significantly influenced by the type I interferon signaling pathway and the JAK-STAT cascade, which is consistent with the results of this study. IBV can inhibit STAT1 transport to block IFN-mediated antiviral gene activation [28] and limit the production of interferon [29] to achieve immune escape. Baicalin can activate the JAK/STAT signaling pathway to exert antiviral effects [30]. In summary, BQ can not only activate the type I interferon signaling pathway but also activate the JAK/STAT signaling pathway to exert antiviral effects. In addition, the JAK/STAT signaling pathway provides a direct mechanism by which cytokines regulate gene expression via immune regulation [31]. It was concluded that BQ may upregulate the expression of type I interferon and the JAK/STAT signaling pathways to protect IBV-infected chickens (Fig. 4).

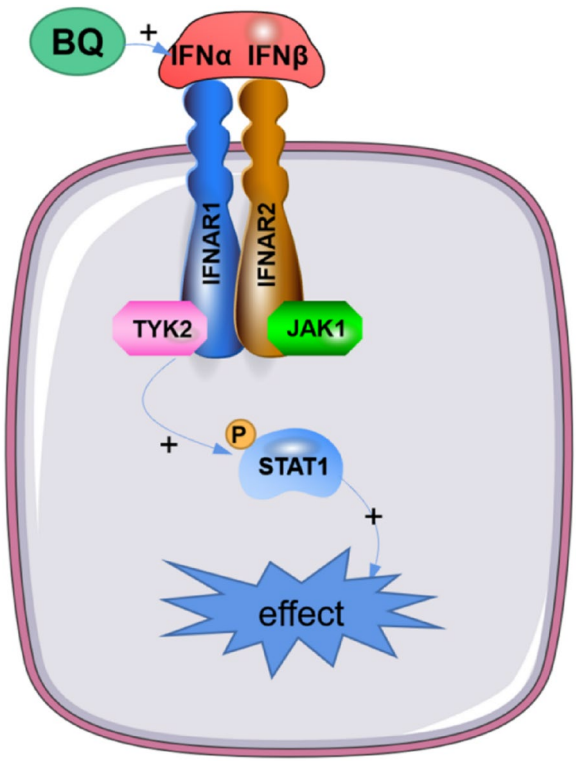

Fig. 4 Summary diagram of the mechanism of action of BQ. BQ affects interferon receptors by regulating type I interferon, affects the JAK-STAT signaling pathway and exerts an effect

Conclusion

Ban-Qin-Fei-Re-Qing oral liquid (BQ) is an effective herbal remedy against IB, and its antiviral activity may be achieved by adjusting the type I interferon signaling pathway and affecting the JAK-STAT signaling pathway. BQ can be used as an effective therapy for IBV infection, and may also provide treatment opitions for other coronavirus infections.

Materials and methods

Preparation of Ban-Qin-Fei-Re-Qing oral liquid

Ban-Qin-Fei-Re-Qing oral liquid (BQ) was obtained by self-preparation. The ingredients Banlangen, Huangqin, Shigao, Beidougen, Jiegeng, Ziwan, Pengsha and Bingpian were all purchased from Tongrentang Chinese Medicine. All the herbs were identified according to the Chinese Pharmacopoeia (2010) (Supplementary Material, Fig. S4). Bo-Ma oral liquid (BM, Batch No.20140802, 1 g of crude medicine in 1 mL of solvent), an effective plant-derived drug for the treatment of IB in chickens, was provided by Jiangsu Muxiangyuan Animal Health Products Co., Ltd. (Jiangsu, China).

Briefly, Banlangen (300 g), Huangqin (120 g), Beidougen (80 g), Jiegeng (120 g), Ziwan (120 g), and Shigao (broken, 150 g) were mixed at a 30:12:8:12:12:15 ratio and boiled with water (10 times water boiled for 2 h for the first time and 8 times water boiled for 1 h for the second time). The decoction was then filtered and concentrated under vacuum to a relative density of 1.10 ~ 1.20 (60 °C). Pengsha (30 g), which was the concentrate, was added to the filtrate and stirred until it dissolved, which was the concentrate. The concentrated solution was then added to Bingpian (5 g), which was dissolved in 4 volumes of ethanol and then added to polysorbate (3 g). Finally, BQ was obtained after mixing with water to 1000 mL.

Baicalin and dauricine were purchased from the National Institutes for Food and Drug Control, China. Chloroform, isopropanol, and ethanol were obtained from Shanghai Sinopharm Chemical Reagent Co., Ltd. TRIzol reagent and PrimeScript reverse transcriptase reagent kits for the gene chip were purchased from TaKaRa. The real-time system was obtained from Thermo Fisher. The primers were synthesized by HIBIO (Hangzhou, China).

Chromatographic conditions of BQ by HPLC

A mixed system of methanol, water and phosphoric acid (47:53:0.2) was used as the mobile phase to detect baicalin in BQ, derived from Huangqin. The column used was a C_{18} column (250 mm × 4.6 mm, 5 μm). The injection volume was 10 μL. The detection wavelength was 280 nm. Baicalin was prepared in a 40 μg/mL solution with methanol as a reference substance. The preparation process of BQ was as follows: 5 mL of BQ solution was accurately measured into a 25 mL volumetric flask (with water used as the solution), 1 mL of BQ was absorbed to a 50 mL volumetric flask (with methanol used as the solution), and the solution was filtrated to obtain the test product.

Similarly, a mixed system of acetonitrile and 0.05% triethylamine (45:55) was used as the mobile phase to detect dauricine in BQ, derived from Beidougen. The column and injection volume were the same as those described earlier. The detection wavelength was 284 nm. Dauricine was prepared in a 100 μg/mL solution with methanol as a reference substance. To prepare the sample, 10 mL of BQ solution was accurately measured into a 25 mL volumetric flask, and then blended and filtered to obtain the test product.

Animals, grouping, and procedure

Experiment I: to determine the antiviral and growth performance effects of BQ in chicken embryo model of IBV infection

Ten-day-old SPF chicken embryos were provided by Sinopharm Yangzhou Vac Biological Engineering Co., Ltd. The chicken embryos were randomly divided into 6 groups: Control group (mock-infected, saline only, $n=16$); Model group (infected, IBV JS/2010/12 only, $n=15$); BM group (positive drug, 72% (*v/v*) BM and IBV JS/2010/12, $n=15$); BQ low dose group (BQL, 0.4 g/mL BQ and IBV JS/2010/12, $n=15$); BQ medium dose group (BQM, 0.6 g/mL and IBV JS/2010/12, $n=16$); BQ high dose group (BQH, 0.8 g/mL and IBV JS/2010/12, $n=16$). Two hours before the herbal remedy (BM or BQ) was infected, the model chicken embryos were created by infection with the virus (100 EID_{50}) administered via the allantoic cavity. The chicken embryos were observed twice a day for 8 days, and those that died within 24 h were discarded, which resulted in an unequal number of eggs in the actual experiment (as shown above). All surviving embryos in the infected group showed varying degrees of developmental curl, embryo atrophy, amnion thickening, yolk sac shrinkage, and epidermal edema, which indicated that the animal model was successfully established. At the end of the trial, allantoic fluid was collected from each chicken embryo using a sterile syringe for qPCR. Subsequently, the number of normally growing embryos, embryo weight, and embryo length were recorded after all the embryos were executed.

Experiment II: to evaluate BQ safety

At 10 days old, the weighed 120 Xinsu green-shelled chick roosters were randomly assigned to four groups: receiving no medicine (Control, water only, $n=30$), receiving the recommended dose of BQ (BQ-1, 2.5 ml/L mixed water, $n=30$), receiving 3 times the recommended dose of BQ (BQ-3, 7.5 ml/L mixed water, $n=30$), and receiving 5 times the recommended dose of BQ (BQ-5, 12.5 ml/L mixed water, $n=30$). Within each BQ group, chickens were administered the medicine in a completely random order. The medicine was administered for five consecutive days.

After the first dose, each chick rooster was monitored for 12 days. Then, routine blood parameters and serum biochemical indicators were examined. The histopathologic alterations of the heart, liver, spleen, lung, kidney, stomach, intestines, bursa of Fabricius and each of these organs were assessed during the autopsy, along with their corresponding weights. A statistical organs index (organ index = viscera live wet weight/body weight × 100) was calculated.

Experiment III: to determine antiviral effect of BQ in chicks infected with IBV

One-day-old Partridge shank chickens were purchased from Changzhou Sandeli Livestock and Poultry Breeding Co., Ltd. The chickens were debeaked at 5 days old and raised for 10 days (100–140 g) before being infected with IBV (referring to the modeling method used for SPF Leghorn chickens). The infected chickens were then randomly divided into 3 groups: Control group (mock-infected, $n=100$), BM group (positive drug, treated with 9 mL/L BM, $n=300$), and BQ group (treated with 2.5 mL/L BQ, $n=300$). The order in which the chickens received the medicine was completely random.

All chickens were observed twice a day for 10 days after 1 day of virus injection. On the 6th day after virus injection, chickens from each group were randomly sampled to collect cloaca cotton swabs, which were subsequently placed in finger tubes containing 1 mL of sterilized PBS for qPCR. The number of surviving/dead chickens and the score of clinical symptoms in each group for each day, the sum of surviving/dead chickens in each group, and the number of ineffective/effective/significant effective/cured chickens among the surviving chickens were recorded. The clinical symptom scoring standard was as follows: + 0, no symptoms (cured); + 1, mild degree of symptoms (significantly effective); + 2, moderate degree of symptoms (effective); + 4, severe degree of symptoms

(ineffective); and + 5, death (death). Subsequently, statistical analysis of the replication of IBV in each group was performed. The researchers did the animal administration (P.W. and T.H.), result judgment (Y.F. and X.X.), and final data analysis (D.M. and X.G.) with blinding.

The IBV JS/2010/12 strains used in Experiments I and III, respectively, were obtained from the State Key Discipline Open Laboratory of Preventive Veterinary Medicine, Yangzhou University Veterinary College. The egg infectious dose (EID_{50}) measured by the Reed-Muench method was $10^{-6.5}$/0.1 mL. All experiments were approved by the China Agricultural University Laboratory Animal Welfare and Animal Experimental Ethical Inspection Committee (NO. AW82210202-2). All experimental protocols were carried out following the relevant guidelines and regulations published by China Agricultural University. All experiments complied with the above guidelines. For all experiments, the RAND function in the Microsoft Excel was used for generating random numbers.

qRT-PCR

1 mL of allantoic fluid or clot cotton swabs was collected and stored in a −70 °C refrigerator, and the number of viruses was determined by qRT-PCR. The RNA of allantoic fluid or clot cotton swabs was extracted by Trizol (TAKARA). Reverse transcription-polymerase chain reaction (RT-PCR) was performed as follows: DEPC, M-MLV, M-MLV buffer, RNA inhibitor, oligodT, and dNTP were mixed at a ratio of 19:2:8:1:4:8, 10.5 μL was added to the RNA, and the mixture was incubated at 42 °C for 1 h and 70 °C for 10 min.

The 20 μL mixture (cDNA, 2 × PCR buffer, primer F, primer R, TaqMan probe and water mixed at a ratio of 5:25:2:2:1:15) was incubated at 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The sense primer 5'-GCTTTTGAGCCTAGCGTT-3', anti-sense primer 5'-GCCATGTTGTCACTGTCTATTG-3', and sense TaqMan probe FAM-CACCACCAGAACCTG TCACCTC-BHQ were used to amplify the IBV gene transcripts.

Gene expression profiling analysis

Breast cancer cells (MCF-7), either untreated or treated with BQ (0.075 mg/mL) for 6 h, were harvested, and total RNA was extracted using an RNeasy® Kit (QIAGEN). The quantification of total RNA in the samples was conducted using a NanoDrop ND-2100 (Thermo Scientific), and the RNA integrity was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies). Following the successful RNA quality assessment, the sample labeling, chip hybridization, and elution were carried out following the standard procedure. Briefly, total RNA was converted to double-stranded cDNA via reverse transcription, and then biotin-labeled cRNA was synthesized. The labeled cRNA was hybridized with the chip. After elution and staining, the original image was obtained by scanning. The above experiments were performed by Shanghai Ouyi Biomedical Technology Co., Ltd. The chip used in the project was an Affymetrix Human Genome U133 Plus 2.0 chip. Array images were analyzed using Affymetrix GeneChip Command Console (version 4.0, Affymetrix) to obtain raw data. After obtaining the raw data, basic analysis was conducted using Genespring software (version 13.1; Agilent Technologies). The raw data were normalized through the RMA algorithm.

The MCF-7 cells were obtained from Wuhan Biofavor Biotech Service Co., Ltd. and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in 5% CO_2. Differentially expressed genes were identified based on specific criteria. Genes with fold change values ≥ 2.0 were classified as upregulated or downregulated, and changes with *P*-values ≤ 0.05 were considered to indicate statistical significance. Heatmaps were generated using MultiExperiment Viewer.

Analysis of the gene chip

GO and KEGG analyses of differentially expressed genes were performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID, version 6.8, https://david.ncifcrf.gov/). The important GO and KEGG terms were identified by Fisher's exact test, and False Discovery Rate (FDR) was applied to correct the *P*-values.

Statistical analysis

All data are presented as the means ± standard deviations (SDs). One-way analysis of variance (ANOVA) and Duncan's multiple comparisons test were employed for group comparisons, using IBM SPSS statistics software (version 20.0). A group difference with statistical significance was considered when $P < 0.05$, and a group difference with high significance was considered when $P < 0.01$.

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

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Authors' contributions
D.M. and X.G. conducted data analysis and wrote the paper; P.W., F.Y., T.H., Y.F. and X.X. completed the experiments; H.X. and Z.H. designed this study; D.M. and X.G. have contributed equally to this study. All authors read and approved the final manuscript.

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Availability of data and materials
We declare that all data supporting the findings of this study are available within the article.

Declarations

Ethics approval and consent to participate
Ethical approval was reviewed and given by China Agricultural University Animal Ethics Committee document (No. AW82210202-2).

Consent for publication
Not applicable.

Competing interests
No potential conflict of interest was reported by the authors.

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