Effect of intermittent mild cold stimulation on intestinal immune function and the anti-stress ability of broilers

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ABSTRACT A total of 240 healthy 1-day-old Ross 308 male broilers were randomly divided into 3 groups (CS0 group, CS3 group, and CS6 group), with 5 replicates in each group and 16 broilers in each replicate, in order to evaluate the effects of intermittent mild cold stimulation (**IMCS**) on the intestinal immune function and anti-cold stress ability of broilers after acute cold stress. The mRNA expression levels of cytokines and Toll-like receptors (**TLRs**) in the duodenum and jejunum were detected at the end of cold stimulation (36 d), 2 wk after recovery (50 d), and after acute cold stress (Y6). In addition, the mRNA and protein expression levels of heat shock proteins (**HSPs**) were measured before and after acute cold stress. The experimental data were statistically processed using 1-way ANOVA and Duncan's multiple comparisons. The results showed that the mRNA expression levels of IL2, IL8, IFN γ, TLR7, and TLR21 in the duodenum and IL2 and IFN γ in jejunum were significantly higher in the CS6 group than in the CS0 and CS3 groups at 36 d ($P < 0.05$). All TLR levels in the jejunum were significantly lower in the CS3 group than in the CS0 and CS6 groups at 36 d ($P < 0.05$). After 6 h of acute cold stress, in the duodenum, the mRNA expression levels of IL6 and IL8 were significantly decreased in the CS0 and CS6 groups compared to levels at 50 d ($P < 0.05$), while levels in the CS3 group remained stable ($P > 0.05$). Compared with 50 d, the expression level of HSP mRNA in the jejunum in the CS3 group was relatively stable compared to that in the CS0 and CS6 groups after acute cold stress ($P > 0.05$). At the protein level, the HSP60 expression level in the duodenum and HSP40, HSP60, and HSP70 expression levels in the jejunum were significantly higher in the CS3 group than in the CS0 and CS6 groups after acute cold stress ($P < 0.05$). In conclusion, cold stimulation training at 3℃/3 h lower than the conventional feeding temperature can improve the intestinal immune function and anti-stress ability of broilers.

Key words: broiler, intermittent mild cold stimulation, small intestine, immune regulation, cold adaptation

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INTRODUCTION

A low ambient temperature of 10°C to 20°C below the conventional feeding temperature usually causes cold stress to poultry, which has negative impacts on animal production performance, the antioxidant system, the immune system, and the neuroendocrine system and can even lead to animal death in serious cases (Tsiouris et al., 2015; Hu et al., 2021). Therefore, cold weather has become one of the most important factors restricting the development of the livestock and poultry industry in northern China (Dantzer et al., 1983). How to minimize the loss caused by low temperature has become the most concerning problem in livestock production. Studies have shown that cold adaptation can enhance the immune function and disease resistance of animals. Manning et al. (1990) found that early cold stimulation training can improve the antioxidant function of birds. Shender et al. (2007) believed that when broilers of 3 to 4 d of age were exposed to 15°C for 3 h every day, broilers at 21 d of age had high cold resistance and reduced mortality. After cold adaptation at 2°C for 2 wk, the cell-mediated immune function of mice was found to be enhanced (Xu et al., 1992). The disease resistance of cold-adapted mice was significantly enhanced (Banerjee et al., 1999). After repeated immersion in cold water at 14°C for 6 wk (1 h/time, 3 times/wk), cold adaptation can be established, and the immune system is activated to a certain extent (Janský et al., 1996). Therefore, proper cold training for broilers to enhance

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their cold resistance has become a feasible breeding method.

The intestinal tract is the largest surface in the body that contacts the external environment, is the main place where nutrients are absorbed, and is the largest immune organ in the body. Intestinal-associated lymphoid tissue and its secreted immunoglobulins, cytokines, and other immune substances together constitute the intestinal mucosal immune barrier (Shi et al., 2017). In poultry, the digestion and absorption of nutrients mainly occur in the small intestine, and its normal structure and function play an important role in the animal body (Wen et al., 2021). Increasing epidemiological evidence indicated that abnormal ambient temperatures can increase the risk of death in animals by causing intestinal function damage (Song et al., 2017). Some studies have shown that long-term cold stimulation can cause congestion of the duodenum and glandular stomach in chicks, while frequent cold stimulation can lead to gastrointestinal ulcers and other diseases (Huang et al., 2016).

Cytokines can extensively regulate the immune response of the body and participate in inflammatory damage and other pathological processes (Al-Zghoul et al., 2019). Intestinal microbes can influence T cell subsets and differentiate initial T cells into Th1 or Th2 cells through mutual recognition of receptors on the surface of dendritic cells (Zeuthen et al., 2008). However, chronic stress leads to a transformation from Th1-mediated cellular immunity to Th2-mediated humoral immunity, thus affecting the infection process and microbial susceptibility (Verbrugghe et al., 2012). Cold stress can increase the level of pro-inflammatory cytokines. Hangalapura et al. (2006) found that cold stress at 10°C increased IL1β, IL6, and IL12β mRNA expression levels of pro-inflammatory cytokines in peripheral blood leukocytes of chickens. Zhao et al. (2013a) showed that 12°C cold stress significantly increased the expression level of IL4 in the small intestine of broilers. Xu et al. (2019) found that IL6 and TNFα levels increased significantly in the hippocampus of mice after placing the mice in a 4°C environment (3 h/d) for 7 d. One study showed that acute cold stress can inhibit the Th1 response in the ileum, reduce the level of IFN γ, and cause morphological damage, oxidative stress, and inflammation in the ileum of broilers (Su et al., 2018). A study by Wei et al. (2018) showed that IFN γ content in the heart tissue of broilers was significantly reduced when the broilers were subjected to acute cold stress.

Toll-like receptors (**TLRs**) are an important class of protein molecules involved in nonspecific immunity (innate immunity) and act as a bridge connecting nonspecific immunity and specific immunity. TLRs can not only prevent the invasion of microbial pathogens but also regulate immune homeostasis (Takeda et al., 2005). The intestinal innate immune system can recognize pathogens by regulating TLRs on the surface of macrophages, dendritic cells, and epithelial cells to bind lipopolysaccharide of the bacterial cell wall (Medzhitov et al., 1997). The inflammatory reaction makes microbial exotoxins pass through the intestinal barrier to directly stimulate the intestinal immune system (Ledbetter et al., 2000). TLR signaling can also activate the NF-κB pathway and induce the expression of IL6 and IL8, thereby activating innate immune responses and adaptive immune responses (Medzhitov et al., 1997). When the temperature changes sharply, cells maintain homeostasis by regulating the expression of TLR genes. Li et al. (2020) found that after cold stimulation training (43 d, 3°C lower than the control group), the mRNA expression level of TLR7 in the duodenum of broilers was significantly higher than that in the control group, while TLR21 expression was significantly decreased. Basu et al. (2015) fed Calta fry in an environment (10°C, 15°C, and 20°C) lower than the water temperature (25°C) of the control group for 12 h, and the expression of TLR2 decreased with decreasing ambient temperature. Paul showed that the mRNA expression levels of TLR2 and TLR7 in the blood of Black Bengal goats stimulated by long-term cold in winter were lower than those in the mild control group, which reduced the activity of dendritic cells and macrophages and resulted in decreased immune function (Paul et al., 2015).

Heat shock proteins (**HSPs**) are a series of highly conserved molecular chaperones that play important roles in the folding and opening of proteins and the assembly and disassembly of protein complexes (Bernabò et al., 2011). As a typical marker of injury, HSPs are produced at low levels under normal physiological conditions and are produced at high levels under various stress states, such as oxidative stress, toxin exposure, and heat and cold stress, thus protecting cells from the damage caused by various types of stress (Morrow et al., 2015; Zhao et al., 2016). The oxidative stress response caused by cold stress is often accompanied by high expression of HSPs. Zhao et al. (2013b) found that cold stress at 12°C caused an oxidative stress response in broiler hearts and significantly upregulated the expression of HSP27, HSP40, HSP60, and HSP70 in the hearts. Zhao et al. (2014) showed that cold stress at 12°C upregulated the expression levels of HSPs (HSP27, HSP40, HSP60, HSP70, and HSP90) in chicken immune organs. Mohanarao et al. (2014) found that the expression of HSP27 in peripheral blood monocytes of goats was upregulated after 3 h of cold stress in a cold environment of 10°C. Chen et al. (2014) found that HSP70 expression in the heart, liver, and muscle was significantly elevated after 5-wk-old Huainan ephedra chickens were placed in a 2°C environment for 72 to 144 h. In conclusion, the expression level of HSPs can be used as an indicator of the stress response in animals.

The negative effects of low temperature and other stressors on animals have been studied previously, but animals can adapt to the environment (Fu et al., 2022). Therefore, it is particularly important to explore ways to induce cold adaptation in animals and make them resistant to cold stress. It has been shown that long-term cold stimulation training under conditions 3°C lower than the normal temperature can establish cold adaptability in broilers and improve immune function and

cold resistance (Su et al., 2018; Wei et al., 2018). Therefore, to further compare the differences in adaptability and immune regulation induced by different cold stimulation schemes during intermittent mild cold stimulation (**IMCS**), we evaluated the expression levels of TLRs, cytokines, and HSPs in the intestine. Through IMCS training, the adverse effects of acute cold stress on broilers were alleviated.

MATERIALS AND METHODS

Animals and Study Design

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University, Harbin, China (protocol number: IACUCNEAU20150616). The experimental temperature conditions are shown in Figure 1.

Two hundred forty healthy 1-day-old Ross broilers were randomly divided into the cold stimulation 0 h group (**CS0** group), cold stimulation 3 h group (**CS3** group), and cold stimulation 6 h group (**CS6** group). The CS0 group was managed according to the standard feeding temperature during the growth stage of the broiler. A cold stimulation time that was 3°C lower than the feeding temperature for the CS0 group at 9:30 am to 12:30 am and 9:30 am to 15:30 pm was applied. Cold stimulation was conducted when the broilers were 15 to 35 d of age, and cold stimulation training was conducted every 2 d. From 36 to 49 d of age, the feeding temperature for the 3 groups of broilers was maintained at 20°C. At the age of 50 d, all groups were subjected to acute cold stress at 10°C for 6 h (8:00 am−14:00 pm).

Broilers were raised in 3 artificial climate houses with a feeding density of 9 broilers/m^2. For 1 to 14 d, the relative humidity was maintained at 60% to 70%, and for 15 to 50 d, the relative humidity was kept at 40% to 50%. Lighting conditions were 24 h of light: 0 h of dark (**24 L: 0 D**) on 1 to 3 d and 23 L: 1 D on 4 to 50 d, and the dark time was set at 20:00 to 21:00 every day. The light intensity was 40 lux for 0 to 14 d and 15 lux for 15 to 50 d. Chickens were provided free access to feed and water. Broilers were fed the complete starter diet (21.00% crude protein [**CP**], 12.10 megajoules [**MJ**]/kg metabolizable energy [**ME**]) for 1 to 21 d. Broilers were raised on a grower diet (19.00% CP, 12.60 MJ/kg of ME) for 22 to 50 d. Immunization procedures were performed according to the Ross broiler production standards.

Sample Collection

One broiler was randomly selected from 5 replicates per group on day 36 and before acute cold stress at 50 d of age for slaughtering at 08:00 am. After acute cold stress, the broilers were slaughtered at 14:00 pm. The chickens were fasted beginning at 20:00 the day before slaughter. The middle segments of the duodenum and jejunum were quickly collected, washed with precooled normal saline, temporarily stored in liquid nitrogen for quick freezing, and then uniformly stored at -80°C.

RNA Extraction and Gene Expression Analysis

Under the guidance of the manufacturer's instructions, TRIzol (Takara, Dalian, China) was used to extract total RNA from duodenum and jejunum broiler tissue samples. The dried RNA was dissolved in 50 μL diethypyrocarbonate water. The integrity of the RNA was detected via 1.5% agarose gel electrophoresis. The purity of RNA was determined by measuring the OD260/OD280 ratio with a UV spectrophotometer (Thermo Fisher Scientific, Carlsbad, CA). According to the instructions, cDNA was synthesized by reverse transcription of RNA with ReverTra Ace qPCR RT Master Mix and gDNA Remover (Toyobo, Osaka, Japan) and stored at -80°C.

The sequences of the primers used in the experiment are shown in Table 1. Primers were designed with Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA) and synthesized by biotechnology company (Sangon, Shanghai, China). A LightCycler 480II Real-Time PCR system (Roche, Basel, Switzerland) was used

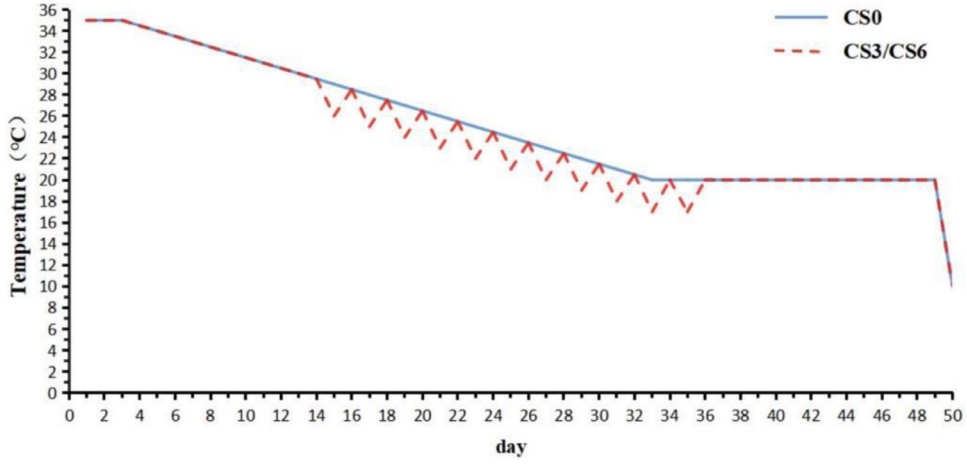

Figure 1. The specific experimental temperature scheme.

Table 1. The primers used in the experiment.

Gene	Serial number	Primer sequences (5'-3')
β-actin	NM_205518.1	F:CACCACAGCCGAGAGAGAAAT R:TGACCATCAGGGAGTTCATAGC
IFN γ	NM_205149.1	F:GAACTGGACAGGGAGAAATGAGA R:ACGCCATCAGGAAGGTTGTT
IL-2	NM_204153.1	F:CTGTATTTCGGTAGCAATG R:ACTCCTGGGTCTCAGTTG
IL-6	NM_204628.1	F:AAATCCCTCCTCGCCAATCT R:CCCTCACGGTCTTCTCCATAAA
IL-8	NM_205018.1	F:GGCTTGCTAGGGGAAATGA R:AGCTGACTCTGACTAGGAAACTGT
IL-17	NM_204460.1	F:GCCATTCCAGGTGCGTGAACTC R:CGGCGGAGGACGAGGATCTC
TLR2	XM_001232192294a	F:GATTGTGGACAACATCATTGACTC R:AGAGCTGCTTTCAAGTTTTCCC
TLR4	NM_001030693.1190a	F:AGTCTGAAATTGCTGAGCTCAAAT R:GCGACGTTAAGCCATGGAAG
TLR5	NM_001024586124a	F:CCTTGTGCTTTGAGGAACGAGA R:CACCCATCTTTGAGAAACTGCC
TLR7	NM_001011688219b	F:TTCTGGCCACAGATGTGACC R:CCTTCAACTTGGCAGTGCAG
TLR21	NM_001030558112a	F:TGCCCCTCCCACTGCTGTCCACT R:AAAGGTGCCTTGACATCCT
HSP27	NM_205290.1	F:ACACGAGGAGAAACAGGATGAG R:ACTGGATGGCTGGCTTGG
HSP40	NM_001199325.1	F:GGGCATTCAACAGCATAGA R:TTCACATCCCCAAGTTTAGG
HSP60	NM_001012916.2	F:AGCCAAAGGGCAGAAATG R:TACAGCAACAACCTGAAGACC
HSP70	NM_001006685.1	F:CGGGCAAGTTTGACCTAA R:TTGGCTCCCACCCTATCTCT
HSP90	NM_001109785.1	F:TCCTGTCCTGGCTTTAGTTT R:AGGTGGCATCTCCTCGGT
HSP110	NM_001159698.1	F:ATCCTAATGGAGTCCCGTAT R:CACCGACATCCTCACTATCT

for qPCR amplification. Each 10 μL reaction system contained 1 μL cDNA template, 0.3 μL each of forward primers and reverse primers, 5 μL THUNDERBIRD SYBR qPCR Mix (Toyobo) and 3.4 μL enzyme-free water. The qPCR amplification conditions involved 3 steps: predenaturation at 95°C for 60 s, repeated 40 cycles of denaturation at 95°C for 15 s, and extension at 60°C for 60 s. Each qPCR was analyzed with a melting curve. The relative mRNA expression levels of target genes were calculated using the $2^{-\Delta\Delta Ct}$ method, with the housekeeping gene β-actin as an internal reference.

Western Blot Analysis

Total protein was extracted from frozen broiler duodenum and jejunum tissues with western IP cell lysis buffer (SparkJade, Harbin, China) containing 1% PMSF (Biosharp, Beijing, China). A BCA protein concentration assay kit (SparkJade) was used to detect the protein concentration, which was adjusted to 4 μg/μL. An equal amount of total protein (32 μg/condition) was placed on a 12.5% gel (SparkJade) for SDS–PAGE. The proteins were transferred to an NC membrane (SparkJade) using semidry transfer equipment (Liuyi, Beijing, China). The membranes were blocked with 5% skim milk at 37°C for 2 h and cleaned with phosphate buffered solution + 0.05% Tween-20, 3 times, and then, specific primary antibodies against HSP40 (1:600, ABclone, Harbin, China), HSP60 (1:400, ABclone), HSP70 (1:1800, ABclone), and β-actin (1:9000, Zenbo, Chengdu, China) were incubated with the NC membranes at 4°C overnight. Then, horseradish peroxidase-labeled goat anti-rabbit IgG (1:9000, Zenbo) was incubated with the membranes. Finally, an enhanced chemiluminescence kit (SparkJade) was used to observe the protein bands with a grayscale scanner (GeneGnomeXRQ, Cambridge, UK). The bands were analyzed using ImageJ (NIH, Bethesda, MD). The relative expression levels of HSPs are expressed as the ratio of the gray value for each target protein to the gray value for β-actin.

Statistical Analysis

Data were analyzed using SPSS 21.0 (IBM, Amunk, NY). After assessment of a normal data distribution with a Kolmogorov–Smirnov test, the mRNA expression levels of cytokines (IL2, IL6, IL8, IL17, and IFN γ), TLRs (TLR2, TLR4, TLR5, TLR7, and TLR21), and HSPs (HSP27, HSP40, HSP60, HSP70, HSP90, and HSP110) and the protein expression levels of HSPs (HSP40, HSP60, and HSP70) in the duodenum and jejunum were compared by applying 1-way ANOVA, and multiple comparisons were conducted using Duncan's test. All results are expressed as the mean ± standard deviation. Differences were considered significant when P values were ≤ 0.05.

RESULTS

Effect of Intermittent Cold Stimulation on the mRNA Expression Levels of Cytokines

The effect of intermittent cold stimulation on the mRNA expression of cytokines in the duodenum and jejunum of broilers is shown in Figures 2 and 3.

In the duodenum, the mRNA expression levels of IL2, IL8, and IFN γ were significantly higher in the CS6 group than in the CS0 and CS3 groups at 36 d ($P < 0.05$). The expression level of IL17 mRNA was significantly lower in the CS6 group than in the CS3 group but significantly higher than that in the CS0 group ($P < 0.05$). At 50 d, compared with the CS0 group and CS3 group, the expression levels of IL2 and IL8 mRNA were significantly higher in the CS6 group ($P < 0.05$). The mRNA expression level of IL17 was significantly lower in the CS3 group than in the CS0 group but significantly higher than that in the CS6 group ($P < 0.05$). The mRNA expression level of IFN γ was significantly lower in the CS3 group than in the CS6 group but significantly higher than that in the CS0 group ($P < 0.05$). There was no significant difference in the expression of IL6 mRNA among the groups at 36 d and 50 d ($P > 0.05$). Compared with 36 d, the expression level of IL2 mRNA was significantly decreased in the CS0 group ($P < 0.05$), and the expression levels of IL6, IL8, and IL17 mRNA were significantly increased at 50 d ($P < 0.05$). The expression level of IL8 mRNA was increased significantly in the CS6 group ($P < 0.05$). The mRNA expression level of IL17 was significantly decreased in the CS3 and CS6 groups ($P < 0.05$). The mRNA expression level of IFN γ was significantly increased in the CS3 group ($P < 0.05$).

In the jejunum, compared with the CS0 group and CS3 group, the mRNA expression levels of IL2 and IFN γ were significantly higher in the CS6 group at 36 d ($P < 0.05$). The expression level of IL6 mRNA was significantly higher in the CS0 group than in the CS3 and CS6

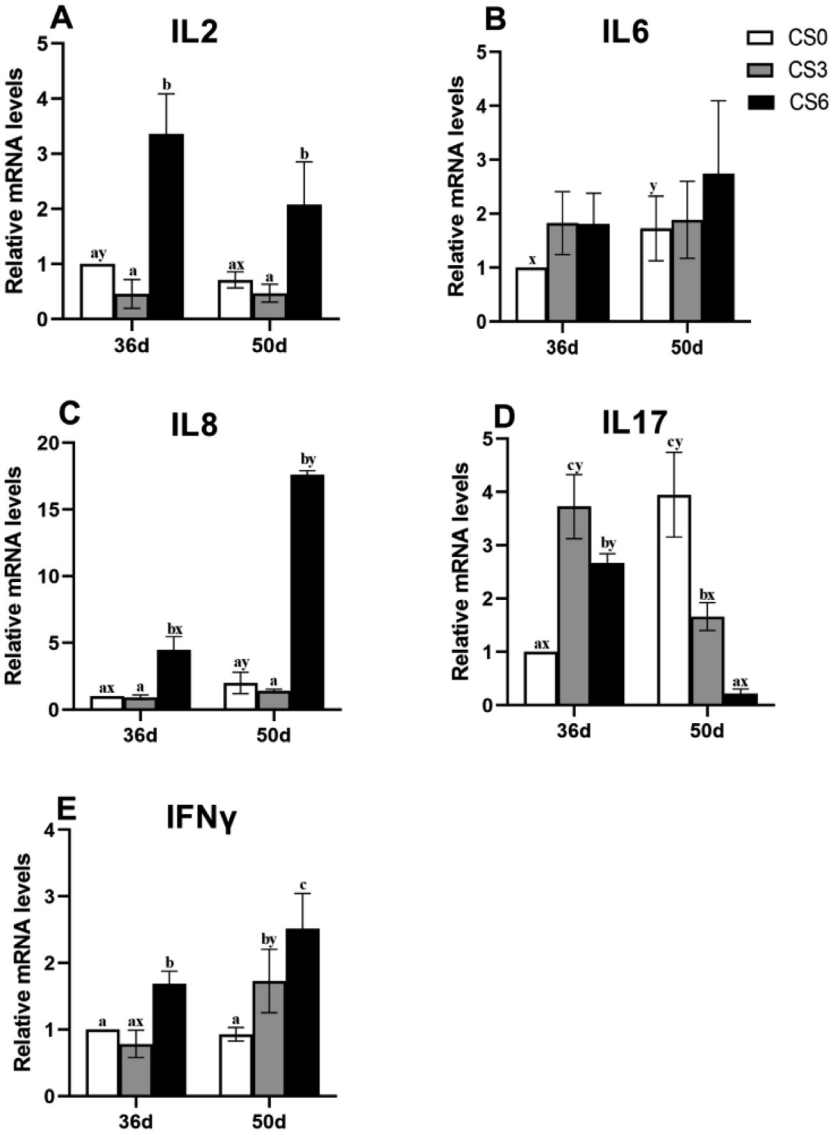

Figure 2. Effect of intermittent cold stimulation on cytokines mRNA expression in duodenum of broilers. Data were presented as mean ± standard deviation. (a-c) indicate significant difference between treatment groups ($P < 0.05$). x and y indicate significant difference 36 d and 50 d ($P < 0.05$).

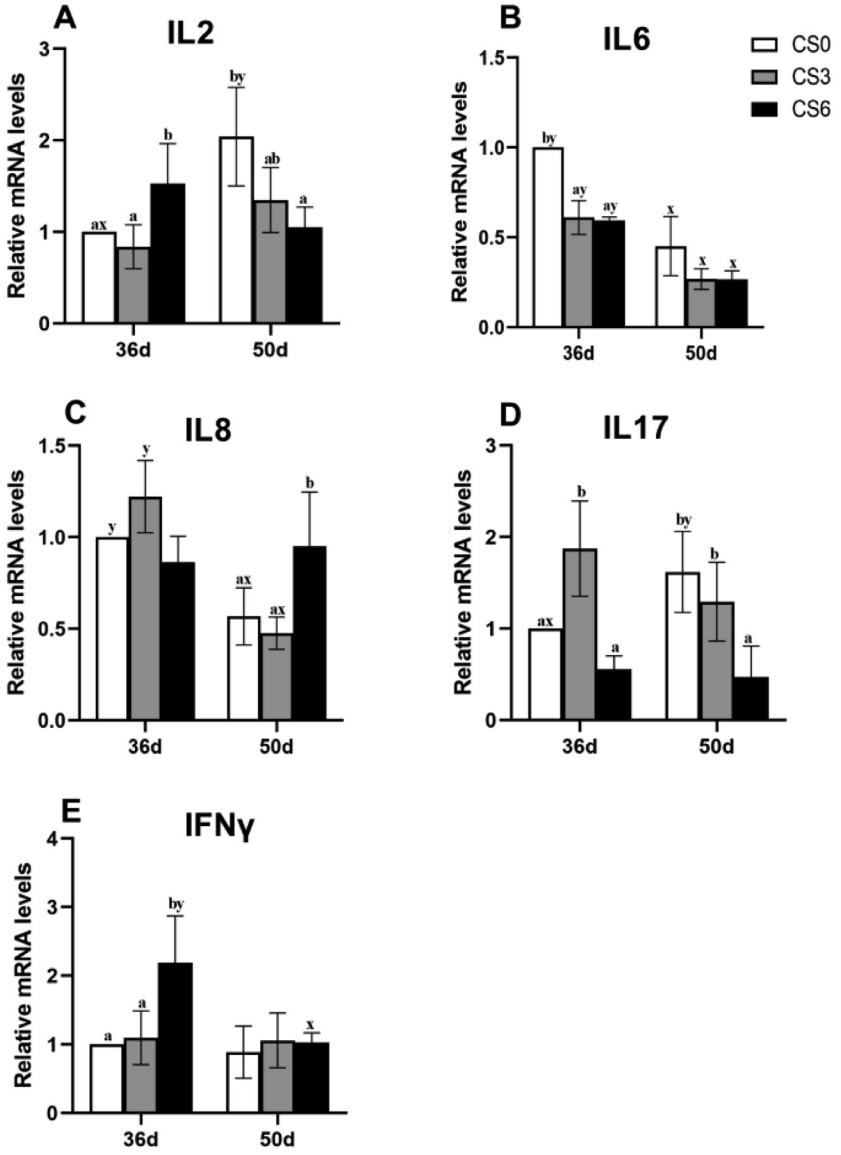

Figure 3. Effect of intermittent cold stimulation on cytokines mRNA expression in jejunum of broilers. Data were presented as mean ± standard deviation. (a-c) indicate significant difference between treatment groups ($P < 0.05$). x and y indicate significant difference 36 d and 50 d ($P < 0.05$).

groups ($P < 0.05$). The expression level of IL17 mRNA was significantly higher in the CS3 group than in the CS0 group and CS6 group ($P < 0.05$). At 50 d, the mRNA expression levels of IL2 and IL17 showed a downward trend with increasing cold stimulation time, and the levels in the CS0 group were significantly higher than those in the CS6 group ($P < 0.05$). The expression level of IL8 mRNA was significantly higher in the CS6 group than in the CS0 group and CS3 group ($P < 0.05$). The mRNA expression levels of IL6 and IFN γ were not significantly different among the groups ($P > 0.05$). Compared with 36 d, the mRNA expression levels of IL2 and IL17 were significantly increased at 50 d in the CS0 group ($P < 0.05$). The expression level of IL6 mRNA in each group was significantly decreased ($P < 0.05$). The IL8 mRNA expression level in the CS0 group and CS3 group and the IFN γ mRNA expression level in the CS6 group were significantly decreased ($P < 0.05$).

Effect of Intermittent Cold Stimulation on TLR mRNA Expression Levels

The effect of intermittent cold stimulation on the mRNA expression levels of TLRs in the duodenum and jejunum of broilers is shown in Figures 4 and 5.

In the duodenum, TLR2, TLR7, and TLR21 mRNA expression levels showed an increasing trend with increasing cold stimulation time at 36 d, and the expression levels in the CS6 group were significantly higher than those in the CS0 group ($P < 0.05$). The expression level of TLR4 mRNA was significantly higher in the CS3 group than in the CS0 and CS6 groups ($P < 0.05$). There was no significant difference in TLR5 mRNA expression among the groups at 36 d ($P > 0.05$). At 50 d, TLR2 mRNA expression was significantly higher in the CS6 group than in the CS0 group and CS3 group ($P < 0.05$). The expression levels

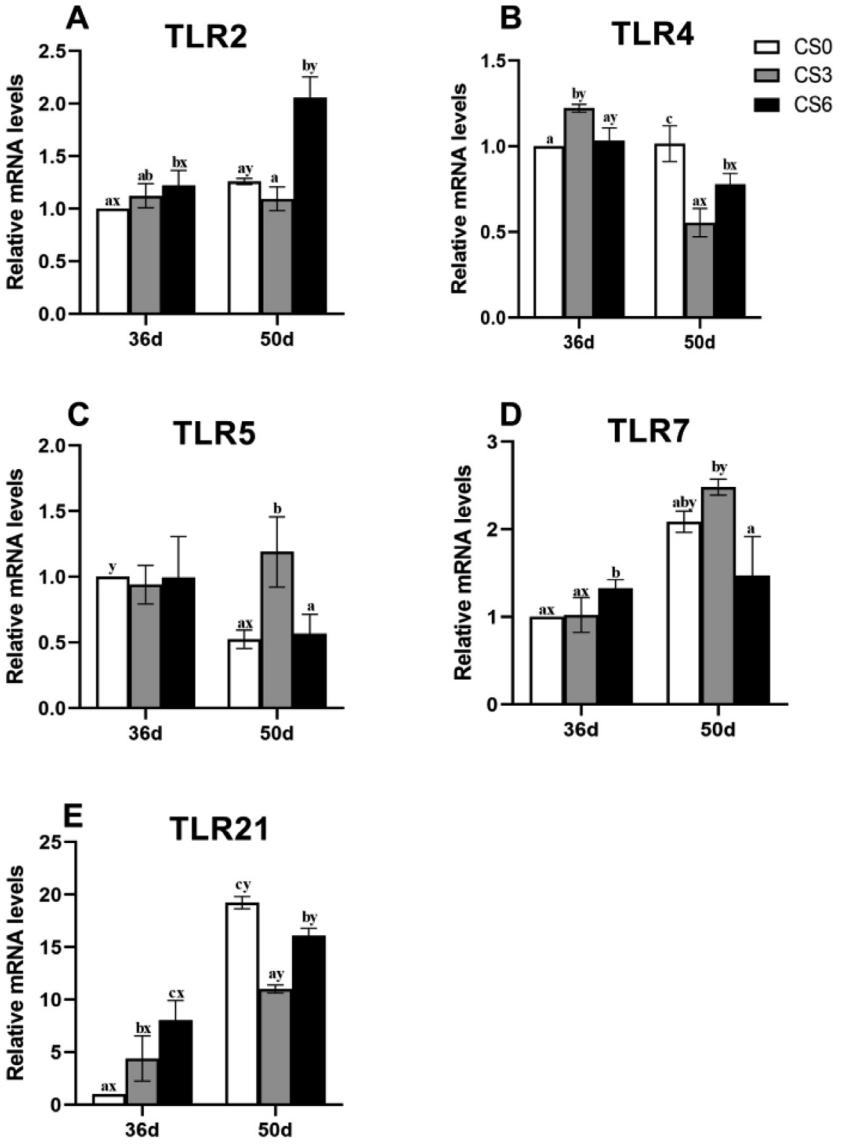

Figure 4. Effect of intermittent cold stimulation on Toll-like receptors (TLRs) mRNA expression in duodenum of broilers. Data were presented as mean ± standard deviation. (a-c) indicate significant difference between treatment groups ($P < 0.05$). x and y indicate significant difference 36 d and 50 d ($P < 0.05$).

of TLR4 and TLR21 mRNA were significantly lower in the CS3 group than in the CS0 and CS6 groups ($P < 0.05$), and levels in the CS0 group were significantly higher than those in the CS6 group ($P < 0.05$). Compared with the CS0 group and CS6 group, the expression level of TLR5 mRNA was significantly higher in the CS3 group ($P < 0.05$). The expression level of TLR7 mRNA was significantly higher in the CS3 group than in the CS6 group ($P < 0.05$). Compared with 36 d, at 50 d, TLR2 mRNA expression levels in the CS0 group and CS6 group, TLR7 mRNA expression levels in the CS0 group and CS3 group and TLR21 mRNA expression levels in all groups were significantly increased ($P < 0.05$). The TLR4 mRNA expression levels in the CS3 group and CS6 group and the TLR5 mRNA expression level in the CS0 group were significantly decreased ($P < 0.05$).

In the jejunum, the mRNA expression levels of TLR2, TLR4, TLR5, TLR7, and TLR21 were significantly lower in the CS3 group than in the CS0 group and CS6 group at 36 d ($P < 0.05$). The expression level of TLR5 mRNA was significantly higher in the CS0 group than in the CS6 group ($P < 0.05$). TLR7 and TLR21 mRNA expression levels were significantly higher in the CS6 group than in the CS0 group ($P < 0.05$). At 50 d, the expression levels of TLR4 and TLR7 mRNA increased with the time of cold stimulation, the expression level of TLR4 mRNA in the CS6 group was significantly higher than that in the CS0 group ($P < 0.05$), and the expression level of TLR7 mRNA was significantly higher in the CS6 group than in the CS0 group and CS3 group ($P < 0.05$). Compared with the CS0 group and CS6 group, the expression level of TLR21 mRNA was significantly higher in the CS3 group ($P < 0.05$). There was no significant difference in the expression level of TLR2 and TLR5 mRNA among the groups ($P > 0.05$). Compared with 36 d, the TLR2 mRNA expression level in the CS3 group was significantly increased at 50 d ($P < 0.05$). The

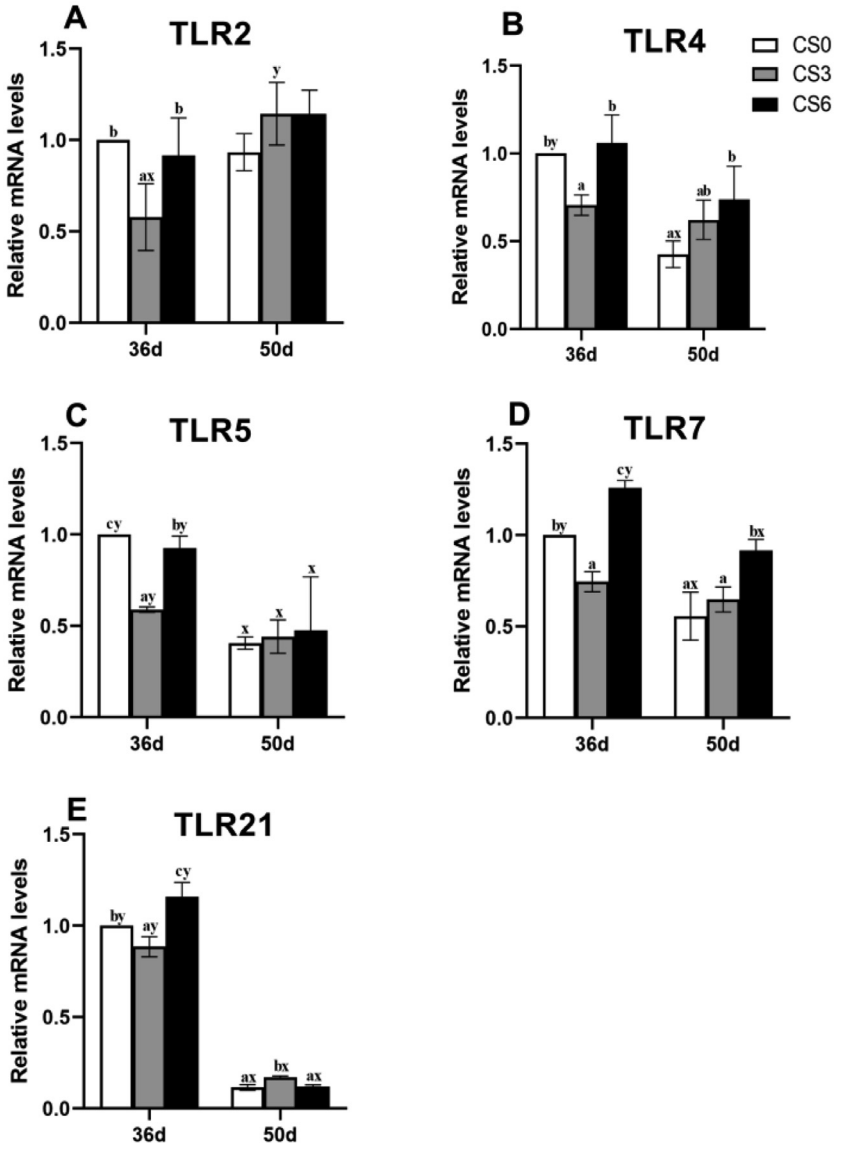

Figure 5. Effect of intermittent cold stimulation on Toll-like receptors (TLRs) mRNA expression in jejunum of broilers. Data were presented as mean ± standard deviation. (a-c) indicate significant difference between treatment groups ($P < 0.05$). x and y indicate significant difference 36 d and 50 d ($P < 0.05$).

expression levels of TLR4 in the CS0 group and TLR7 mRNA in the CS0 and CS6 groups were significantly decreased ($P < 0.05$). The expression levels of TLR5 and TLR21 mRNA in the 3 groups were significantly decreased ($P < 0.05$).

Effects of Acute Cold Stress on the mRNA Expression Levels of Cytokines

The effect of acute cold stress on the mRNA expression of cytokines in the duodenum and jejunum of broilers is shown in Figures 6 and 7.

In the duodenum, the mRNA expression levels of IL2 and IFN γ in Y6 were not significantly different between the groups ($P > 0.05$), and when compared with 50 d, the difference was still not significant ($P > 0.05$). In Y6, the mRNA expression levels of IL6, IL8, and IL17 were significantly higher in the CS3 group than in the CS0 group and CS6 group, but only IL8 and IL17 levels were significantly higher in the CS6 group than in the CS0 group ($P < 0.05$). Compared with 50 d, IL6 and IL8 mRNA expression levels were significantly decreased in the CS0 group and CS6 group after acute cold stress ($P < 0.05$), IL17 mRNA expression levels were significantly decreased in the CS0 group ($P < 0.05$), and IL17 mRNA expression levels were significantly increased in the CS3 group ($P < 0.05$).

In the jejunum, the IL2, IL6, IL8, and IL17 mRNA expression levels in the CS3 group after acute cold stress were significantly higher than those in the CS0 group and CS6 group ($P < 0.05$), but the IL2 mRNA expression level in the CS0 group was significantly higher than that in the CS6 group ($P < 0.05$), and the IL8 and IL17 mRNA expression levels in the CS6 group were significantly higher than that in the CS0 group ($P < 0.05$). The mRNA expression level of IFN γ was significantly higher in the CS6 group than in the CS3 group but significantly lower than that in the CS0 group ($P < 0.05$).

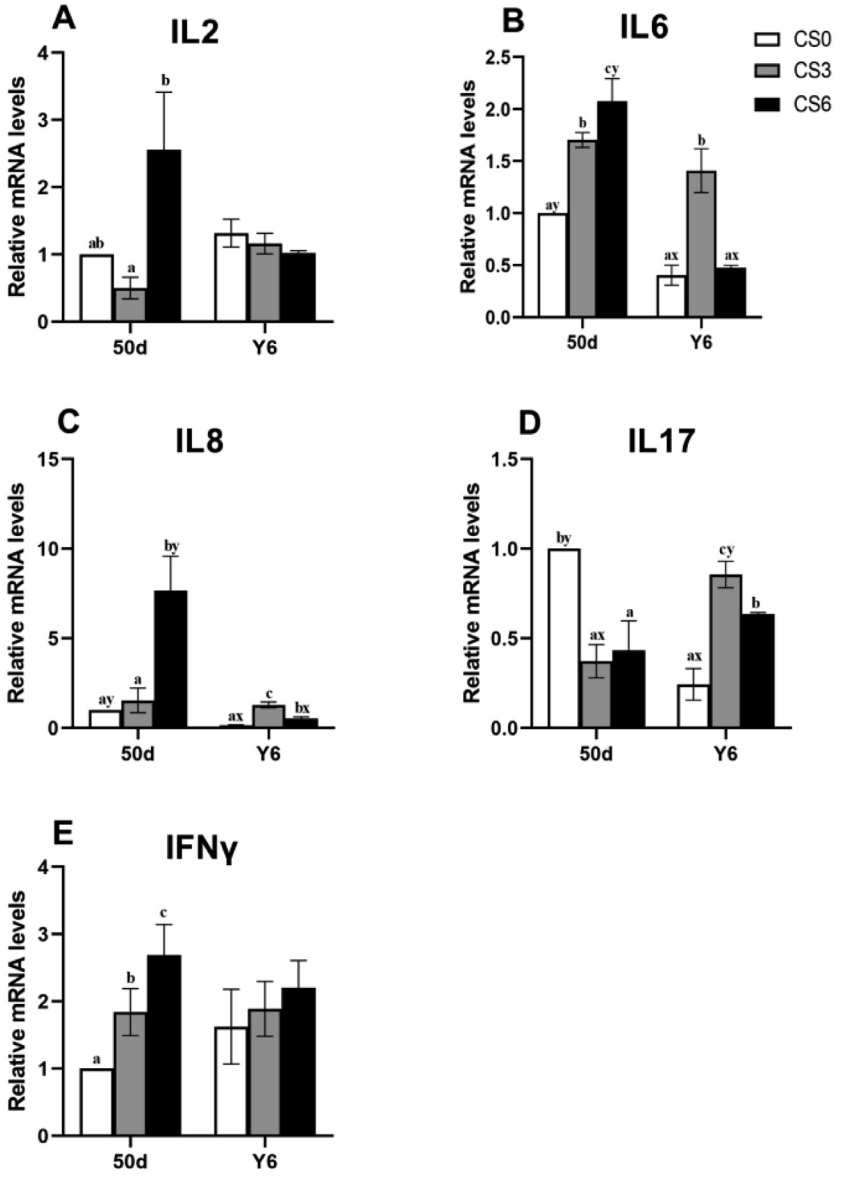

Figure 6. Effect of acute cold stress on cytokines mRNA expression in duodenum of broilers. Data were presented as mean ± standard deviation. (a-c) indicate significant difference between treatment groups ($P < 0.05$). x and y indicate significant difference 50 d and Y6 ($P < 0.05$).

Compared with 50 d, in Y6, IL2 and IL6 mRNA expression levels were significantly increased in the CS0 and CS3 groups ($P < 0.05$), and the IL8 mRNA expression level in the CS3 group and the IFN γ mRNA expression level in the CS0 and CS6 group were also significantly increased ($P < 0.05$). IL8 mRNA expression was significantly decreased in the CS0 and CS6 groups, and IL17 mRNA expression was significantly decreased in the CS0 and CS3 groups ($P < 0.05$).

Effects of Acute Cold Stress on TLR mRNA Expression Levels

The effect of acute cold stress on the mRNA expression of TLRs in the duodenum and jejunum of broilers is shown in Figures 8 and 9.

In the duodenum, TLR4 and TLR21 mRNA expression in the CS3 group in Y6 was significantly higher than that in the CS0 group and the CS6 group ($P < 0.05$). The mRNA expression level of TLR7 was significantly higher in the CS3 group than in the CS0 group ($P < 0.05$), but the difference was not significant compared with the CS6 group ($P > 0.05$). There was no significant difference in the expression of TLR2 and TLR5 mRNA among all groups in Y6 ($P > 0.05$). Compared with 50 d, the level of TLR4 mRNA expression was significantly reduced in the CS0 group and the CS6 group after acute cold stress ($P < 0.05$), the TLR7 and TLR21 mRNA expression levels were significantly reduced in the CS0 group ($P < 0.05$), and the TLR21 mRNA expression level was significantly increased in the CS3 group ($P < 0.05$).

In the jejunum, TLR2 mRNA expression levels after acute cold stress were inversely correlated with intermittent cold stimulation time, and the differences were significant among all groups ($P < 0.05$). The TLR5 mRNA expression level also decreased with

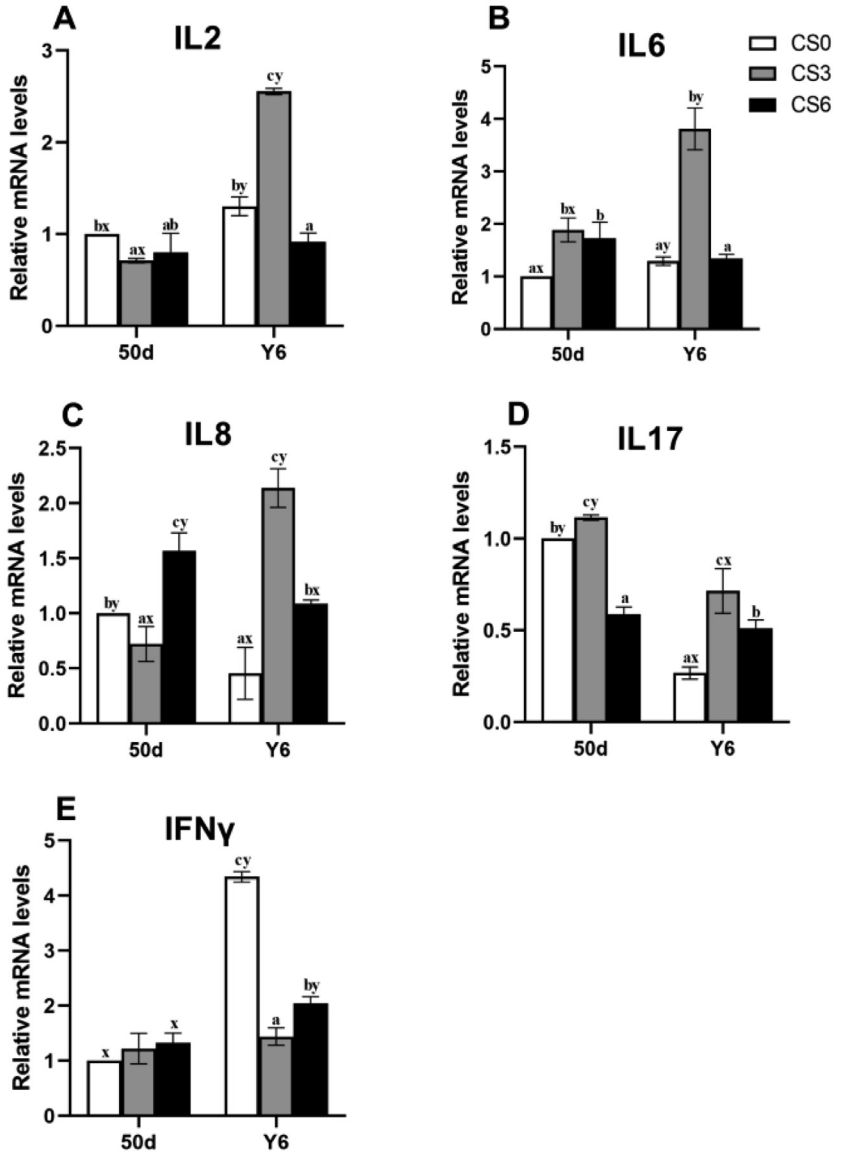

Figure 7. Effect of acute cold stress on cytokines mRNA expression in jejunum of broilers. Data were presented as mean ± standard deviation. (a-c) indicate significant difference between treatment groups ($P < 0.05$). x and y indicate significant difference 50 d and Y6 ($P < 0.05$).

increasing intermittent cold stimulation time, but only that in the CS6 group was significantly lower ($P < 0.05$). The level of TLR7 mRNA expression was significantly higher in the CS3 group than in the CS0 group ($P < 0.05$), and the level of TLR21 mRNA expression was significantly higher in the CS3 group than in the CS0 group and the CS6 group ($P < 0.05$). There was no significant difference in the expression of TLR4 mRNA among the groups ($P > 0.05$). Compared with 50 d, the TLR2 mRNA expression level was significantly reduced in the CS6 group after acute cold stress ($P < 0.05$). The TLR4 mRNA expression level was significantly decreased in each group ($P < 0.05$). The TLR2 and TLR5 mRNA expression levels were significantly increased in the CS0 group ($P < 0.05$). The TLR7 and TLR21 mRNA expression levels were significantly reduced in the CS0 group and CS6 group ($P < 0.05$).

Effects of Acute Cold Stress on Expression Levels of HSP mRNA

The effect of acute cold stress on the mRNA expression of HSPs in the duodenum and jejunum of broilers is shown in Figures 10 and 11.

In the duodenum, the mRNA expression levels of HSP27, HSP40, HSP70, and HSP110 at 50 d showed an increasing trend with increasing intermittent cold stimulation time, and the differences between groups were significant ($P < 0.05$). The HSP60 mRNA expression level was significantly higher in the CS6 group than in the CS0 group and the CS3 group ($P < 0.05$). The HSP90 mRNA expression level showed a trend of first increasing and then decreasing with increasing intermittent cold stimulation time, the differences between the groups were significant, and the expression level in the CS6 group was significantly higher than that in the CS0

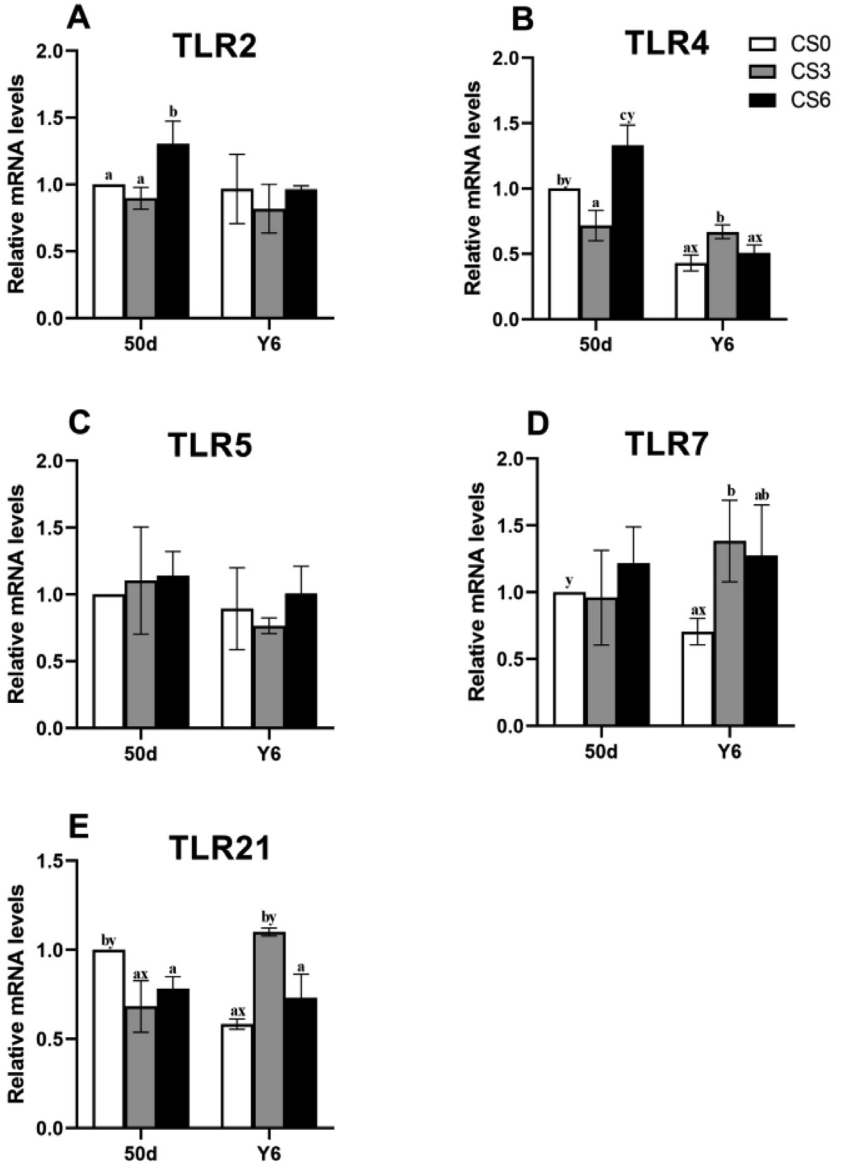

Figure 8. Effect of acute cold stress on Toll-like receptors (TLRs) mRNA expression in duodenum of broilers. Data were presented as mean ± standard deviation. (a-c) indicate significant difference between treatment groups ($P < 0.05$). x and y indicate significant difference 50 d and Y6 ($P < 0.05$).

group ($P < 0.05$). After acute cold stress, all HSPs showed a trend of first decreasing and then increasing with increasing intermittent cold stimulation time, but not all showed a significant difference trend. The expression level of HSP27 mRNA was significantly higher in the CS6 group than in the CS3 group but significantly lower than that in the CS0 group ($P < 0.05$). Compared with the CS0 group and CS3 group, the expression levels of HSP40 and HSP70 mRNA were significantly higher in the CS6 group ($P < 0.05$). The expression level of HSP60 mRNA was significantly lower in the CS3 group than in the CS0 group and the CS6 group ($P < 0.05$). The expression level of HSP90 mRNA was significantly higher in the CS0 group than in the CS3 group but significantly lower than that in the CS6 group ($P < 0.05$). The expression level of HSP110 mRNA was significantly higher in the CS0 group than in the CS3 group and the CS6 group ($P < 0.05$). There was no significant difference in the level of HSP mRNA expression between the other groups ($P > 0.05$). Compared with 50 d, the expression levels of HSP27, HSP40, HSP70, HSP90, and HSP110 mRNA after acute cold stress were significantly decreased in the CS3 and CS6 groups ($P < 0.05$). HSP60 mRNA expression levels were also significantly reduced in the CS3 group ($P < 0.05$). The HSP27, HSP40, HSP60, and HSP110 mRNA expression levels were significantly upregulated in the CS0 group ($P < 0.05$).

In the jejunum, the expression levels of HSP27, HSP90, and HSP110 mRNA at 50 d were significantly higher in the CS6 group than in the CS0 and CS3 groups ($P < 0.05$). The HSP40 mRNA expression level in the CS6 group was significantly higher than that in the CS3 group but significantly lower than that in the CS0 group ($P < 0.05$). The level of HSP70 mRNA expression was significantly higher in the CS6 group than in the CS0 group ($P < 0.05$). The expression level of HSP40 mRNA was significantly lower in the CS3 group than in the CS0 group, but the expression level of HSP110 mRNA was

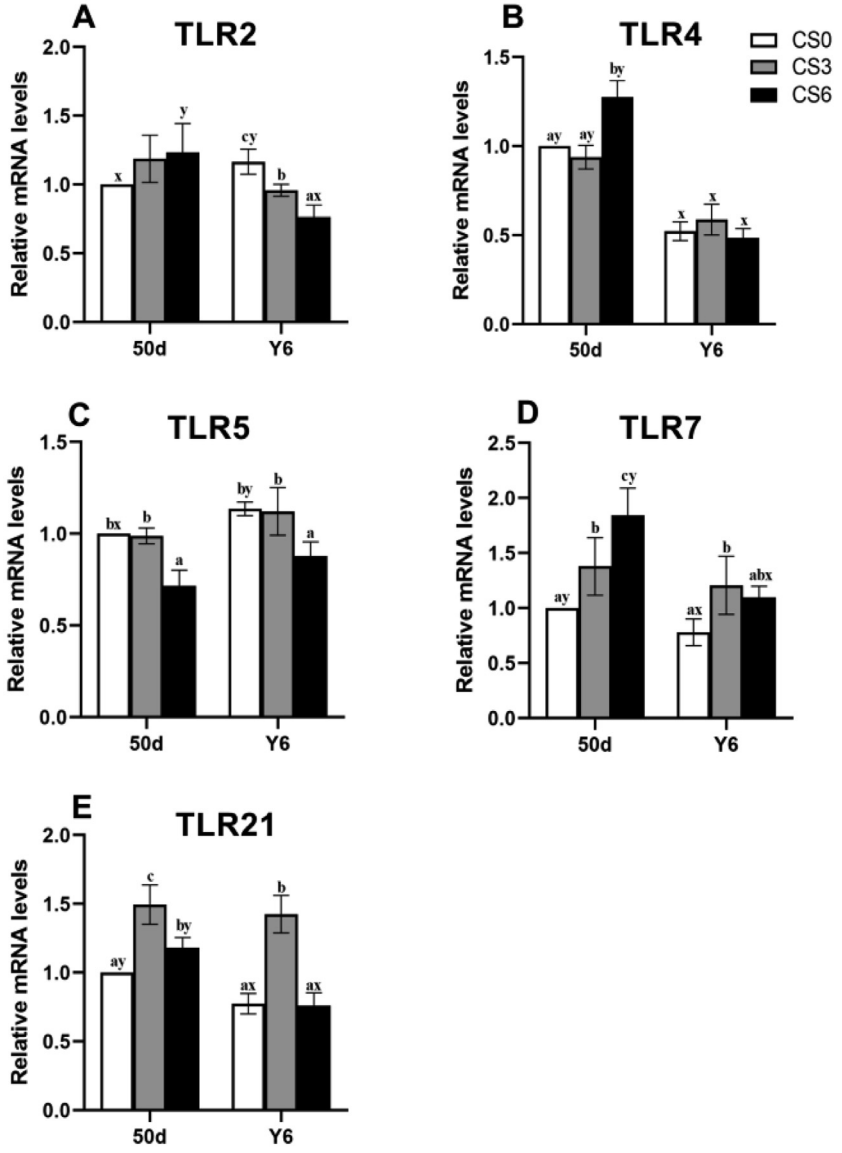

Figure 9. Effect of acute cold stress on Toll-like receptors (TLRs) mRNA expression in jejunum of broilers. Data were presented as mean ± standard deviation. (a-c) indicate significant difference between treatment groups ($P < 0.05$). x and y indicate significant difference 50 d and Y6 ($P < 0.05$).

significantly higher than that in the CS0 group ($P < 0.05$). There was no significant difference in the level of HSP mRNA expression among the other groups ($P > 0.05$). After acute cold stress, the levels of HSP40, HSP90, and HSP110 mRNA expression were significantly higher in the CS6 group than in the CS0 and CS3 groups ($P < 0.05$). The HSP60 and HSP70 mRNA expression levels showed a decreasing and increasing trend, respectively, with prolonged intermittent cold stimulation time, and the differences among the groups were significant ($P < 0.05$). The HSP mRNA expression levels were not significantly different between the remaining groups ($P > 0.05$). Compared with 50 d, HSP27 and HSP60 mRNA expression levels after acute cold stress were significantly elevated in the CS0 group ($P < 0.05$). The expression level of HSP40 mRNA was significantly reduced in the CS0 group but significantly elevated in the CS6 group ($P < 0.05$). The HSP70 and HSP110 mRNA expression levels were significantly decreased after acute cold stress in all groups ($P < 0.05$). The HSP90 mRNA expression level was significantly decreased in the CS0 and CS6 groups after acute cold stress ($P < 0.05$). The differences in HSP mRNA expression levels between the remaining groups before and after acute cold stress were not significant ($P > 0.05$).

Effects of Acute Cold Stress on Expression Levels of HSP Proteins

The effect of acute cold stress on the protein expression of HSPs in the duodenum and jejunum of broilers is shown in Figures 12 and 13.

In the duodenum, the HSP40 protein expression level at 50 d was significantly higher in the CS6 group than in the CS0 group and the CS3 group ($P < 0.05$). HSP60 showed a trend of first increasing and then decreasing with increasing cold stimulation time in each group,

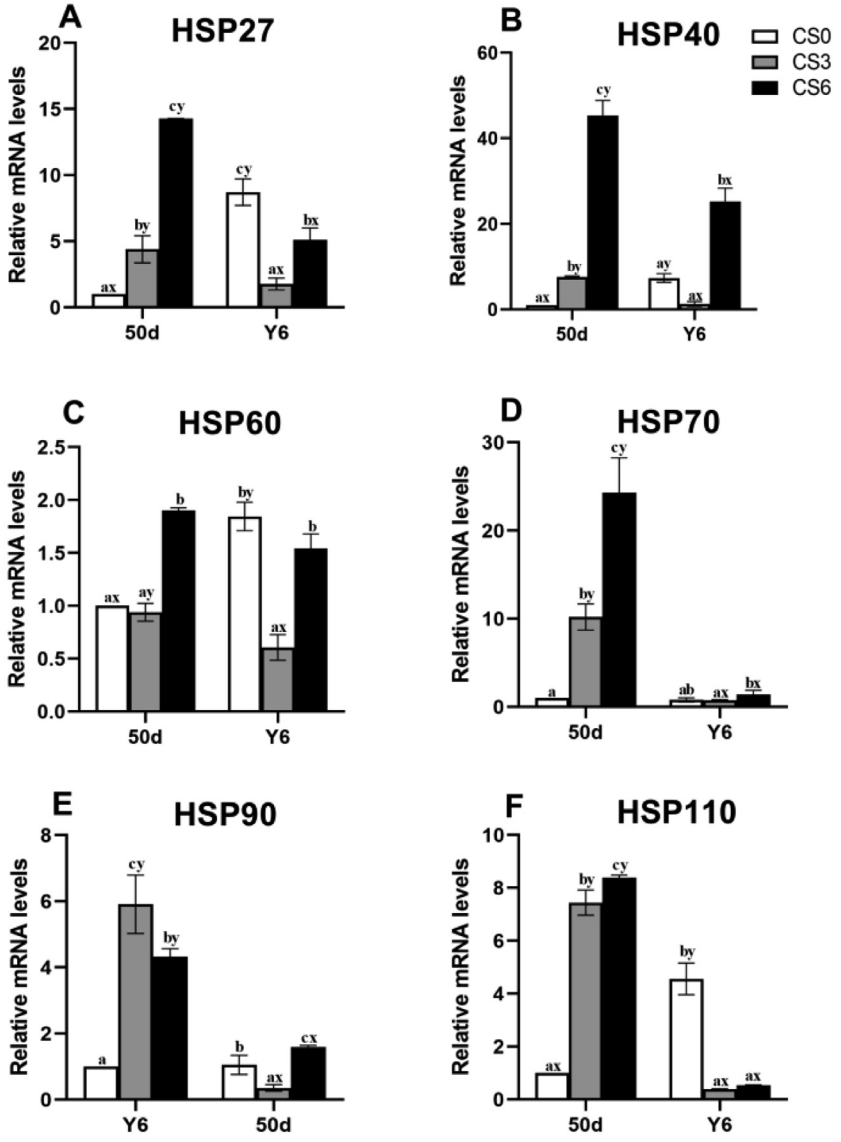

Figure 10. Effect of acute cold stress on heat shock protein (HSPs) mRNA expression in duodenum of broilers. Data were presented as mean ± standard deviation. (a-c) indicate significant difference between treatment groups ($P < 0.05$). x and y indicate significant difference 50 d and Y6 ($P < 0.05$).

while HSP70 showed an upward trend, and the differences among the groups were significant ($P < 0.05$). After acute cold stress, the HSP40 protein level was significantly lower in the CS3 group than in the CS0 group ($P < 0.05$). The expression level of HSP60 protein showed a trend of first increasing and then decreasing with prolonged cold stimulation time, and the expression level in the CS0 group was higher than that in the CS6 group, and the differences among the groups were significant ($P < 0.05$). The HSP70 protein expression level showed a downward trend with prolonged cold stimulation time, and the differences among groups were significant ($P < 0.05$). Compared with 50 d, HSP40 protein expression levels were significantly reduced in all groups after acute cold stress ($P < 0.05$). The level of HSP60 protein expression was significantly elevated in the CS0 and CS3 groups ($P < 0.05$) and significantly decreased in the CS6 group ($P < 0.05$). HSP70 protein expression levels were significantly elevated in the CS0 group ($P < 0.05$) and significantly decreased in the CS3 and CS6 groups ($P < 0.05$).

In the jejunum, the expression level of HSP40 protein at 50 d was significantly higher in the CS0 group than in the CS3 group ($P < 0.05$) but significantly lower than that in the CS6 group ($P < 0.05$). The level of HSP60 protein expression was significantly higher in the CS3 group than in the CS0 group and the CS6 group ($P < 0.05$). The HSP70 protein expression level was significantly lower in the CS3 group than in the CS0 group and the CS6 group ($P < 0.05$). After acute cold stress, HSP40, HSP60, and HSP70 expression levels all showed a tendency to first rise and then fall with prolonged cold stimulation time, and there were significant differences among the groups ($P < 0.05$). Compared with 50 d, the HSP40 and HSP70 protein expression levels in the CS3 group were significantly higher after acute cold stress ($P < 0.05$), while the HSP60 protein expression

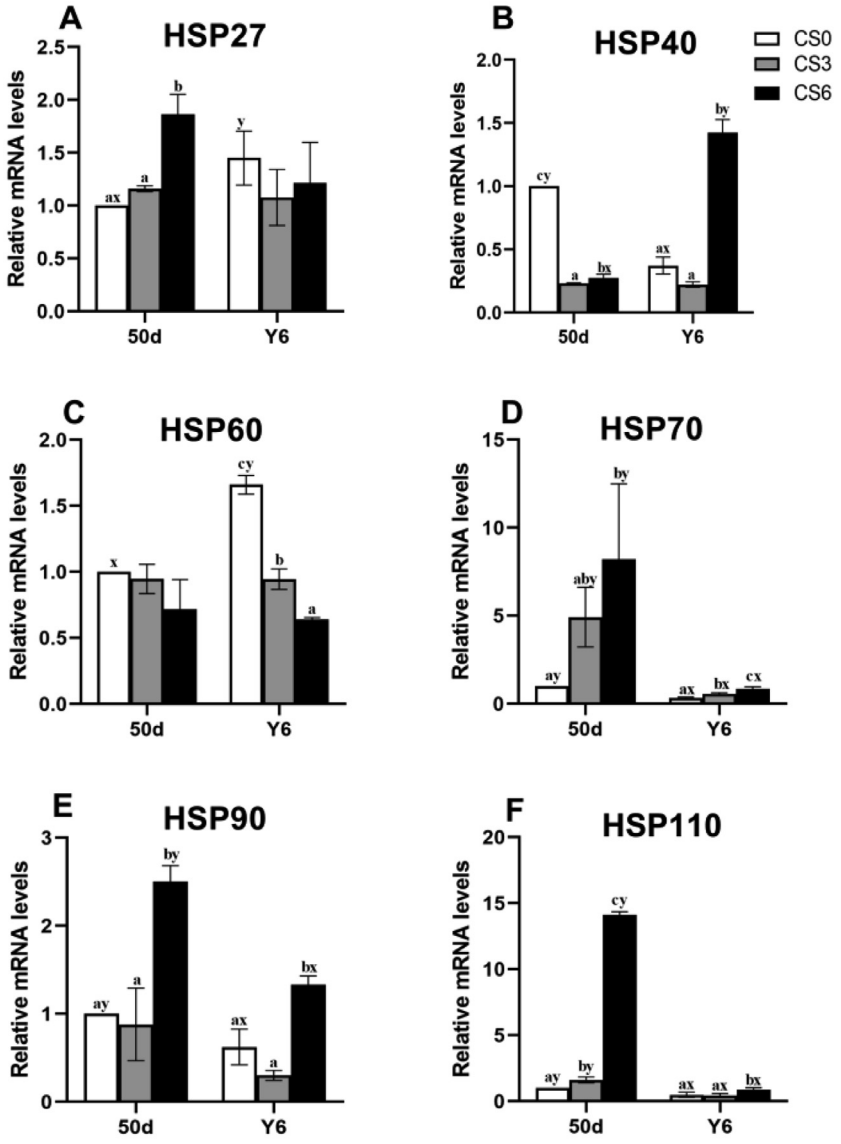

Figure 11. Effect of acute cold stress on heat shock protein (HSPs) mRNA expression in jejunum of broilers. Data were presented as mean ± standard deviation. (a-c) indicate significant difference between treatment groups ($P < 0.05$). x and y indicate significant difference 50 d and Y6 ($P < 0.05$).

level was significantly reduced ($P < 0.05$). The expression levels of HSP40 and HSP60 proteins were significantly decreased in the CS0 and CS6 groups ($P < 0.05$). The HSP70 protein expression level was significantly elevated in the CS6 group ($P < 0.05$).

DISCUSSION

Low temperature is an important factor affecting the development of animal husbandry, and sudden changes in ambient temperature can bring huge economic losses. It is generally believed that adverse environments, such as cold, have a negative impact on immune function (Olfati et al., 2018; Shah et al., 2020a,b), leading to immune imbalance and causing inflammation or inflammatory diseases (Zhao et al., 2013a; Hu et al., 2021). However, previous studies have demonstrated that appropriate cold stimulation training in the early growth stage of animals can help them adapt to cold environments and improve their immune function and anti-stress ability (Su et al., 2019; Liu et al., 2020). In previous research by our team, intermittent cold stimulation training during early development increased the immune capacity and cold tolerance of the bursa, spleen, and thymus of broilers (Wei et al., 2018; Su et al., 2019; Liu et al., 2020; Xue et al., 2021). This study revealed that early intermittent cold stimulation training (cold stimulation for 3 h at 3°C lower than the standard feeding temperature) regulates the immune function of the duodenum and jejunum of broilers by regulating the expression of cytokines and TLRs and instills good maintenance ability during the recovery period and after acute cold stress. Changes in HSP levels after acute cold stress confirm that intermittent cold stimulation improves the cold tolerance of broilers.

Cytokines play an important role in the inflammatory response and immune cell differentiation (Raquel et al.,

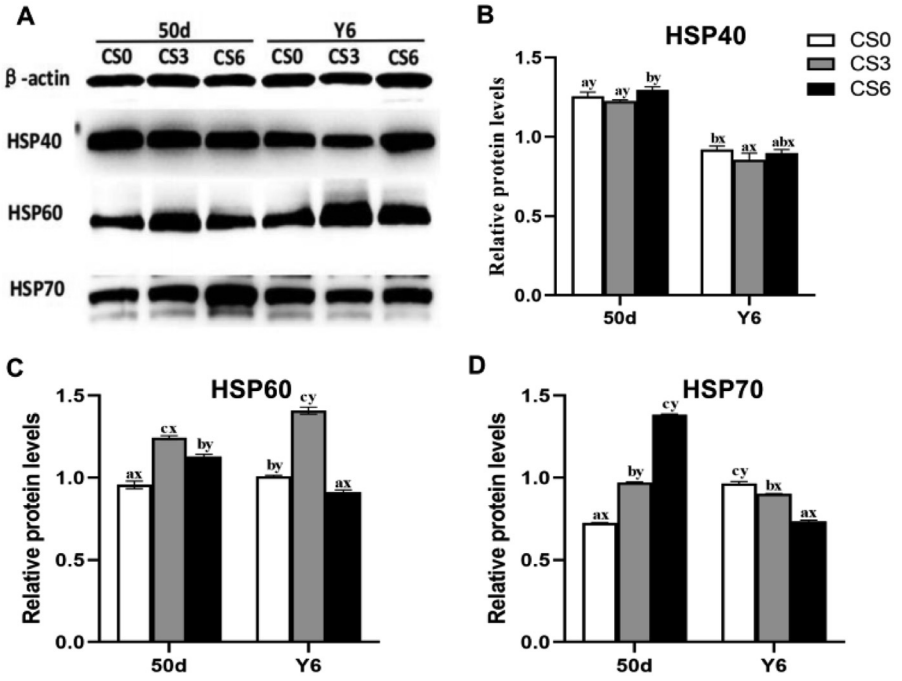

Figure 12. Effect of acute cold stress on heat shock protein (HSPs) protein expression in duodenum of broilers. Data were presented as mean ± standard deviation. (a-c) indicate significant difference between treatment groups ($P < 0.05$). x and y indicate significant difference 50 d and Y6 ($P < 0.05$).

2018). Th0 cells can differentiate into Th1 and Th2 cells when they are stimulated by antigen. IFN γ and IL2 secreted by Th1 cells mediate cellular immunity, while IL4 and IL10 secreted by Th2 cells mediate humoral immunity. An imbalance in Th1/Th2 cells is closely related to inflammation (Shieh et al., 2015). IL2, IL8, IL17, and IFN γ are important pro-inflammatory factors, IL4 is an anti-inflammatory factor, and IL6 has been reported to have pro-inflammatory and anti-inflammatory effects (You et al., 2011). Studies have found that cold stimulation can increase the expression of pro-inflammatory factor genes (Webel et al., 1997). This is similar to the results of our study; the IL2, IL8, IL17, and IFN γ mRNA expression levels in the

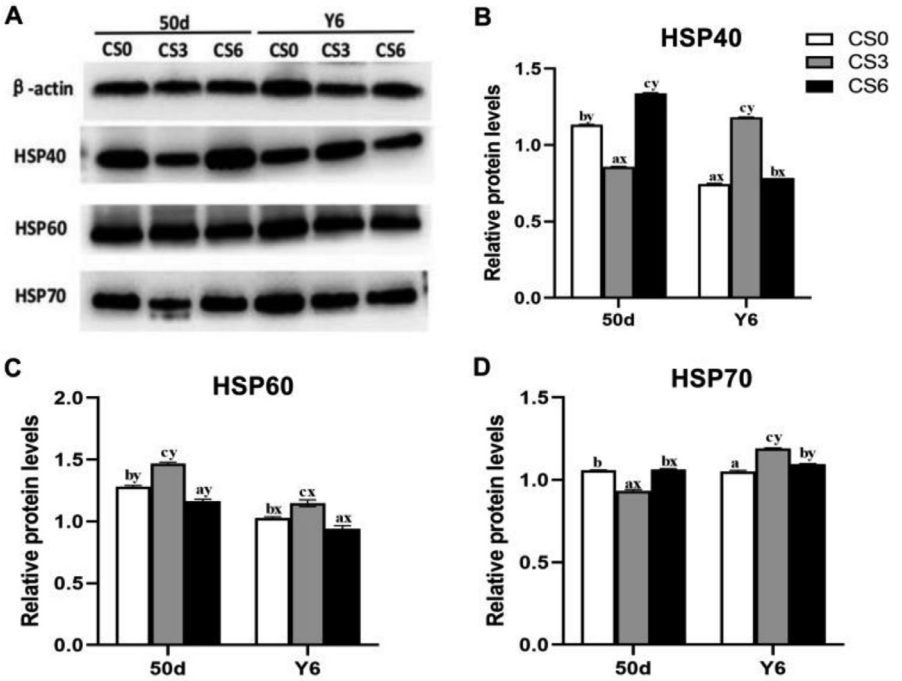

Figure 13. Effect of acute cold stress on heat shock protein (HSPs) protein expression in jejunum of broilers. Data were presented as mean ± standard deviation. (a-c) indicate significant difference between treatment groups ($P < 0.05$). x and y indicate significant difference 50 d and Y6 ($P < 0.05$).

duodenum were significantly higher in the CS6 group than in the CS0 group at 36 d. Thiel et al. (2017) pointed out that IFN γ release in rats increased during the first 1 to 2 d in a microgravity environment and decreased on the 12th d when the body adapted to the new environment. Similarly, in our study, except for IL17, the mRNA expression levels of other cytokines in the CS3 group were not significantly different from those in the CS0 group. This finding suggests that 6 h of cold stimulation induced an inflammatory response in broilers, while 3 h of cold stimulation induced cold adaptation at 36 d. This was further confirmed 2 wk after recovery from cold stimulation. At 50 d, the expression levels of some cytokines were significantly higher in the CS6 group than in the CS0 and CS3 groups. Hangalapura reported that cold stimulation can enhance Th1-mediated cellular immunity in chickens, thereby increasing the expression levels of IL2 and IFN γ (Hangalapura et al., 2003). This is consistent with our results showing that the mRNA expression levels of IL2 and IFN γ in the jejunum were significantly higher in the CS6 group than in the CS0 and CS3 groups at 36 d. Two weeks after recovery at the end of cold stimulation (50 d), no significant difference was observed between the CS3 group and CS0 group in any of the cytokine levels, while the mRNA expression level of IL2 was significantly lower in the CS6 group than in the CS0 group, and the mRNA expression level of IL8 was significantly higher than that in the CS0 group. This finding indicates that cold stimulation for 6 h can cause intestinal inflammation in broilers at 50 d. Su et al. (2018) showed that cold stimulation at 3°C below normal temperature effectively alleviated the increase in pro-inflammatory cytokines caused by subsequent cold stress in the broiler ileum, which was similar to our findings. After 6 h of acute cold stress, the duodenum of broilers in the CS0 and CS6 groups was affected by cold stress, and the mRNA expression levels of IL6 and IL8 were significantly decreased, while levels in the CS3 group remained stable. After acute cold stress, the mRNA expression levels of some cytokines in the jejunum were significantly higher in the CS3 group than in the CS0 and CS6 groups, which was different from the inflammation level in the duodenum, possibly because the jejunum is more sensitive to cold and can produce a stronger immune response during cold stress. Therefore, after cold training at 3°C for 3 h in the early stage, broilers can resist the short-term negative effects of cold stress on the body and adapt to the cold environment during acute cold stress.

The intestinal tract is constantly challenged by microbial pathogens. The recognition and binding of pathogen-related molecular pattern proteins by TLRs triggers a series of signaling cascades that activate related cytokines and play an important role in the innate immune response (Kawai et al., 2010; St Paul et al., 2013; Abdel-Mageed et al., 2014). Al-Zghoul et al. (2019) found that TLR4 mRNA expression was upregulated in the spleen and liver of broilers under acute heat stress (40°C for 7 h). Short-term acute heat stress at 38°C for 10 h significantly upregulated the expression of TLR4 mRNA in the spleen of broilers (Huang, 2017). Our study found that the TLR2, TLR7, and TLR21 mRNA expression levels in the duodenum were significantly higher in the CS6 group than in the CS0 group after intermittent cold stimulation. Liu et al. (2020) showed that after 21 d of IMCS, the expression level of most TLRs was lower in the cold stimulation group than in the control group. Previous studies by Quinteiro-Filho et al. (2017) showed that chronic thermal stimulation (10 h/d for 6 d) prior to sampling reduced TLR2 expression in the spleen of broilers. This is consistent with our findings that all TLR expression levels in the jejunum of broilers were significantly lower in the CS3 group than in the CS0 and CS6 groups after intermittent cold stimulation treatment. This suggests that the broilers may have adapted to this low-temperature environment; thus, the TLR expression level was low. A study by Zhou et al. (2014) showed that acute cold stress at -20°C caused neonatal mice to form necrotizing enteritis, which significantly increased the mRNA expression levels of TLR2 and TLR4. In our study, after 6 h of acute cold stress, the mRNA expression level of most TLRs in the duodenum in the CS3 group and CS6 group was not significantly different from that at 50 d, while that in the CS0 group decreased significantly. In the jejunum, most TLR mRNA expression levels in the CS3 group were not significantly different before and after acute cold stress, while those in the CS0 group and CS6 group decreased significantly after acute cold stress. This indicates that the body autoimmune regulation ability of broilers is stronger after the early 3 h of cold stimulation, and the ability to resist stress is also enhanced.

Heat shock proteins are a series of highly conserved molecular chaperones involved in the regulation of innate and adaptive immune responses (Srivastava et al., 2002). As typical markers of injury, HSPs can protect cells from various types of stress through their proliferation and differentiation (Zhao et al., 2016). Injury to various tissues and organs is often accompanied by high expression of HSPs (Khoso et al., 2015; Liu et al., 2015). Zhao et al. (2014) showed that chronic cold stress at 12°C led to oxidative stress in the thymus and spleen of broilers and significantly increased the expression levels of HSP27, HSP40, HSP60, HSP70, and HSP90 in immune organs. In the present study, at 50 d, the expression levels of most HSP mRNAs were significantly higher in the CS6 group than in the CS0 group and CS3 group in the duodenum and jejunum. These results indicate that the CS6 group still showed significantly higher levels of inflammation and intestinal damage than the CS0 and CS3 groups after 2 wk of recovery. Wei et al. (2018) demonstrated that 24 h of acute cold stress after 34 d of chronic cold stimulation (3°C lower than control) significantly upregulated HSP40 expression in the heart of broilers. In the present study, after 6 h of acute cold stress, HSP levels in the duodenum changed significantly in all groups. In the jejunum, the mRNA expression levels of almost all HSPs were significantly changed in the CS0 and CS6 groups compared with those before acute cold stress, while levels in the CS3 group were relatively

stable. This further verifies that the expression of HSPs caused by stress in animal tissues is highly tissue specific, as suggested by Hoekstra et al. (1998), and indicates that broilers have stronger cold resistance after long-term cold training at a lower temperature of 3°C for 3 h. At the protein level, the HSP60 expression level in the duodenum and HSP40, HSP60, and HSP70 expression levels in the jejunum were significantly higher in the CS3 group than in the CS0 and CS6 groups after acute cold stress. This is consistent with the results of Zeng et al. (2014), who showed that the gene expression level of HSP40 in the liver of ducks with different qualities was significantly upregulated after heat stress at 39°C/1 h to alleviate the damage caused by stress. This further confirms that cold training at a 3°C lower temperature induced strong cold resistance in broilers.

CONCLUSION

Early IMCS can regulate the function of immune organs by inducing the expression of cytokines, TLRs, and HSPs, thus enhancing immune ability. In this study, cold stimulation training at a temperature 3°C lower than the conventional feeding temperature enhanced the immune function of broilers and improved their adaptability to cold environments. Moreover, the 3 h intermittent cold stimulation program made broilers more resistant to cold stress. The results of this experiment not only provide a scientific basis for establishing cold adaptation technology in production but also provide a theoretical basis for regulation of the immune function of broilers in cold environments. In future research, we will explore the molecular mechanism of cold adaptation at the cellular level.

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DISCLOSURES

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

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