Chai Hu oral liquid enhances the immune functions of both spleen and bursa of Fabricius in heat-stressed broilers through strengthening TLR4-TBK1 signaling pathway

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ABSTRACT Heat stress can affect the poultry production and immune status of broilers. Heat stress disrupts intestinal integrity and increases intestinal inflammation, which is related with body immune dysfunction. Chai Hu oral liquid used as an antipyretic and anti-inflammatory drug is widely used in exogenous fever of poultry, but its resistance to heat stress and the mechanism is still unclear. In this study, a chronic heat stressed broilers model was established to explore the mechanisms of broilers' immune function changes and the effects of Chai Hu oral liquid. In this study, a total of 480 broilers were randomly divided into 6 groups with 80 replicates. Heat stress (**HS**) group broilers were stressed at 35 ± 2°C for 5 or 10 consecutive d with 6 h/d. Heat stressed (for 5 or 10 d) broilers were given with Jieshu KangreSan (Positive), Chai Hu oral liquid high, middle and low dosage (CH-High, CH-Mid, CH-Low) by oral administration. Birds in control group were treated with the same volume of PBS only in 25 ± 2°C. All birds were sacrificed at last heat stress challenged day. Changes in immune function were assessed by immune organs index, serum IFN-γ level, gene and protein expressions of immune factors in spleen and bursa of Fabricius. Results from this experiment showed that heat stress enhanced the immune organs' edema by directly increased the organs indexes of spleen and bursa of Fabricius in broilers. Heat stress for 10 d also increased bursa of Fabricius HSP70 protein level and significantly lowered the spleen and bursa of Fabricius proteins expressions of IFN-α, IFN-β, and IFN-γ in broilers. The IFN-β and IFN-γ protein levels in spleen and bursa of Fabricius also decreased in heat stressed broilers for 5 d. The gene and protein expressions of TLR4 and TBK1 markedly decreased in spleen and bursa of Fabricius of broilers treated with chronic heat stress. Chai Hu oral liquid reduced edema of immune organs and elevated TLR4-TBK1 signaling pathway to release immune factors. Above results indicated that chronic heat stress induced impaired immune function by inhibiting TLR4-TBK1 signaling pathway, and Chai Hu oral liquid had effective protection of body's immune ability by enhancing this signaling pathway.

Key words: heat stress, immunity, Chai Hu oral liquid, broiler, TLR4

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INTRODUCTION

With the rise of the global temperatures, heat stress (**HS**) has become the main environmental factor in poultry industry due to its negative effects on animal health and livestock production (He et al., 2018), which causes economic loss of over 100 million dollars in the United States (St-Pierre et al., 2003). It is reported that broiler chicken exposure to HS can alter physiological homeostasis, such as systemic immune dysregulation, endocrine and electrolyte disorders (Sohail et al., 2010), which result in decreased growth performance and increased morbidity and mortality (Rimoldi et al., 2015). Moreover, the reduced growth performance may relate to decreased feed intake, suppressed intestinal nutrient transporters and immune response by injuring intestines and immune organs (Habashy et al., 2017). Previous studies have reported that HS affects vital organs of broiler chickens, such as lower relative weights of thymus and spleen and dysfunction of the small intestine (Yi et al., 2017). Besides this, the size of immune-related organs such as the spleen, thymus, and lymphoid organs are also regressed in the heat-stressed birds

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(Quinteiro-Filho et al., 2010; Ghazi et al., 2012). In general, HS on broilers show an immunosuppressing effect, but it is underlying mechanism remains unclear. So, the understanding of immunological disorders in heat-stressed broiler chickens is of great importance to the poultry industry.

Innate immune cells identify danger substance through surface expressed pattern recognition receptors (**PRRs**). The activated PRRs can initiate TLR4 signaling pathway that trigger the release of inflammatory cytokines. It is reported that TLR2 and TLR4 (TLRs, Toll-like receptors) mediated immune dysfunction in heat-stressed pigs (Ju et al., 2011). The mRNA levels of TLR4 were increased in the jejunum and ileum of heat stressed chickens (Varasteh et al., 2015). The TANK-binding Kinase 1 (**TBK1**) is involved in distinct signaling pathways induced by various stimuli, such as environmental stress, inflammatory cytokines. Once activated, TBK1 can activate several downstream cellular signaling cascades, such as IRF-3, IRF-7 and the nuclear factor kappa B (NF-κB), which leads to the production of proinflammatory cytokines and interferons (**IFN**) (Shibata et al., 2011). So, TBK1 and TLR4 may play important roles in immunomodulation, but the mechanisms were rarely reported in broilers under HS. Therefore, it is important to explore the immunosuppression status through restraining the activation of TLR4-mediated TBK1 pathway.

Chai Hu oral liquid, a patented traditional Chinese medicine (**TCM**), has been widely used as an antipyretic and anti-inflammatory drug. In the clinic, it can be used to treat common cold, paramyxoviridae infections, infectious bovine rhinotracheitis (**IBR**), and proventriculitis. Previous studies have disclosed its components as radix bupleuri, scutellaria baicalensis, ginger processed pinellia, codonopsis pilosula, radix glycyrrhizae, etc. Radix Bupleuri has been widely used in TCM for over 2000 yr, many valuable and important activities have been discovered, such as anti-inflammatory (Xie et al., 2012), antiviral (Chiang et al., 2003), hepatoprotection (Wang et al., 2013a), and immunoregulation (Ying et al., 2014). It has been used in many traditional Chinese prescriptions, such as Xiao Chai Hu Tang, Chai Hu Shu Gan Yin, and Chai Hu oral liquid (Chen et al., 2011). *Scutellaria baicalensis* Georgi has been widely used to treat various diseases including inflammation, hypertension, hepatitis, tumors, diarrhea, and bacterial and viral infections (Lee et al., 2011). Inflammation is an important part of immune pathogenesis and is a response to injury, infection, and stress. Although Chai Hu oral liquid is widely used as an anti-inflammatory drug in poultry industry, there is little experimental data on its immune effect. Based on these findings, we hypothesized that Chai Hu oral liquid could modulate the innate immunity in spleen and bursa of Fabricius in heat-stressed broilers to inhibit inflammation. However, direct evidence for supporting this hypothesis is missing. Therefore, the major objective of this study is to investigate the effect of Chai Hu oral liquid on the activation of innate immunity and its related mechanisms in heat-stressed broilers.

MATERIALS AND METHODS

Animals

All animal work was conducted in accordance with the guidelines for the care and use of experimental animals established by the Ministry of Science and Technology of the People's Republic of China (Approval number: 2006-398), and was approved by the Laboratory Animal Management Committee of Foshan University.

A total of 480 one-day-old Ma chickens were obtained from a commercial hatchery (Foshan Nanhai Poultry Corporation, Foshan, China). One-day-old chickens were housed in climate-controlled rooms at the Foshan Technology University, Guangdong, China. Chickens were maintained in isolator chambers, containing high efficiency particulate air (**HEPA**) filters, from the first posthatching day (ED1) until the last experimental day (ED21). The chickens freely received water and were fed as described below. The humidity was monitored and controlled (at not less than 60%).

Group Formation and Heat Stress

At the ED21, the broilers were randomly and equally allocated into 2 independent experiments. Experiment 1: The treatments were as follows: control group (Control); Heat stress group (Heat Stress-5d); Heat stress + Jieshu KangreSan group (Positive), Heat stress + Chai Hu oral liquid high dosage group (CH-High); Heat stress + Chai Hu oral liquid middle dosage group (CH-Mid); Heat stress + Chai Hu oral liquid low dosage group (CH-Low) with 40 birds in each group. Birds in control group were treated with PBS only in 25 ± 2°C. Birds in other groups were challenged with 35 ± 2°C heat stress for 5 consecutive d with 6 h in each day. Ten g Jieshu KangreSan was added per 1 kg feed in positive birds. 125, 100 and 75 mL Chai Hu oral liquid were separately added per 100 kg water in CH-High, CH-Mid and CH-Low broilers. The drugs were given with broilers as the same day with heat stress and for 5 consecutive d. Experiment 2: The groups and treatments were the same as experiment 1. But the time of heat stress and drug administration were changed to 10 d.

Broilers had free access to experimental diets and drinking water. A combination of daylight and artificial light was used, with a 12-h light/dark cycle. At d 26 or 31 (after 5 or 10 d of heat stress challenge), blood samples were taken from the jugular vein for measurement. The broilers were then killed by CO_2 inhalation and the samples of spleen and bursa of Fabricius were collected.

Enzyme-linked Immunosorbent Assays

We collected blood samples from all chickens' jugular veins before euthanasia. The plasma samples were separated from the whole blood by centrifugation 3,000 × g, 10 min at 4°C, and then the serum was harvested for use in enzyme-linked immunosorbent assays (**ELISAs**).

Serum IFN-γ level was measured by ELISA according to the manufacturer's instructions.

Western Blot Analysis

Standard western blot analysis was performed on spleen and bursa of Fabricius tissues of broilers. Briefly, tissues were homogenized with protein lysis buffer, then protein concentration was determined using the BCA protein assay kit. For the detection, primary antibodies against TLR4, TBK1, p-TBK1, IFN-α, IFN-β, IFN-γ and β-actin; Cell Signaling Technology, Danvers, MA) for 2 h at room temperature. The corresponding secondary antibodies (goat antirabbit IgG HRP or rabbit antimouse IgG HRP, Santa Cruz Biotechnology, Dallas, TX) were incubated for 1 h at room temperature. The level of protein expression was analyzed by comparing the density of binds using the software, Image J.

Gene Expression

A 5 cm segment of spleen and bursa of Fabricius tissues were snap-frozen in liquid nitrogen and stored at -80°C until mRNA extraction, gene expression measurement. Total RNA was extracted from tissue samples using the Trizol Reagent (Hlingene Corporation, Shanghai, China) according to the manufacturer's guidelines, and 10 ug of RNA was used for cDNA synthesis using UEIris Ⅱ RT-PCR System for First-Strand cDNA Synthesis (Jiangsu, China) to examine expression of Hsp70, TLR4 and TBK1. RNA was spectrophotometrically quantified (A260) and its integrity was verified by agarose gel electrophoresis.

All-in-one TM qPCR mix kit (GeneCopoeia) in accordance with the manufacturer's protocol for the lightCycler 480 Real-time PCR system (Applied Biosystems, Life Technologies, Carlsbad, CA). The PCR efficiency and melting curves were checked to ensure consistent amplification of a single PCR product. Gene expression was normalized to β-actin (internal reference) and presented as relative fold change compared with control. All samples were tested in triplicate.

Real-time PCR was performed using specific primers for Hsp70, TLR4 and TBK1. The primers used in RT-PCR are listed in Table 1.

Statistical Analysis

Data were presented as mean ± standard deviation (SD). All data were analyzed using SPSS 17.0 for one-way ANOVA. Differences was considered statistically significant at $P < 0.05$, obviously significant at $P < 0.01$.

Table 1. The primers used in RT-PCR.

Gene	Gene (bp)	Primers Sequence (5' to 3')
TLR4 for	132	TCTTTCAAGGTGCCACATCCA
TLR4 rev		AGCGACGTTAAGCCATGGAA
TBK1 for	149	ACTGAGGTTGTCATCAGACTGG
TBK1 rev		GAGCTGGAGAGACGCAACAA
β-actin for	82	ACGTCTCACTGGATTTCGAGCAGG
β-actin rev		TGCATCCTGTCAGCAATGCCAG

RESULTS

Chai Hu Oral Liquid Enhanced Serum IFN-γ Level in Heat-Stressed Broilers

As shown in Figure 1, compared with control group broilers, HS for 5 d enhanced the serum IFN-γ level ($P < 0.01$) in heat stress (HS) group chicken, and CH-High for 5 d significantly increased IFN-γ level ($P < 0.01$) compared with HS group. However, the serum IFN-γ level in broilers had no change between control and HS (10 d) group, but CH groups (CH-High, CH-Mid, CH-Low) for 10 d obviously raised serum IFN-γ level ($P < 0.01$, $P < 0.01$, $P < 0.01$, respectively) compared with HS group. Our results showed that CH can increase the IFN-γ level in serum of heat-stressed broilers.

Chai Hu Oral Liquid Decreased Immune Organs Index in Heat-Stressed Broilers

As shown in Figure 2A, compared with control group broilers, HS for 5 and 10 d obviously increased the spleen index ($P < 0.01$, $P < 0.01$, respectively) in HS group broilers. Compared with HS group, CH groups (CH-High, CH-Mid, CH-Low) for 5 d significantly decreased spleen index. There was a similar trend in the bursa of Fabricius index between 5 and 10 d of HS, and CH-High for 5 d decreased bursa of Fabricius index (Figure 2B, $P < 0.01$). These results suggested that CH can reduce broilers' immune organs index induced by heat stress.

Chai Hu Oral Liquid Increased Spleen IFN-α, IFN-β and IFN-γ Levels in Heat-Stressed Broilers

In this study, we explored changes in the level immune cytokines in the spleen of broilers with heat stress. As shown in Figure 3A, compared with control group broilers, HS for 5 d decreased the spleen IFN-β and IFN-γ levels ($P < 0.01$, $P < 0.01$, respectively) in HS group broilers. Compared with HS group, CH groups (CH-H, CH-M, CH-L) for 5 d obviously enhanced spleen IFN-α, IFN-β, and IFN-γ levels. Compared with control group broilers, HS for 10 d decreased the spleen IFN-α, IFN-β, and IFN-γ levels ($P < 0.01$, $P < 0.01$, $P < 0.01$, respectively) in HS group broilers. Compared with HS group, CH groups (CH-H, CH-M, CH-L) for 10 d obviously enhanced spleen IFN-α level, CH groups (CH-H, CH-M) for 10 d increased spleen IFN-β level, and CH groups (CH-M, CH-L) for 10 d enhanced spleen IFN-γ levels. These results suggested that CH can increase spleen immunity in heat stressed broilers.

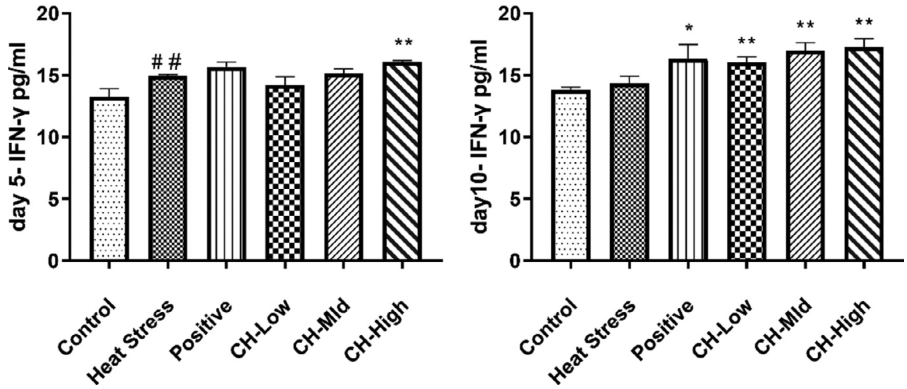

Figure 1. The effects of Chai Hu oral liquid on serum IFN-γ level in heat-stressed broilers. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$, vs. heat stress group, (n = 10).

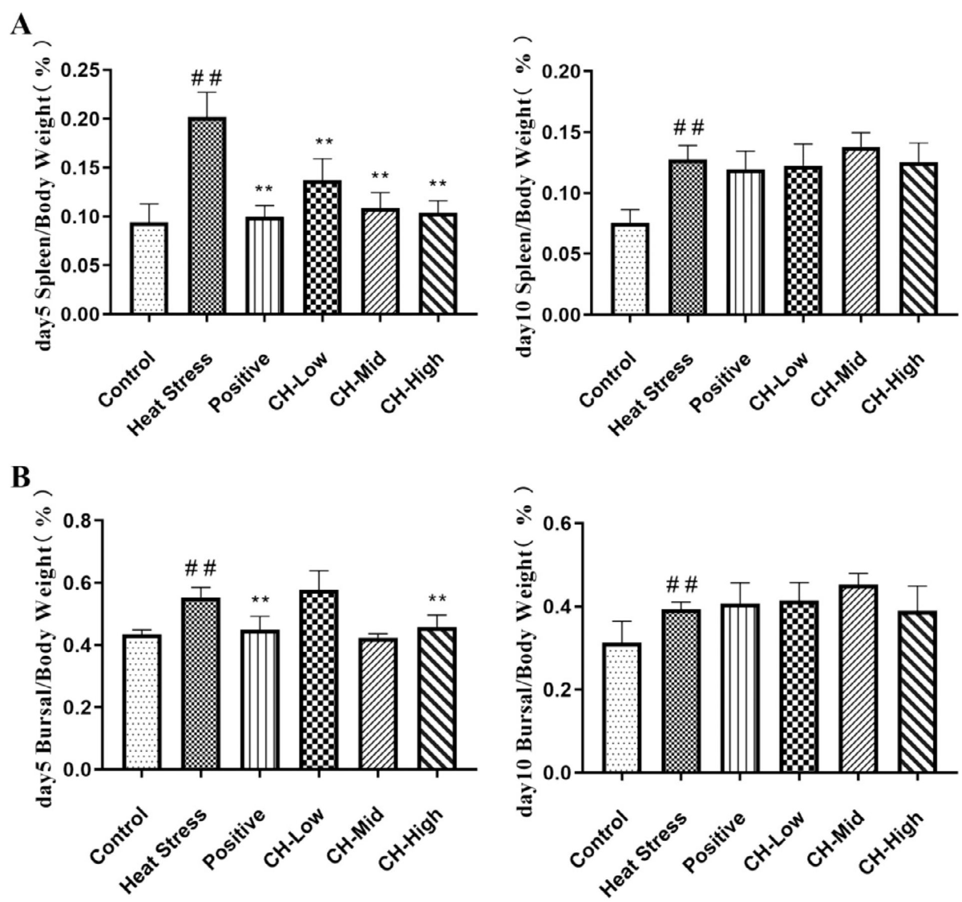

Figure 2. The effects of Chai Hu oral liquid on spleen and bursa of Fabricius index in heat-stressed broilers. (A) Day 5 and 10 Spleen/Body Weight (10%). (B) Day 5 and 10 Bursa/Body Weight (10%). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$, vs. heat stress group, (n = 10).

Chai Hu Oral Liquid Elevated Spleen TBK1 Protein Expression Levels in Heat-Stressed Broilers

We examined the changes of TBK1 and p-TBK1 protein expressions to test their relationship with heat stress. TBK1 phosphorylation can lead to the production of type I interferon. Results showed that compared with control group, TBK1 protein expressions in spleen were enhanced and p-TBK1 level was inhibited in HS 5 d group broilers (Figure 4A, $P < 0.01$, $P < 0.05$, respectively). Heat stress 5 d significantly inhibited TBK1 activation in spleen compared with those in the control group broilers. Compared with HS group, CH obviously decreased TBK1 level and enhanced p-TBK1 level in heat-stressed broilers. As shown in Figure 4B, TBK1 and p-TBK1 levels in spleen were decreased in HS 10 d group broilers compared with control group birds (Figure 4B, $P < 0.01$, $P < 0.01$, respectively). CH

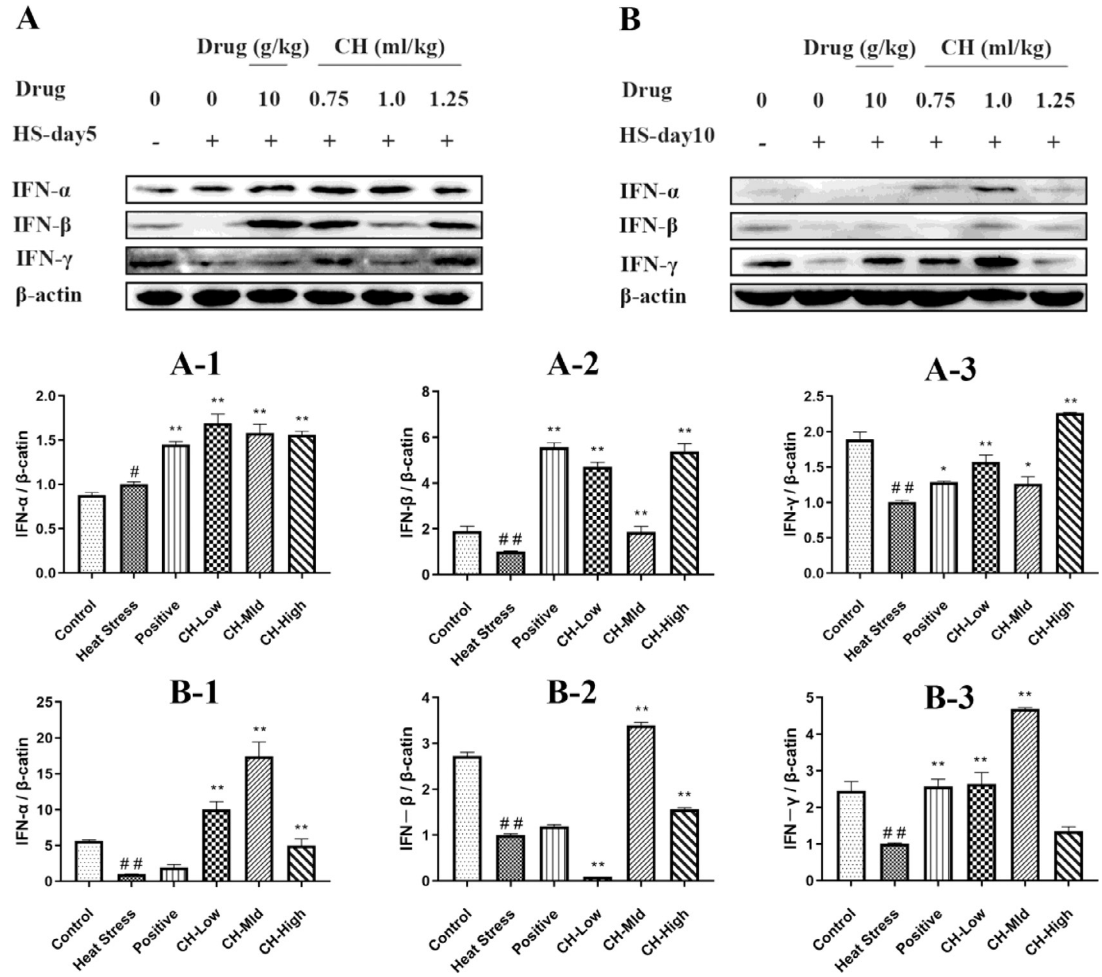

Figure 3. The effects of Chai Hu oral liquid on spleen IFN-α, IFN-β, and IFN-γ levels in heat-stressed broilers. (A) Spleen IFN-α, *IFN-β* and IFN-γ protein levels in heat stress for 5 days of broilers. (B) Spleen IFN-α, *IFN-β* and IFN-γ protein levels in heat stress for 10 days of broilers. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$, vs. heat stress group.

for 10 d significantly elevated TBK1 and p-TBK1 levels in heat-stressed broilers. These results suggested that CH administration for 5 and 10 d can enhance spleen immunity in heat-stressed broilers.

Chai Hu Oral Liquid Improved Spleen Gene Expressions Levels of TBK1 and TLR4 in Heat-Stressed Broilers

As shown in Figure 5, compared with control group, HS for 5 d enhanced the spleen TBK1 and TLR4 genes expressions ($P < 0.01$, $P < 0.01$) in HS group broilers. Compared with HS group, CH groups for 5 d decreased TBK1 and TLR4 genes levels. However, HS for 10 d significantly inhibited the spleen TBK1 and TLR4 gene expressions (Figure 5, $P < 0.01$, $P < 0.01$) compared with control group broilers. CH groups for 10 d obviously enhanced spleen TBK1 and TLR4 gene levels. There had a same trend change of spleen gene expressions between TLR4 and TBK1 in heat stressed broilers.

Chai Hu Oral Liquid Raised Bursa of Fabricius IFN Levels in Heat-Stressed Broilers

In Figure 6, we found that INF-β and INF-γ expressions were decreased in the bursa of Fabricius for 5 d ($P < 0.05$, $P < 0.01$) after heat stress. Under the influence of heat stress for 10 d, the expressions of IFN-α, INF-β, and INF-γ significantly inhibited in the bursa of Fabricius ($P < 0.01$, $P < 0.05$, $P < 0.01$). CH for 5 and 10 d can obviously increase IFN levels in heat stressed broilers. These results suggested that CH can enhance bursa of Fabricius immune function in heat stressed broilers.

Chai Hu Oral Liquid Elevated Bursa of Fabricius TBK1 Levels in Heat-Stressed Broilers

Compared with control group, heat stress for 5 d significantly decreased TBK1 and p-TBK1 expressions in

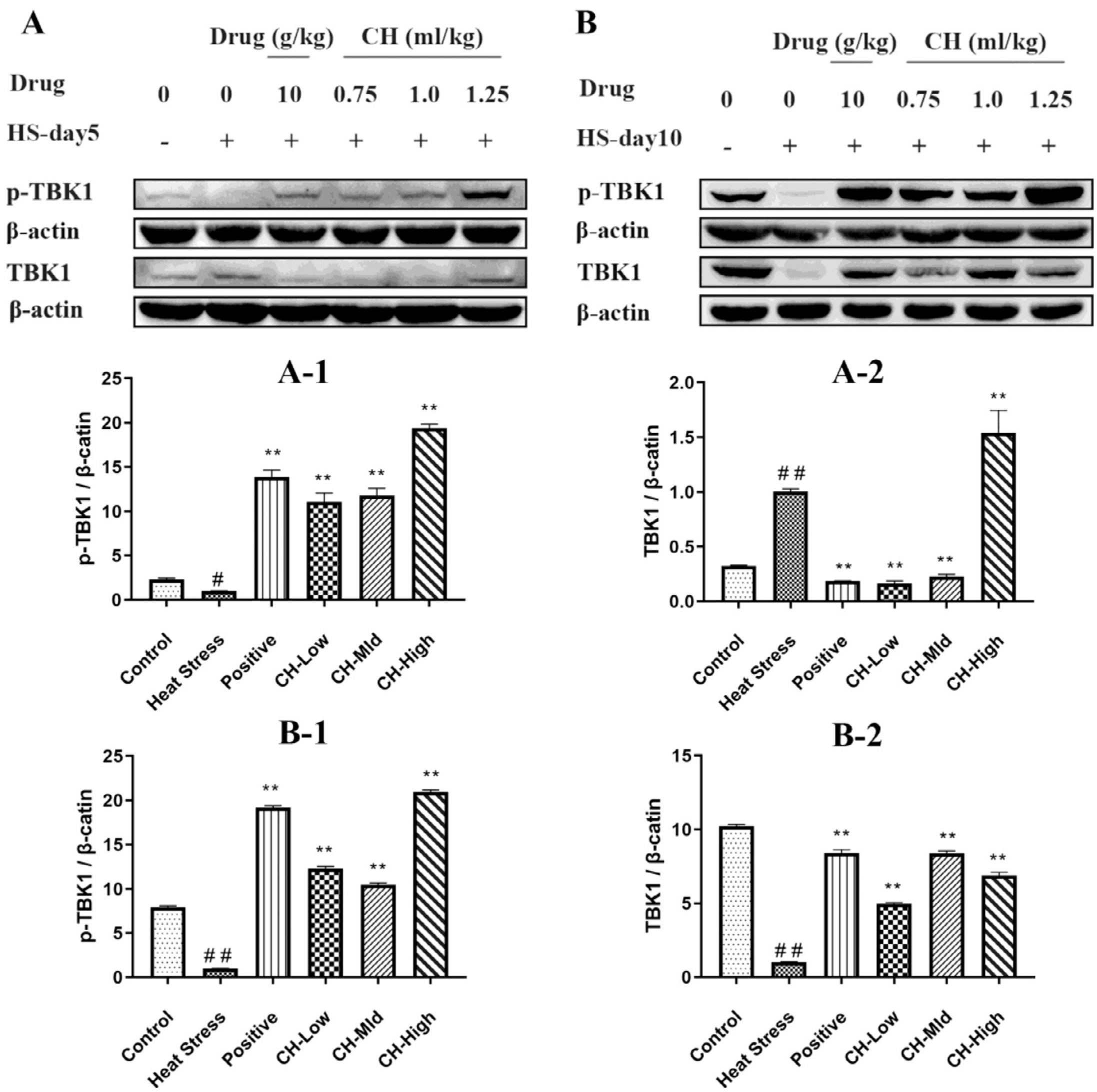

Figure 4. The effects of Chai Hu oral liquid on spleen TBK1 and p-TBK1 levels in heat-stressed broilers. (A) Spleen p-TBK1 and TBK1 protein levels in heat stress for 5 days of broilers. (B) Spleen p-TBK1 and TBK1 protein levels in heat stress for 10 days of broilers. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$, vs. heat stress group.

the bursa of Fabricius of broilers (Figure 7A, $P < 0.01$, $P < 0.01$). TBK1 and p-TBK1 expressions were decreased in the bursa of Fabricius for given with CH (CH-Mid, CH-High) for 5 d and CH (CH-Low, CH-Mid, CH-High) for 10 d after heat stress. However, heat stress for 10 d tremendously increased TBK1 and p-TBK1 expressions in the bursa of Fabricius of broilers (Figure 7B, $P < 0.01$, $P < 0.01$). Compared with HS 10 d group, CH decreased bursa of Fabricius TBK1 and elevated p-TBK1 levels in broilers. Thus, our results indicated that CH for 10 d can increase interferon expressions in heat stressed broilers through enhancing the TBK1 activation.

Chai Hu Oral Liquid Decreased Bursa of Fabricius HSP70 Protein Levels in Heat-Stressed Broilers

In Figure 8, we found that TLR4 protein level in bursa of Fabricius was decreased after heat stress for 5 d and increased for 10 d, while CH obviously decreased TLR4 protein level in broilers with heat stress for 5 and 10 d (Figure 8A). Under the influence of heat stress for 10 d, the expressions of HSP70 were significantly increased in bursa of Fabricius ($P < 0.011$) of broilers, but there was no change of HSP70 level in broilers with heat stress for 5 d, while CH-High obviously decreased HSP70 protein level in broilers with heat stress for 10 d. These results suggested that CH can reduce broilers' bursa of Fabricius damage induced by heat stress.

Chai Hu Oral Liquid Enhanced Bursa of Fabricius TBK1 and TLR4 Gene Levels in Heat-Stressed Broilers

The gene expressions of TBK1 and TLR4 in bursa of Fabricius were examined. As shown in Figure 9A, compared with control group, heat stress for 5 d increased TBK1 gene level ($P < 0.01$). TBK1 and TLR4 gene levels were decreased in the bursa of Fabricius for given

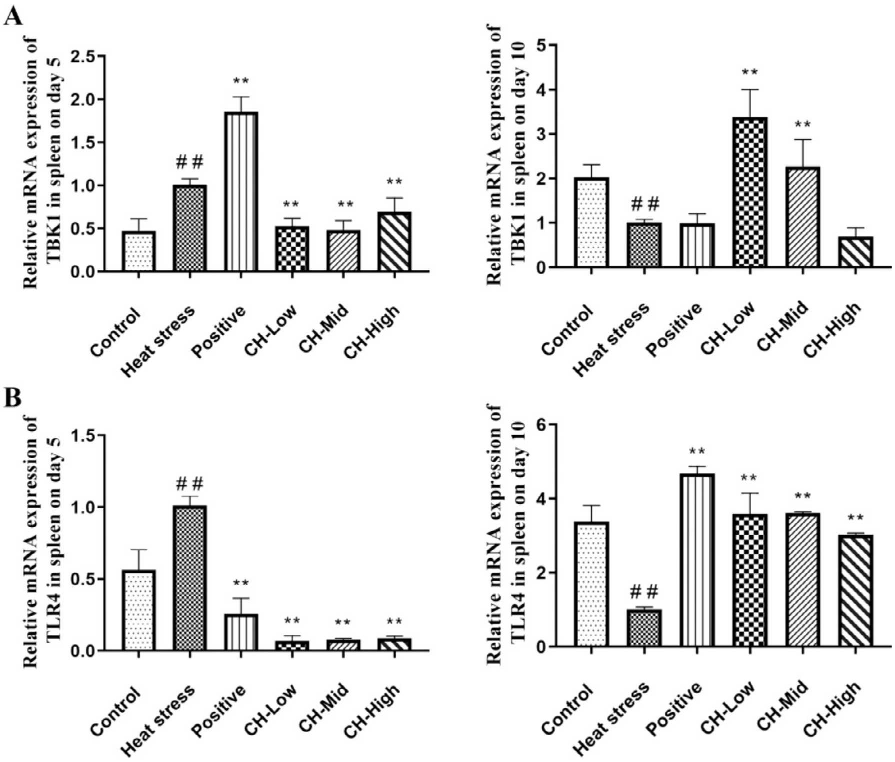

Figure 5. The effects of Chai Hu oral liquid on spleen TBK1 and TLR4 gene levels in heat-stressed broilers. (A) Spleen TBK1 gene levels in heat stress for 5 and 10 days of broilers. (B) SpleenTLR4 gene levels in heat stress for 5 and 10 days of broilers. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$, vs. heat stress group.

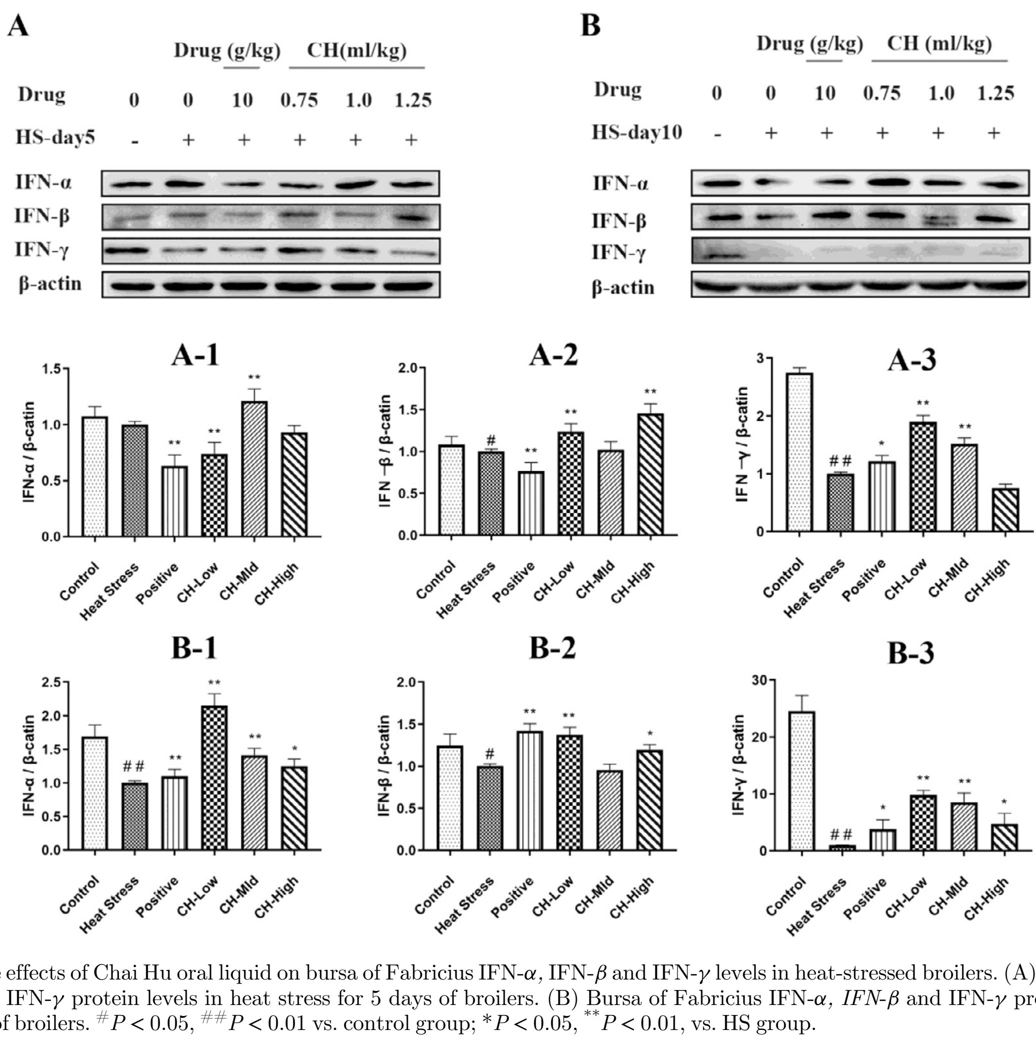

Figure 6. The effects of Chai Hu oral liquid on bursa of Fabricius IFN-α, IFN-β and IFN-γ levels in heat-stressed broilers. (A) Bursa of Fabricius IFN-α, *IFN-β* and IFN-γ protein levels in heat stress for 5 days of broilers. (B) Bursa of Fabricius IFN-α, *IFN-β* and IFN-γ protein levels in heat stress for 10 days of broilers. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$, vs. HS group.

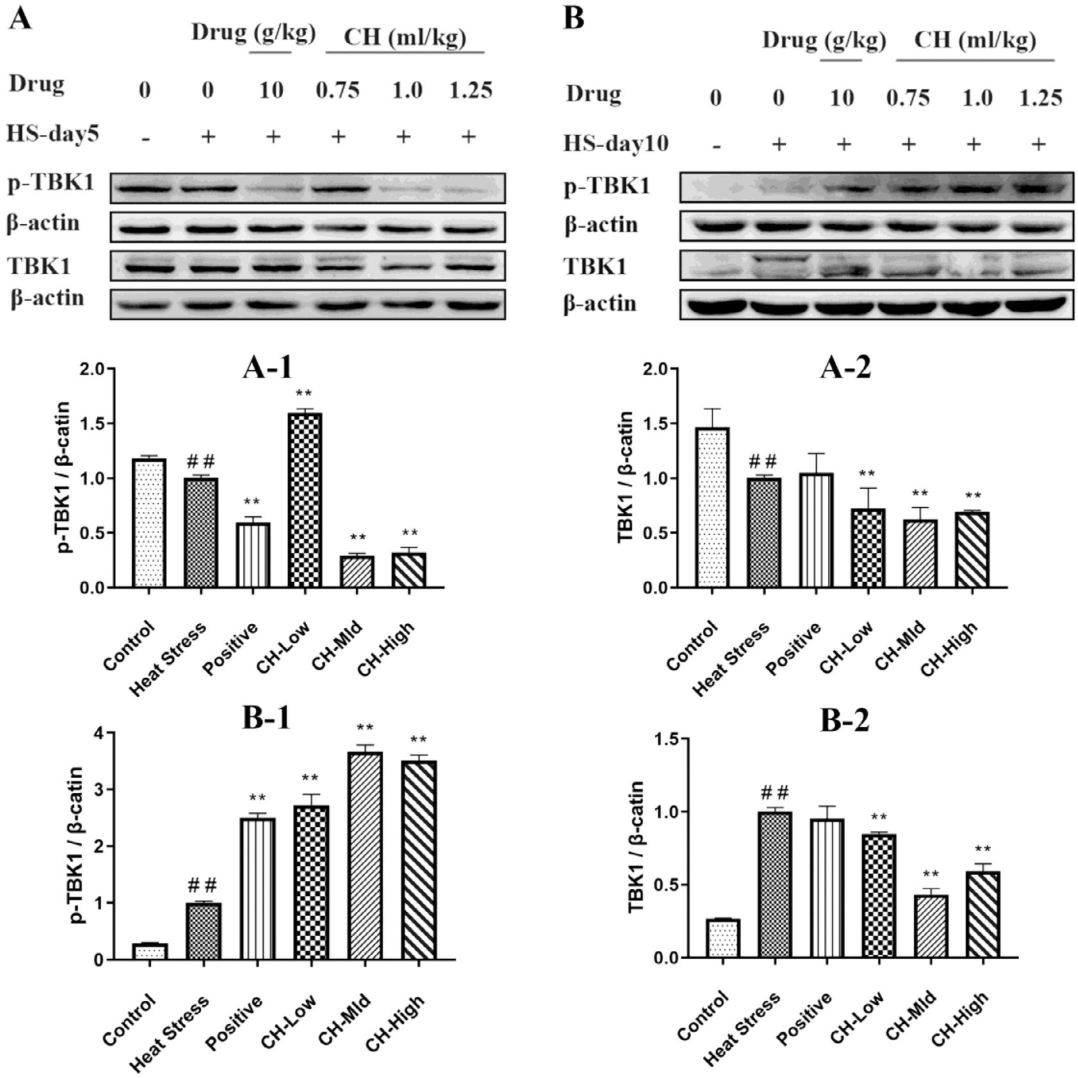

Figure 7. The effects of Chai Hu oral liquid on bursa of Fabricius TBK1 and p-TBK1 levels in heat-stressed broilers. (A) Bursa of Fabricius p-TBK1 and TBK1 protein levels in heat stress for 5 days of broilers. (B) Bursa of Fabricius p-TBK1 and TBK1 protein levels in heat stress for 10 days of broilers. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$, vs. heat stress group.

with CH for 5 d after heat stress. However, heat stress for 10 d tremendously decreased TBK1 and TLR4 gene expressions in the bursa of Fabricius of broilers (Figure 9B). Compared with HS 10 d group, CH-High elevated bursa of Fabricius TBK1 gene level and CH-Low enhanced TLR4 gene level in broilers treated with heat stress.

DISCUSSION

For most poultry, growth rate, productivity, and many biochemical parameters are affected when the ambient temperature exceeds 20°C (Alagawany et al., 2017). High ambient temperature can cause various harmful effects on the physiological and production characteristics of poultry, which may be related with decreased poultry immunity and immunological parameters (Amedy et al., 2011; Kumari and Nath., 2018). It is reported that broilers exposed to chronic heat stress showed reduced performance parameters, and decreased immunity (Amedy et al., 2011). Spleen and bursa of Fabricius are important immune organs in broilers. The bursa of Fabricius is an avian-specific primary immune tissue that is responsible for B lymphocyte development and diversity in the antibody repertoire (Niu et al., 2009; Ratcliffe and Härtle., 2014; Jahanian and Rasouli., 2015).Heat stress for 5 d can significantly induce edema of immune organs in heat stressed broilers with higher levels of spleen and bursa of Fabricius index compared with control group broilers, and CH obviously relieve the edema of above immune organs. The results suggested that CH may enhance immune function in heat stressed broilers.

In this study, we aimed to first delineate whether heat stress can worse immune suppression in spleen and bursa of Fabricius of broilers. Heat stress has been found to modulate the splenic and intestinal gene expressions of several different cytokines in broilers (Ohtsu et al., 2015; Varasteh et al., 2015). Cytokines enable cell communication during immunological development and immune response. With respect to avian immune function, IFN-γ is considered to be an immunoregulatory cytokine, which can enhance cell-mediated immunity. It has been

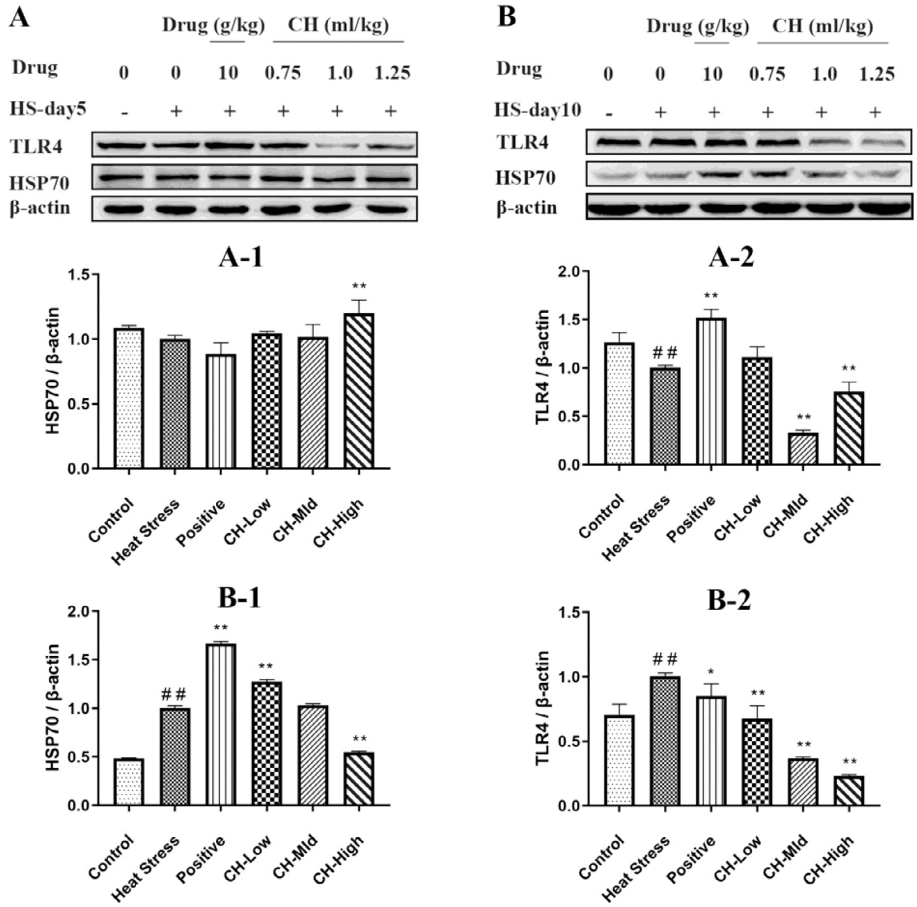

Figure 8. The effects of Chai Hu oral liquid on bursa of Fabricius TLR4 and HSP70 protein levels in heat-stressed broilers. (A) Bursa of Fabricius TLR4 and HSP70 protein levels in heat stress for 5 days of broilers. (B) Bursa of Fabricius TLR4 and HSP70 protein levels in heat stress for 10 days of broilers. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$, vs. heat stress group.

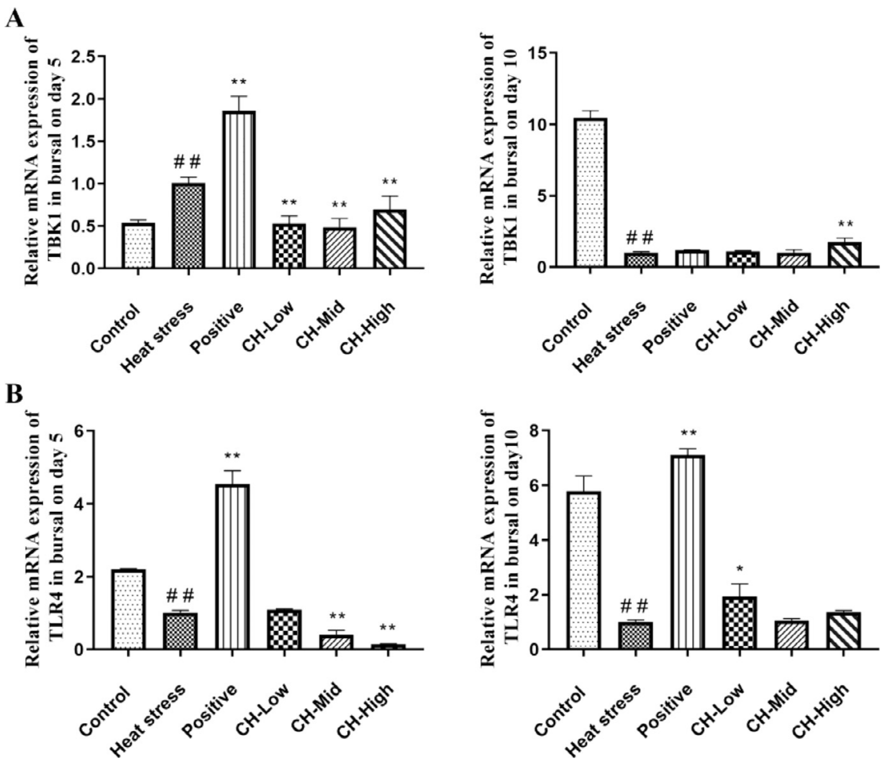

Figure 9. The effects of Chai Hu oral liquid on bursa of Fabricius TBK1 and TLR4 gene levels in heat-stressed broilers. (A) Bursa of Fabricius TBK1 gene levels in heat stress for 5 and 10 days of broilers. (B) Bursa of Fabricius TLR4 gene levels in heat stress for 5 and 10 days of broilers. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$, vs. heat stress group.

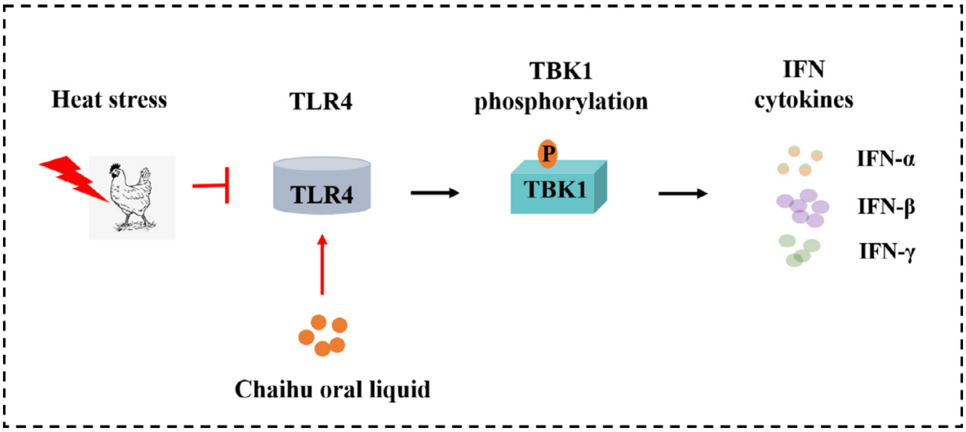

Figure 10. Induction of type I IFNs in spleen and bursa of Fabricius in heat-stressed broilers. Type I IFNs can be induced via TLR4. Chaihu oral liquid induced the production of type I IFNs via TLR4 /TBK1 signaling pathway.

reported that IFN-γ is down-regulated in the poultry gut after heat stress (Ohtsu et al., 2015). Type I interferons suppress bacterial infections by inducing the production of interferon-stimulating genes, regulating tissue integrity and inhibiting excessive inflammatory responses (Boxx and Cheng., 2016). No previously published data could be found regarding the expression of type-I interferons under heat stress conditions in broilers. In this study we found that heat stress significantly decreased protein levels of IFN-β and IFN-γ in spleen and bursa of Fabricius of broilers with heat stress. Our results are consistent with those reported by Liu et al., who showed that IFN-γ was lower in the spleen of pigeons exposed to avermectin (Liu et al., 2014a). We observed higher expressions of IFN-α, IFN-β and IFN-γ in the spleen and bursa of Fabricius in broilers treated with Chai Hu oral liquid. The drugs worked for 10 d had better protection for immune function. These results indicated that heat stress can aggravate immunosuppression by decreasing IFN-α, IFN-β and IFN-γ in spleen and bursa of Fabricius of broilers.

Heat stress can also lead to increasing expression of Toll-like receptors (**TLRs**), especially Hsp70, which specifically binds TLR2 and TLR4 and induces immunomodulatory effects such as cytokines and chemokines (Ju et al., 2014). HSP70 is an important class of nonspecific cytoprotective proteins that plays an important role in regulating tolerance to various stresses and resistance to stress (Mahmoud et al., 2004). The TLR4 mediated signal pathway is activated in response to stress, particularly in heat stress of broilers (Huang., 2017). In our study, the protein expression of HSP70 after heat stress was obviously increased in spleen and bursa of Fabricius of broilers compared to the control group broilers. Interestingly, we found that the protein expression trend of HSP70 was the same as that of TLR4. Chai Hu oral liquid especially for 10 d significantly enhanced the gene and protein expression of TLR4 and also elevated HSP70 protein level in spleen and bursa of Fabricius of broilers. As a result of heat stress, cells are able to adjust their metabolism to the changing environmental conditions by modifying TLR4 gene and protein expressions.

Previous studies have shown that after a viral infection, a protein called TBK1 sends a signal that induces a protective program that inhibits virus replication (Tojima et al., 2000). In the absence of TBK1, pathogens such as *Salmonella, enteropathogenic E. coli*, and *group A streptococci* are able to escape from closed host vacuoles and grow at high levels in the host cytoplasm (Perrin et al., 2004). The independent branch of MyD88 in TLR4 signaling is mediated by the adaptor molecule TRIF, leading to activation of TBK1. TBK1-IKKε mediates type I interferon expression, and subsequent interferon-responsive genes (Doyle et al., 2002). The importance of the MyD88-dependent pathway in antibacterial immunity is well known, but the functional contribution of TBK1-dependent signaling in heat stress is still unclear. In this study, we found that heat stress decreased the TBK1 gene and protein level in spleen and bursa of Fabricius of broilers. Interestingly, we found that the gene and protein expression trend of TBK1 was the same as that of TLR4, which suggested that heat stress caused immune suppression by decreasing the TLR4-TBK1 signaling pathway (Figure 10).

In TCM, Chai Hu oral liquid has effects of anti-inflammatory, antiviral, hepatoprotection and immunoregulation, and is mainly used to treat liver diseases, alleviate the symptoms of the common cold, such as fever, chills, and chest pain (Zuo et al., 2013). With the development of TCM modernization, more Radix Bupleuri preparations have been developed, such as Xiao Chai Hu tablets, Chai Hu dripping pills, Chai Hu injection, and Chai Hu Shu Gan pills (Li et al., 2014a). Among them, Chai Hu oral liquid is widely used to treat exogenous fever or fever caused by influenza or common cold in poultry. In this study, Chai Hu oral liquid administration can inhibit broilers' spleen and bursa edema and lead to increased release of immune factors. The change in the trend of TBK1 after Chai Hu oral liquid of broilers with heat stress is consistent with the change of interferon and TLR4, indicating that Chai Hu oral liquid can improve the body's immune ability to fight heat stress. This change may be regulated by TLR4-TBK1 signaling pathway.

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DISCLOSURES

No potential conflict of interest was reported by the authors.

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