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Effect of *Echinacea purpurea* (L.) Moench and its extracts on the immunization outcome of avian influenza vaccine in broilers

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ABSTRACT

Ethnopharmacological relevance: *Echinacea purpurea* (L.) Moench (EP) is a perennial herbaceous flowering plant with immunomodulatory effects. However, the immunomodulatory effects of EP on broilers after vaccination are still unclear.

Aim of the study: The aim is to study the effect of EP and *Echinacea purpurea* (L.) Moench extracts(EE) on avian influenza virus (AIV) immunity, and further explore the potential mechanism of immune regulation.

Materials and methods: Broilers were fed with feed additives containing 2% EP or 0.5% EE, and vaccinated against avian influenza. The samples were collected on the 7th, 21st, and 35th day after vaccination, and the feed conversion ratio (FCR) was calculated. Blood antibody titer, jejunal sIgA content, tight junction protein, gene and protein expression of TLR4-MAPK signaling pathway were also detected.

Results: The results showed that vaccination could cause immune stress, weight loss, increase sIgA content, and up-regulate the expression of tight junction proteins, including zonula occludens-1 (ZO-1), Occludin, and Claudin-1, as well as the genes of Toll-like receptor 4 (TLR4), myeloid differentiation primary response 88 (MyD88), receptor-associated factor 6 (TRAF6), activator protein 1 (AP-1) protein gene expression on TLR4-mitogen-activated protein kinase (MAPK) signaling pathway, and the protein expression of MyD88, extracellular regulated protein kinases (ERK), and c-Jun N-terminal kinase (JNK). EP and EE could increase the body weight of broilers, further improve antibody titers, decrease FCR, increase sIgA levels, up-regulate the expression of tight junction proteins, including ZO-1, Occludin, and Claudin-1, as well as the genes of TLR4, MyD88, TRAF6, and AP-1 and the protein expression of MyD88, ERK, and JNK in the TLR4-MAPK signaling pathway.

Conclusion: In conclusion, EP and EE can increase the broiler's production performance and improve vaccine immune effect through the TLR4-MAPK signaling pathway.

1. Introduction

Food security and safety is the major concern when consider animal-origin protein. In the era of a total ban on antibiotics in feed, with the increase in animal infectious diseases and the rapid mutation of pathogens; the effectiveness of a large number of natural and safe herbal medicines in enhancing animal resistance, reducing drug resistance, and improving economic efficiency is needed (Anwar et al., 2023; Gul and Alsayeqh, 2022) EP is medicinal herbs indigenous to the United States and Canada. Native Americans used preparations of Echinacea roots to treat a variety of conditions associated with inflammatory and allergic disease, including swollen gums, inflamed skin, sore throats, and gastrointestinal disorders (Gulledge et al., 2018). EP also has a long history of medicinal use for mainly infections, indicated in bacterial and viral infections, and as an "anti-toxin" for snakebites and blood poisoning (Williamson, E. M, 2003). Other traditional uses listed include nasopharyngeal catarrh, pyorrhoea (periodontitis) and tonsillitis, as a

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Abbreviations

AIV	avian influenza virus
AP-1	activator protein 1
BTV	bluetongue virus
EE	*Echinacea purpurea* (L.) Moench extracts
EP	*Echinacea purpurea* (L.) Moench
FCR	feed conversion ratio
group C	control group
group V	vaccine control group
group VE	*Echinacea purpurea* group
group VEE	*Echinacea purpurea* extract group
HA	hemagglutinin
HCoV-229E	Echinacea on human coronavirus 229E
HI	hemagglutination inhibition
IRAK1	interleukin 1 receptor-associated kinase 1
JEV-RdRp	Japanese encephalitis virus-RNA dependent RNA polymerase
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MyD88	myeloid differentiation primary response 88
RT-PCR	real-time PCR
SCFA	short-chain fatty acid
TLR4	Toll-like receptor 4
TNF	tumor necrosis factor
TRAF6,	receptor-associated factor 6
ZO-1	occludens-1

supportive treatment for influenza-like infections and recurrent infections of the respiratory tract and lower urinary tract, and, externally, for poorly healing superficial wounds (Barnes et al., 2005). EP is a perennial herbaceous flowering plant that contains dozens of active ingredients, including alkylamides, caffeic acid derivatives, polysaccharides, and glycoproteins (Awortwe et al., 2021; Ren et al., 2023). Alkylamides in EP are mainly responsible for anti-inflammatory, immune regulation, and macrophage regulation (Vieira et al., 2023). These Alkylamides can make mast cell degranulation and calcium influx, thereby treating allergic and inflammatory responses mediated by mast cells (Travis et al., 2018). Caffeic acid derivatives in EP include chicoric acid, caftaric acid, and chlorogenic acid (de Oliveira et al., 2021). They mainly have antioxidant and anti-ulcer, anti-inflammatory, anti-allergic, and antiviral effects (Ravazzolo et al., 2022; Ye et al., 2019). Polysaccharides in EP have the effects of anti-tumor, anti--oxidation, antibacterial, anti-virus, immune regulation, hypoglycemic, liver protection, and gastrointestinal protection (Jiang et al., 2021; Liu et al., 2020, 2022; Shi et al., 2021). Numerous studies have shown that Echinacea has immunomodulatory, anti-inflammatory, antiviral, antibacterial, and antioxidant therapeutic effects. EP stimulates the immune system in three ways: by activating phagocytosis, stimulating fibroblasts, and increasing respiratory movement. These actions can increase the movement ability of white blood cells (Khattab et al., 2019; Michele et al., 2018; Park et al., 2018; Paulovicova et al., 2022; Seckin et al., 2018). EP also activates the immune system by increasing the number, function and mobility of various immune cells, including neutrophils, polymorphonuclear leukocytes, and NK cells, further enhancing innate immunity, and playing an anti-inflammatory role (Khalaf et al., 2019; Khattab et al., 2019; Thomsen et al., 2018). Therefore, it is believed that the most important active ingredient in EP is responsible for enhancing immunity and anti-inflammatory effects (Aarland et al., 2017; Pillai et al., 2007; Sousa et al., 2018; Sultan et al., 2014). However, there is still insufficient research on EP and EE. Most studies on its immunomodulatory mechanism include *in vitro* cell experiments and are rarely further verified *in vivo* in animals. Furthermore, there are few studies on the application of EP in poultry, especially its preventive effect as a feed additive.

AIV is a common infectious disease in the poultry breeding industry. The detection rate of H5, H7, and H9 subtype AIV in Chinese chickens without vaccination was 39.26% (Song et al., 2021). AIV also poses the risk of antigenic drift and introduction of new viruses (Hartaningsih et al., 2015; Lee et al., 2022). It is also easy to reassort with other subtypes, producing new recombinants such as the H7N9 virus. Although, the live attenuated H9N2 vaccine provides good multiple immunity, including humoral immunity, cellular immunity, and mucosal immunity, the risk of reassortment between the vaccine strain and the wild-type strain remains a concern (Chen et al., 2020). Additionally, avian influenza is a zoonotic disease, and people who are in direct contact with live poultry are susceptible to the disease. Since, the first case of novel H7N9 infection was reported, there have been five H7N9 outbreaks in China. In the fifth wave of the epidemic, a highly pathogenic H7N9 strain appeared. At the same time, the H7N9 virus continues to accumulate and mutate, and the affinity of the human respiratory epithelial cell sialic acid 2–6 receptor is enhanced (Bortolami et al., 2022). There have been five outbreaks of novel H7N9 infection in China since the first reported case. In the fifth wave of the outbreak, a highly pathogenic strain of H7N9 emerged. Additionally, the H7N9 virus has accumulated mutations and now has an increased affinity for the human respiratory epithelial sialic acid 2–6 receptor, making immune protection in chickens crucial (Wu et al., 2020). However, due to the high variability and individual differences of the virus, animals may not be able to mount an effective immune response, resulting in immune failure. As a result, the development of a cheap, safe, and effective immune adjuvant has significant potential applications (Ren et al., 2022b). EP also demonstrates antiviral activity. EP has a significant inhibitory effect on coronavirus. The half-maximal inhibitory concentration of EP on human coronavirus 229E (HCoV-229E) is 3.2 μg/mL, and MTT assays on Huh-7, Vero, and Vero E6 cells show irreversible inactivation of HCoV-229E (Saifulazmi et al., 2022). EE exhibit profound antiviral activity against several viruses, including human and avian influenza viruses, H3N2-type IV, H1N1-type IV, herpes simplex, and rhinoviruses, and reverse virus-induced proinflammatory responses (Dobrange et al., 2019). Compounds screened from narrow-leaved EP are protein inhibitors of Japanese encephalitis virus-RNA dependent RNA polymerase (JEV-RdRp), exhibiting inhibitory effects on JEV(Yadav et al., 2022). Adding EP to feed at 5 g/kg can increase the antibody level of chickens after inoculation with Newcastle disease vaccine (Gado et al., 2019). Oral administration of EP can significantly reduce the number of diarrhea days in calves vaccinated with a bluetongue virus (BTV) vaccine, indicating that oral EP stimulates the local intestinal immune system and enhances vaccine efficacy (Ayrle et al., 2021). In addition, IMU (based on EP and *Nigella sativa)* significantly increases vaccine protection rates, HI antibody titers, and phagocytic activity of heterophilic granulocytes while reducing stress-induced viral shedding and viral titers. Oral administration of 1% IMU for six weeks enhances the immune response after AI-H9N2 vaccine inoculation and reduces the pathogenicity of infected stressed chickens (Eladl et al., 2019).

2. Materials and methods

2.1. Plant material

Echinacea purpurea (L.) Moench, this name is reported by Asteraceae as an accepted name in the genus Echinacea and family Asteraceae (The World Flora Online, http://www.worldfloraonline.org./taxon/wfo-0000036347). The record derives from TICA which

reports it as an accepted name (record GCC-AADAD3F3-3650 -4DDD-BB13-E9573CBE0045). The information about the preparation method and effective components of EP is the same as that of this article published in our laboratory(Gu et al., 2023).

2.2. Experimental layout/plan

All the experimental procedures were carried after approval by the Animal Ethics Committee of South China Agricultural University (License Number: SYXK, 2022-0136), and were in accord with the requirements of the National Institutes of Health Guide for the Care and use of laboratory animals. After a one-week adaptation, a total of 80 broiler birds (Fast-Growing Yellow Broilers) were randomly divided into four groups with 20 birds in each group. The experiment included a control group (group C) and vaccine control group (group V),fed on a basal diet with and without AIV immunization respectively. While, other groups, the EP group (group VE), and the EE group (group VEE) additionally received supplementation of EP and EE at 20 g/kg and 5 g/kg respectively in basal diet alongside AIV vaccine at the age of 9 days. The weight was measured at 7, 21, and 35 days after vaccination, and the average daily weight gain, average daily feed intake, and feed conversion ratio (FCR) were calculated for each group.

2.3. Detection of antibody titers by hemagglutination-hemagglutination inhibition (HA-HI) test

HA-HI test was performed as described previously by Fayyaz with some modifications (Fayyaz et al., 2023) Briefly, at 7th, 21st, and 35th days after vaccination, 6 broilers were randomly selected from each group for blood collection and the serum was separated for the hemagglutination-hemagglutination inhibition test. The 4-unit antigens were prepared based on the hemagglutinin (HA) titer. The hemagglutination inhibition (HI) tests were performed by double diluting sera with PBS. The diluted sera were mixed with an equal volume of 4-unit HA antigens and incubated at 37 °C for 20 min. Then, 1% (v/v) chicken erythrocytes were added and incubated at 37 °C for 20–30 min. The HI titer was determined as the maximum serum dilution that completely inhibited hemagglutination. The antibody titers were determined against H5N2 rHN5801, rGD59, and H7N9 rHN7903 strains.

2.4. Real time-PCR analysis

The primers and sequences of real-time PCR (RT-PCR) are shown in Table 1. Total RNA was extracted from 100 mg of jejunum using RNA isolation reagent (Vazyme, China). The total RNA was isolated using chloroform and precipitated with isopropanol. The residual isopropanol was cleaned with ethanol. Total RNA concentration was measured by Microvolume UV–Vis spectrophotometer. (NanodropTM One; Thermo Fisher Scientific, Madison, WI, USA). Reverse transcription was performed using approximately 5 μg of total RNA into cDNA using HiScript III RT SuperMix for qPCR (Vazyme, China). The mixture contained 1 μL cDNA primer, and used the ChamQ University SYBR qPCR Master Mix (Vazyme, China) in the real-time PCR detection system (QuantStudioTM 5; Thermo Fisher Scientific, Waltham, MA, USA). The relative expression level of genes was calculated by 2–11 (Ct) method, and GAPDH was used as the internal reference gene. The results were expressed as normalized mRNA levels of reference genes.

2.5. Western blot analysis

Western blot assay was performed as described previously by Wang L with some modifications (Wang et al., 2023). The antibodies used for Western blot are shown in Table 2. Approximately 100 mg of jejunum was lysed with RIPA lysis buffer (Meilunbio, China) and 1 mM protease inhibitor (PMSF) (Meilunbio, China) at 4 °C. The protein concentration was determined using a BCA protein concentration determination kit (Yamay biotech, China). After that, the samples were diluted with 5 × SDS-PAGE loading buffer and boiled for 8 min. An equal amount of protein sample (10 μg) was loaded and separated by 12.5% SDS-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% skimmed milk powder in Tris-buffered saline containing Tween (TBST) for 1 h, followed by overnight incubation with primary antibodies. The membrane was washed three times with TBST and then incubated with secondary antibody for 1 h. The signal was detected using an electro chemiluminescent liquid (ECL) (Meilunbio, China). The gray value of each band was quantified using ImageJ software and normalized.

2.6. Statistical analysis

The data were analyzed using GraphPad Prism 8.0 and SPSS 26.0. The independent sample *t*-test was used to analyze differences between groups, and data were expressed as mean ± standard deviation (SD). Differences between groups were analyzed by one-way analysis of variance (ANOVA), and statistical significance was considered as $P < 0.05$.

3. Results

3.1. Effect of EP and EE on growth performance of broilers immunized with AIV vaccine

The results of body weight and FCR are shown in Table 3. There was no significant difference in body weight among all treatment groups on days 7 and 21 after vaccination ($P > 0.05$). On day 35, body weight was significantly higher in group VEE compared to group V ($P < 0.05$).

Table 2
List of antibodies used for Western blot.

Name	Company	Cat. no.	Dilution
TLR4 Rabbit pAb	Bioss	bs-20379R	1:1000
MyD88 Rabbit pAb	Novus Biologicals	NB100-56698	1:1000
ERK1/2Rabbit pAb	Abmart	T55487	1:1000
JNK1/2/3Rabbit pAb	Abmart	T40073	1:1000
TRAF6Rabbit pAb	ABclonal	A16991	1:1000
GAPDHRabbit pAb	ABclonal	AC001	1:9000
Goat anti-Rabbit IgG	Zenbio	H&L	1:5000

Table 1
The nucleotide sequence of primers used for real-time PCR.

Gene	5′-primer (F)	bp	3′-primer (R)	bp
β-actin	GCACCTAGCACAATGAAA	18	GATAGAGCCTCCAATCCA	18
Occludin	CGTCATGCTCATCGCCTCCATC	22	TTGAGGTAGGTGCTGCCGTAGG	22
Claudin1	GACCAGGTGAAGAAGATGCGGATG	24	CGAGCCACTCTGTTGCCATACC	22
ZO-1	TCTTCCTCCTCCCGCTTCTTCAC	23	AGAGATGGTGGTGTAGGCAGTGG	23
TLR-4	CATCCCAACCCAACCACAGTAGC	23	CCACTGAGCAGCACCAATGAGTAG	24
MyD88	GTACTTACGAAGGAAGCAGCAGGAG	25	ATGCCCATCAGCTCTGAAGTCTTTG	25
TRAF6	AGGAATGAACTGGCACGACACATG	24	GAAGAGGGCAGGCTCAAATGGTAG	24
AP-1	CGCCTCATCATCCAGTCCAACG	22	TGGTTCTGCTTGTGCAAGTCCTC	23

Table 3
The effects of EP and EE on growth performance of broilers on days 7,21,35.

	Body Weight			FCR		
	7d	21d	35d	7d	21d	35d
C	257.400 ± 6.462	743.740 ± 122.215	1375.600 ± 92.604^{ab}	2.220 ± 0.249^{a}	2.231 ± 0.134^{a}	3.195 ± 0.072^{a}
V	259.700 ± 9.464	702.040 ± 36.517	1290.700 ± 154.793^{b}	2.355 ± 0.174^{a}	1.977 ± 0.666^{a}	2.543 ± 0.061^{c}
E	263.800 ± 20.554	753.200 ± 44.386	1394.500 ± 209.444^{ab}	1.745 ± 0.108^{b}	2.222 ± 0.286^{a}	2.586 ± 0.071^{c}
EE	264.800 ± 20.554	752.940 ± 88.891	1538.500 ± 122.376^{a}	1.801 ± 0.147^{b}	1.321 ± 0.528^{b}	2.825 ± 0.274^{b}

All data were presented as mean ± SD(n = 6). Means with different superscripts (abc) within the same column differ significantly ($P < 0.05$).

On day 7 after vaccination, the FCR of the VE and VEE groups was significantly lower compared to the groups C and V ($P < 0.05$). Similarly, on day 21, the FCR of group VEE was significantly reduced compared to the C, V, and VE groups ($P < 0.05$) while, on day 35, the FCR of V, VE, and VEE groups significantly decreased compared to C group ($P < 0.05$). Besides, the V and VE groups also showed a significant reduction compared in FCR compared to the VEE group ($P < 0.05$).

3.2. Effect of EP and EE on antibody of broilers immunized with AIV vaccine

The results of the effects of EP and EE on alteration of antibody levels are shown in Fig. 1. No significant differences were recorded in the levels of rHN58, rHN7903 and rGD59 antibodies among all groups before vaccination. However, after vaccination, the antibody levels of rHN58 and rGD59 for groups V, VE, and VEE showed a significant increase on day 7 ($P < 0.01$), day 14 ($P < 0.01$), day 21 ($P < 0.001$), day 28 ($P < 0.001$), and day 35 ($P < 0.001$) compared to those of group C. Besides, at 35th day, the antibody levels of rHN58 in group VE expressed significantly higher value compared to those in group V($P < 0.05$). Similarly, the antibody levels of rHN7903 were significantly higher in group V compared to control on day 7 ($P < 0.05$) while, all groups including V, VE, and VEE exhibited a significant increase for day 14 ($P < 0.001$), day 21 ($P < 0.001$), day 28 ($P < 0.001$) and day 35 ($P < 0.001$) for the same antibody compared to group C. Additionally, the antibody levels of rGD59 were significantly higher compared to group V at 7th and 14th day for VE group ($P < 0.01$) and for both VE and VEE groups on 21st day ($P < 0.01$). Likewise, antibody levels of rHN7903 in VE and VEE groups were also extremely higher than those in group V on 28th day ($P < 0.001$).

3.3. Effect of EP and EE on intestinal immunity of broilers immunized with AIV vaccine

As represented in Fig. 2, on day 7, the concentration of sIgA in intestinal mucosa was significantly higher in the group VEE compared to the groups V, C, and VE ($P < 0.01$). On day 21, there were no significant differences among all groups ($P > 0.05$). On day 35, the concentration of sIgA in intestinal mucosa for groups V, VE, and VEE was significantly higher compared to group C ($P < 0.05$).

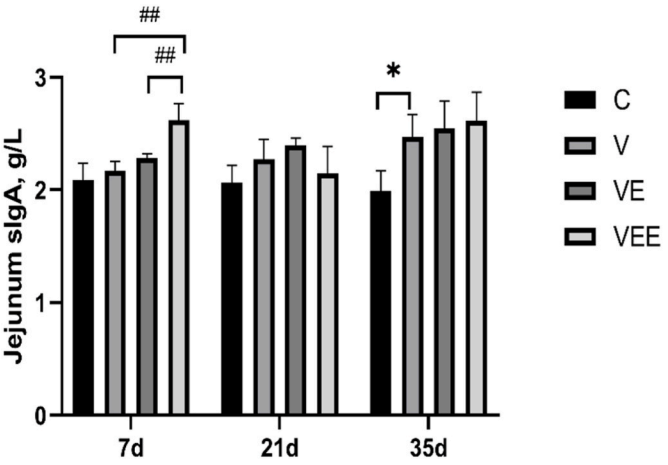

Fig. 2. EP and EE affecting the concentration of sIgA in intestinal mucosa. All data were presented as mean ± SD(n = 6). $^{*}p < 0.05$,$^{**}p < 0.01$,$^{***}p < 0,001$ compared to the group C; $^{\#}p < 0.05$,$^{\#\#}p < 0.01$,$^{\#\#\#}p < 0,001$ compared to the group V and intergroup.

Fig. 3 depicts the expression of tight junction proteins ZO-1, Occludin, and Claudin-1. On day 7, the expression of ZO-1 and Occludin was significantly higher in the VEE group ($P < 0.01$) compared to V, C, and VE groups while, the gene expression of Claudin-1 was not significantly different between the groups ($P > 0.05$); On the day 21, the gene expression of ZO-1 and Occludin in VEE group was significantly higher ($P < 0.05$) as compared to V, VE, and C groups. Besides, the gene expression of Claudin-1 was significantly higher in groups VE and VEE ($P < 0.05$) than that in group C. However, no significant difference was recorded in the gene expression of ZO-1 and Occludin among each group on day 35, but the gene expression of Claudin-1 was significantly higher in group VE ($P < 0.05$) than that in group C.

Fig. 4 shows the gene expression of TLR4, MyD88, TRAF6, and AP-1. On day 7, the gene expression of MyD88 was significantly higher in the V, VE, and VEE groups ($P < 0.01$) than in group C; while, the gene expression of TRAF6 and AP-1 remained non-significant among groups ($P > 0.05$). On day 21, the gene expression of MyD88, TLR4 and AP-1 remained insignificant among all groups ($P > 0.05$). Also, the gene expression of TRAF6 was significantly higher in group VEE ($P < 0.01$) compared to groups V and C. On day 35, the gene expression of TLR4 was significantly higher in groups VE and VEE ($P < 0.05$) than in groups V and C.Likewise, compared with the group C, the gene expression of MyD88 significantly increased in group VEE ($P < 0.05$). For gene expression of TRAF6, there was no significant difference among all groups ($P > 0.05$). Gene expression of AP-1 significantly increased in the group VEE compared with the other groups ($P < 0.05$).

As shown in Fig. 5, on day 7, the relative protein expression of MyD88 was significantly higher in group VE compared to group V ($P <$

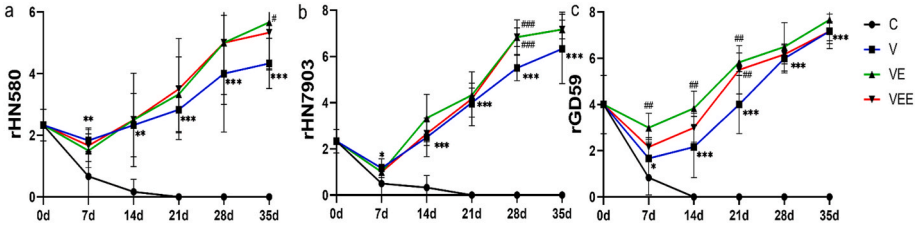

Fig. 1. The effects of EP and EE on the levels of antibodies in broilers on days 7,14,21,28,35:a) rHN580, **b)** rHN7903, **c)** rGD59. All data were presented as mean ± SD(n = 6). $^{*}p < 0.05$,$^{**}p < 0.01$,$^{***}p < 0,001$ compared to the group C; $^{\#}p < 0.05$,$^{\#\#}p < 0.01$,$^{\#\#\#}p < 0,001$ compared to the group V.

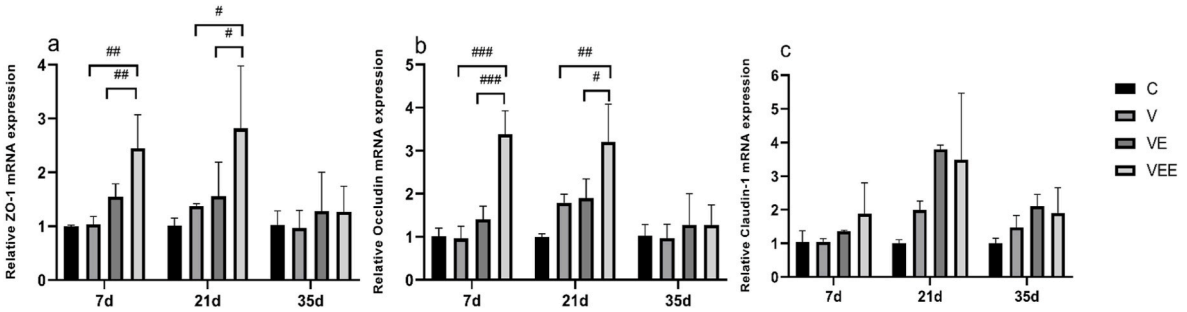

Fig. 3. EP and EE affects the expression levels of tight junction protein in jejunum: a) relative ZO-1 mRNA expression, **b)** relative Occludin mRNA expression, **c)** relative Claudin-1 mRNA expression. All data were presented as mean ± SD(n = 6). $^{*}p < 0.05$,$^{**}p < 0.01$,$^{***}p < 0{,}001$ compared to the group C; $^{\#}p < 0.05$,$^{\#\#}p < 0.01$,$^{\#\#\#}p < 0{,}001$ compared to the group V and intergroup.

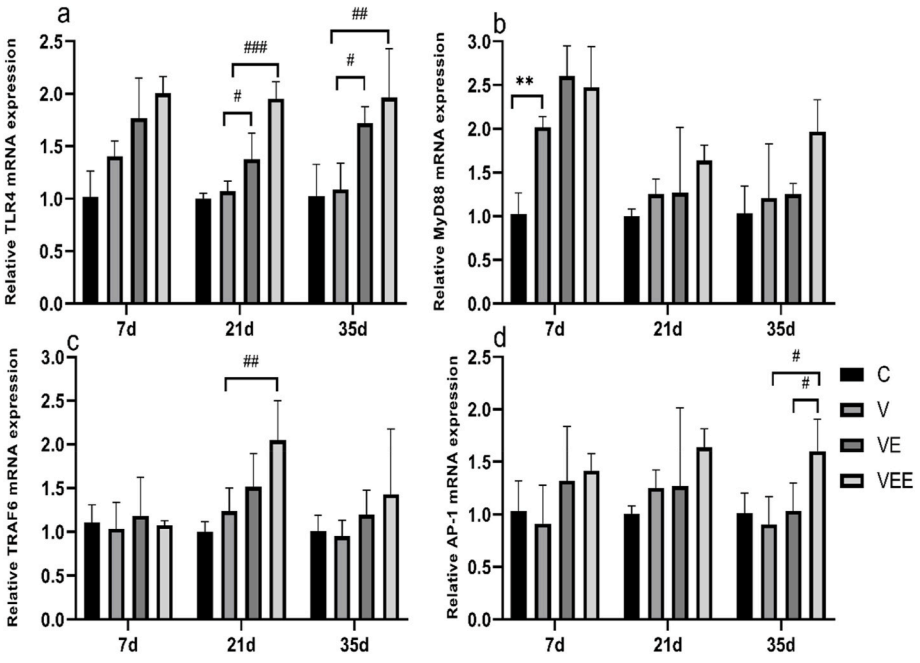

Fig. 4. EP and EE affects the immune gene expression in jejunum: a) relative TLR4 mRNA expression, **b)** relative MyD88 mRNA expression, **c)** relative TPAF6 mRNA expression, **d)** relative AP-1 mRNA expression. All data were presented as mean ± SD(n = 6). $^{*}p < 0.05$,$^{**}p < 0.01$,$^{***}p < 0{,}001$ compared to the group C; $^{\#}p < 0.05$,$^{\#\#}p < 0.01$,$^{\#\#\#}p < 0{,}001$ compared to the group V and intergroup.

0.05), and significantly higher in the group V compared to the group C ($P < 0.05$). Also, the relative protein expression of TRAF6 and ERK was pointedly higher ($P < 0.001$) in the group V compared to groups C, VE, and VEE. The relative protein expression of JNK was also significantly higher in group VE ($P < 0.05$) than in group C on day 7The results also showed that the relative protein expression of TLR4 was significantly higher in group VE ($P < 0.05$) than VEE, V, and C groups on day 21; the relative protein expression of ERK was significantly higher in groups VE and VEE ($P < 0.01$) relative to the group V; the relative protein expression of JNK was significantly higher in groups VE and VEE ($P < 0.05$) relative to the group V. On day 35, the relative protein expression of MyD88 was significantly higher in the group VEE ($P < 0.05$) than in the other groups, and the relative protein expression of ERK was significantly higher in the group VEE compared to the group V ($P < 0.05$).Besides, the relative protein expression of JNK was significantly higher in the group VEE compared to the groups V and VE ($P < 0.01$).

4. Discussion

Immunopotentiators are often combined with vaccines in order to enhance the effectiveness of vaccines. As research on the mechanisms of traditional Chinese medicine deepens, some studies have shown that traditional Chinese medicine has immune modulating effects and is considered as an ideal immune enhancer(Wang et al., 2022). Although, AIV vaccination is an effective and mandatory method in poultry production and breeding, the vaccine itself can cause a short-term decline in production performance, which may not be restored within the relatively short growth cycle of broilers (Orso et al., 2021).

In our research, the dose range was preliminarily determined by literature(Li et al., 2022; Lee et al., 2013), and the dosage of EP 20 mg/kg and EE 5 mg/kg was determined by our pre-experiment. Our results indicated that supplementation of EP and EE augment the broiler production performance. Our results were in line with the study of Hashem, M.A. who reported that adding EP to the diet can increase the weight and immune response of chicks (Hashem et al., 2020). Similarly, Q. C. Ren found that adding purified Echinacea polysaccharide to the diet can linearly increase the final live weight and daily body weight gain of broilers (Q.C. Ren, 2020). In another study, Awad reported that, adding 2.5, 5.00, and 7.5 g/kg of EP to the diet had a significant effect on egg production, egg weight, egg production rate, feed intake, and feed conversion rate of ducks (Awad et al., 2021). The results showed that AIV vaccination of broilers in one group without supplementation of EP

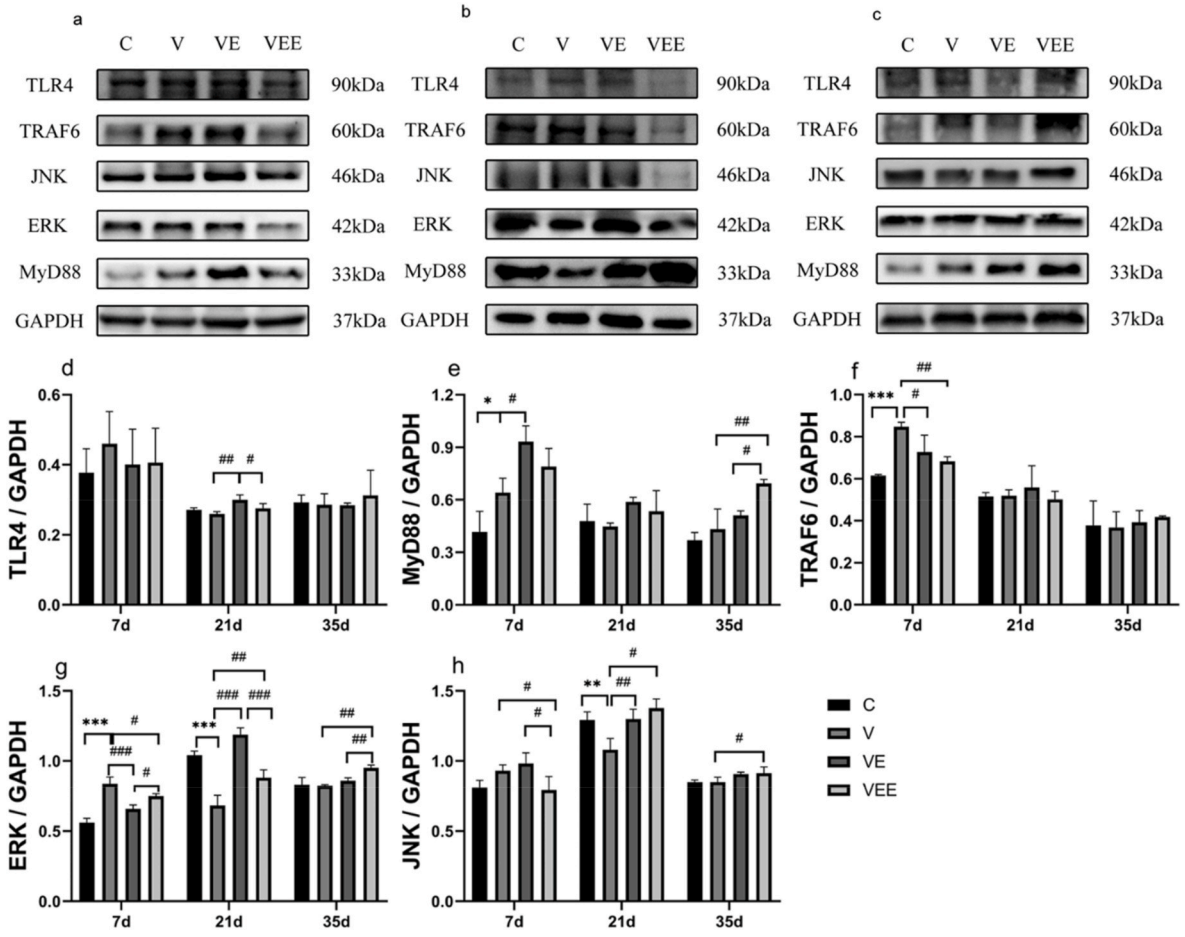

Fig. 5. Effects of *EP and EE* on the relative expression of jejunal proteins in broilers after immunization: a), b), c) is Western blot detects protein expression bands on days 7,21,35; **d), e), f), g), h)** is the level of the protein expression: TLR4,MyD88,TPAF6,ERK,JNK. All data were presented as mean ± SD(n = 6). $^{*}p < 0.05$, $^{**}p < 0.01$,$^{***}p < 0,001$ compared to the group C; $^{\#}p < 0.05$,$^{\#\#}p < 0.01$,$^{\#\#\#}p < 0,001$ compared to the group V and intergroup.

and EE caused a decline in performance parameters, and did not recover at the end of the trial. However, it is evident from our results that adding EP or EE to the diet could improve the weight loss caused by the vaccine and the increase in FCR. Furthermore, even addition of EE is sufficient to improve the body weight significantly compared to that of unvaccinated broilers and to make the FCR significantly lower than that of unvaccinated broilers. This could be related to the increased gene expression of jejunum tight junction proteins ZO-1, Occludin, and Claudin, which can promote intestinal absorption.

The intestinal surface is covered by abundant number of closely adhered simple columnar epithelial cells. Both, epithelial cells and lymphocytes work together in the mucosal immune system. The main participants in this immune system are polymeric immunoglobulins, especially dimeric IgA (Zhang et al., 2023). The lipophilic extract of EP can promote the maturation and migration of mouse dendritic cells by regulating JNK, P38 MAPK, and nuclear factor kappa-B (NF-κB) pathways (Yang et al., 2018), thereby inducing the proliferation and activation of T ($CD4^+$ and $CD8^+$) and B cells, further inducing T helper cell differentiation and secretion of cytokines, and promoting the secretion of sIgA (Cai et al., 2022; Hussain et al., 2023). The results showed that adding EP and EE to the diet caused broilers to secrete sIgA earlier and in greater quantities, indicating that EP and EE could produce intestinal immunity faster and enhance intestinal immunity.

There is a complex multi-protein network between intestinal epithelial cells, and intestinal tight junction proteins can play a crucial role in maintaining intestinal barrier stability. Typical tight junction proteins, such as ZO1, Claudin-1, and Occludin, can achieve a selective permeability barrier, which is more conducive to the absorption of nutrients (Nazir et al., 2022; Tomaszewska et al., 2021). Polyphenols in EP have a positive regulatory effect on gut microbiota and short-chain fatty acid (SCFA)-producing bacteria (Ren et al., 2022a). SCFAs can promote the interaction between the SP1 transcription factor and Claudin-1 promoter increasing protein abundance (Brandejs et al., 2022). In addition to stimulating claudin expression, SCFAs also accelerate the assembly of tight junctions through calcium/calcium-dependent protein kinase β-mediated AMP-activated protein kinase (Lei et al., 2022; Mátis et al., 2022; Zhang et al., 2021). Yong Y showed that ZO-1 and Claudin-4 were associated with phosphorylated p38 and ERK1/2 in the duodenum of heat-stressed pigs (Yong et al., 2021). Similarly, it has been reported by Hu R that activation of TLR4-mediated inflammatory pathways, such as NF-κB, may potentially correlate with the expression of tight junction proteins (Hu et al., 2020). The results indicated that the expression of ZO1 and Occludin protein was enhanced in broilers fed with EE, which was significantly higher than that in the vaccine group. This indicates that EP and EE promotes the nutrient absorption and improves the production performance of broilers.

Serum virus antibody levels are often used as indicators of the antiviral ability. Kim HaRim showed that EP could reduce the immunosuppression caused by cyclophosphamide, by stimulating spleen lymphocytes, particularly T and B lymphocytes, and by regulating the NK cell activity (Kim et al., 2021). Moreover, EP can regulate the dendritic cells by regulating key cells and promoting cell fluidity and chemotaxis (Yin et al., 2010). The immune activity of EP may be attributed to chicoric acid, which can enhance the function of $CD4^+$ T

cells and control NK cell activity, thereby improving immune response (Park et al., 2021). Moreover, Echinacea polysaccharides have immunomodulatory effects on dendritic cells, lymphocyte proliferation, and cytokines secretion (Yang et al., 2018)(Yao et al., 2019) In addition to its immunomodulatory activity, EP also has a very active killing effect on membrane-bound viruses such as AIV (Hudson J, 2011). In another study, Declerck Ken revealed that commercial EP's antiviral effect was particularly prominent during the initial virus exposure (Declerck et al., 2021). The results showed that EP and EE could significantly increase AIV antibody levels, resulting in faster antibody production by broilers and enhanced antiviral immunity against AIV.

Currently, research on the mechanism of EP is focused on cAMP, NF-κB, p38/MAPK, and other pathways (Declerck et al., 2021; Li et al., 2014, 2017), and less on TLR4 in broilers. Landmannshowed that cichoric acid can affect the gene expression of MyD88, interfering with the activation of TLR4-dependent signaling cascades. Likewise, Hou showed that EP can significantly upregulate AP-1 through JNK, playing an anti-inflammatory and hepatoprotective role (Hou et al., 2011). Similarly, the same immune pathway was observed in lipopolysaccharide (LPS)-infected chicks (Zhang et al., 2023). Some pharmacological studies on the TLR4 signaling pathway have shown that activated TLR4 can recruit MyD88, subsequently, MyD88 attracting interleukin 1 receptor-associated kinase 1 (IRAK1), IRAK4 and tumor necrosis factor (TNF), TRAF6 (You et al., 2022). Additionally, EP can induce phosphorylation of JNK and ERK whereas, JNK can further activate innate immunity through JNK-STAT1 signaling. IFN signaling will induce various IFN-responsive genes to produce antiviral effects (Lee S, 2019). In another study, Yali Li disclosed that EP does not lead to phosphorylation of ERK in mouse dendritic cells, which differs from our experimental results, and may be caused by different species and cells (Li et al., 2017). Our experiment showed that EP and EE could activate the TLR4-MAPK signaling pathway, promote the gene and protein expression of TLR4, MyD88, TRAF6, and AP-1 in the pathway, and also increase the protein expression of JNK and ERK. Significant differences were observed in mRNA levels of signaling factors at different time points after vaccination, indicating that EP and EE can regulate the TLR4-MAPK signaling pathway and improve and prolong broilers' immunity to AIV vaccine.

5. Conclusion

Vaccination against AIV can cause immune stress in broilers and reduce production performance, but it does not have a significant impact on the TLR4-MAPK signaling pathway. This suggests that the immune stress caused by the vaccine can be controlled and is only reflected in the production performance of broilers. For AIV-immunized broilers, adding EP and EE to the diet can enhance production performance by improving the mechanical barrier of the intestinal tract and enhancing the intestinal immunity and immune response to vaccines through regulation of the TLR4-MAPK signaling pathway.

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CRediT authorship contribution statement

Xinyue Wang: were responsible for the study conception and design, revised the manuscript, were involved in the drafting of the manuscript. **Jiaxin Chen:** were responsible for the study conception and design, revised the manuscript, were involved in the drafting of the manuscript. **Yanzi Chan:** were responsible for the study conception and design. **Sihan Li:** were responsible for the study conception and design. **Menglin Li:** were responsible for the study conception and design. **Fei Lin:** were responsible for the study conception and design. **Khalid Mehmood:** were responsible for the study conception and design. **Asif Idrees:** were responsible for the study conception and design. **Renzhao Lin:** were responsible for the study conception and design. **Yalin Su:** were responsible for the study conception and design. **Chunkai Wang:** were responsible for the study conception and design. **Dayou Shi:** revised the manuscript, were involved in the drafting of the manuscript.

Declaration of competing interest

All authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

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