Effect of mild intermittent cold stimulation on thymus immune function in broilers

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ABSTRACT This study aims to assess the effect of intermittent and mild cold stimulation (**IMCS**) on thymus function and the ability of 1-day-old male Ross 308 broilers to withstand cold. Four hundred broilers were reared under normal and mild cold temperatures at 3°C below the normal feeding temperature and were subjected to acute cold stress (**ACS**) at 10°C on d 50 at 7 am for 6 h, 12 h, and 24 h. We determined the expression levels of toll-like receptors (**TLRs**), cytokines and avian β-defencins (**AvBDs**), encoding genes in thymus of broilers at 22, 36, 43, and 50 d of age, and the serum ACTH and cortisol (**CORT**) levels at 50 d of age. At D22 and D36, the mRNA expression levels of TLRs and AvBDs genes in CS groups were generally significantly decreased ($P < 0.05$). The lowest expression levels were found in birds submitted to intermittent and mild cold stimulation training for 5 h (**CS5 group**) on d 22 and 36 of development ($P < 0.05$). At D43 and D49 after IMCS, mRNA expression levels of most *TLRs* and *AvBDs* were significantly lower than those in CC group ($P < 0.05$), and that mRNA expression levels of all *TLRs* and most *AvBDs* in CS5 group had the same change trend with age as those in CC group ($P > 0.05$). At D22 and D36, mRNA expression levels of different cytokines in each CS groups were different ($P < 0.05$). mRNA expression levels of *IL-2, IL-4, IL-6, IL-8, IL-17,* and *IFN-α* all reached the highest values in the CS5 group at D36 ($P < 0.05$). The levels of ACTH and CORT in all IMCS-treated birds changed in varying degrees after ACS, but there was no significant change in CS5 group ($P > 0.05$). Collectively, different cold stimulation schemes could modulate thymus immune function of broilers by maintaining homeostasis and enhancing cold resistance. In particular, the optimal cold adaptation scheme was at 3°C below the conventional feeding temperature for 5 h.

Key words: broiler thymus, cold stimulation, immune regulation, hormone regulation, cold adaptation

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INTRODUCTION

The ultimate goal of modern commercial poultry farming is to improve the birds' ability to withstand environmental stress and disease incidence without increasing production costs. Low temperature 10 to 20°C below the conventional feeding temperature affects negatively the performance of broiler chickens, increasing morbidity and mortality in the northern alpine region (Hangalapura et al., 2004; Zhao et al., 2013). When the threshold of autoimmunomodulatory capacity is exceeded, stressors (including cold stimuli) can disrupt homeostasis (Zhao et al., 2013; Chen et al., 2020). Thus, enhancing immune capacity is important to withstand cold stress. Recent studies found that cold acclimation could reduce chilling injury in broilers by increasing antioxidant capacity and disease resistance (Tsiouris et al., 2015; Mohammed et al., 2019). In this context, cold training during early stages of development could improve adaptability in late stages of growth (Bukowiecki et al., 1986; Liu et al., 2020; Xue et al., 2021). Intermittent and mild cold stimulation (**IMCS**) has been suggested as an energy efficient strategy which could achieve the goal of enhancing immune capacity to withstand cold stress. In particular, it has been demonstrated that the use of a 21-d IMCS at feeding temperature, with 1-d or 6-h intervals, did not affect broiler's performance but improved antioxidant capacity (Wei et al., 2018; Su et al., 2019). Our previous experiment found that IMCS at 3°C below normal feeding temperature for 3 h, 4 h, 5 h, and 6 h for 21 d was beneficial to the development of bursal in fabricius, spleen and intestine of broilers, in addition 5 h was the optimal solution

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(Li et al., 2020 ; Liu et al., 2020; Xue et al., 2021). However, it is necessary to further study the spatiotemporal differences in the expression levels of immune-related genes in different organs.

Thymus, as the first lymphoid organ to appear in the development of organisms, accommodates crucial immunologic material related to nonspecific (nonadaptive) and specific (adaptive) immune responses (Reese et al., 2006). The expression of immune-related genes is an important indicator of the performance of immune responses and functions in the body. As sentinels of the immune system, Toll-like receptors (**TLRs)** sense specific microbial ligands and primarily participate in the activation of the adaptive immune response of vertebrate hosts (Ramasamy et al., 2011). In response to drastic temperature changes, cells maintain homeostasis by modulating the expression of TLRs genes (Li et al., 2020 ; Liu et al., 2020; Xue et al., 2021). In addition, TLRs triggers activation cascades to mediate inflammatory and immune responses by recognizing and responding to pathogen-associated molecular patterns (**PAMPs**) (Takeda et al., 2003; Kawai and Akira, 2010). Paul et al. (2015) found that the expression of genes encoding *TLR1-10* was unregulated by season in immune cells of goats submitted to hyperthermia, which led to reduced viral infection. In addition, Vandana et al. (2018) found that expression levels of genes encoding *TLR-1, TLR-4*, and *TLR-5* in mesenteric lymph nodes of goats were significantly decreased after continuous exposure to high temperature, while expression of *TLR-2* significantly increased.

Cytokines expressed rapidly induce a coordinated response of mRNA translation and turnover designed to protect cells against environmental stress-induced damage and assist homeostasis. In addition, cytokines act as key signaling molecules of immune cellular functions, such as directional differentiation and viability (Coondoo, 2011; Toribio-Fernandez et al., 2018). Studies have shown that secretion of interleukin-2 (**IL-2**) and interferon gamma (**IFN-γ**) promoted Th1 cell differentiation, while secretion of interleukin-4 (**IL-4**) facilitated Th2 cell differentiation (Toribio-Fernandez et al., 2018). In addition, interleukin-6 (**IL-6**) regulated Th lymphocyte differentiation by two independent mechanisms, that is, promoting Th1 cell differentiation and inhibiting Th2 cell differentiation (Mayer et al., 2014). Su et al. (2018) found that low temperature stimulation increased *IL-4* and *IL-6* expression levels and decreased *IFN-γ* release, resulting in Th1/Th2 immune cell dysregulation. Scientists have long known that cold stress could reduce cellular immunity in chickens (Regnier and Kelley, 1981,). Considering that the expression of immune-related genes in the thymus is generally reduced, it leads to T cell dysplasia, which ultimately reduces the body's ability to resist cold. Therefore, we suspect that, according to the principle of energy priority, the expression of immune-related genes may be selectively increased and decreased during IMCS to ensure energy balance and normal immunity.

Avian β-defensins (**AvBDs**) are antimicrobial peptides found in chickens, which participate in innate immunity and synergistic inflammatory response (Newcomer and CONNALLY, 1960; Cuperus et al., 2013; Lee et al., 2016; Su et al., 2019). AvBDs enhance body's defenses against pathogens by activating macrophages and promoting the onset of immune response by interacting with inflammatory factors (Newcomer and CONNALLY, 1960). Su et al. (2019) found that broilers submitted to low temperature had higher gene expression levels of *AvBD-7* and *AVBD-10*, which tended to stabilize in late stages of long-term cold stimulation training, suggesting that the organism has the propensity to enact a cold response.

In response to changes in living environment temperature, the body establishes homeostasis by coordinating the response of multiple organs, such as nerve, endocrine, and immune regulation (Li et al., 2017). As one of the major modulators of thermoregulatory systems in endotherms (including chickens), the hypothalamus-pituitary-adrenal axis (**HPA**) is the main regulator of stress response (Imaki et al., 1995). Therein, the levels of adrenocorticotropic hormone (**ACTH**) and cortisol (**CORT**) are important biochemical indicators of stress response in animals (Feng et al., 2021). ACTH has the fastest and strongest stress response, followed by CORT, and both have been reported to be associated with cold stress in mice, chickens and humans (Meltzer, 1983). By redistributing body energy and nutrients to critical and noncritical living biological systems, ACTH and CORT maintain basal and stress-related homeostasis (Feng et al., 2021).

Previous studies showed that continuous cold stimulation of broilers at 3°C below normal temperature could improve immune function and the ability to withstand cold stress at late stages of growth (Li et al., 2017). Therefore, in order to further compare the differences in homeostasis, adaptability and immune regulation of different cold stimulation schemes during IMCS, we evaluated the expression of genes encoding TLRs, cytokines and AvBDs in the thymus as well as the serum levels of ACTH and CORT of broilers.

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). Experimental temperature conditions are shown in Figure 1.

Four hundred one-day-old male Ross 308 broiler chickens were reared for 14 days in normal temperature conditions, 35°C from d 1 to 3, then the temperature was lowered by 0.5°C each day from d 4 to 14. At d 15, all broilers were randomly allocated in 5 experimental

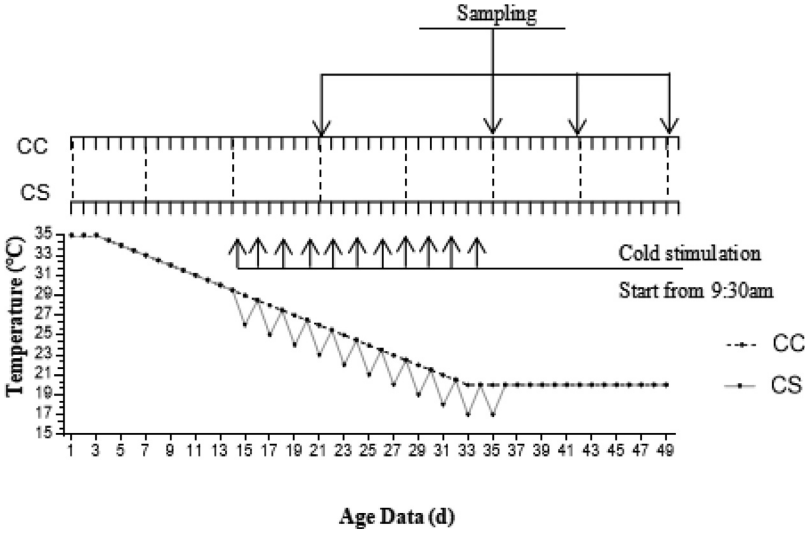

Figure 1. The temperature control in the experiment.

groups, with 16 birds per cage and 5 cages per experimental group. From d 15 to 35, animals in the CC group were maintained under normal temperature, while animals in the CS groups were maintained under 3°C below the conventional feeding temperature every other day (**IMCS**) at 09:30 am for different lengths of time respectively, 3 h (**CS3**), 4 h (**CS4**), 5 h (**CS5**), and 6 h (**CS6**). From d 36 to 49, both CC and CS groups were reared at 20°C. On d 50, all broilers were subjected to acute cold stress (**ACS**) at 10°C at 7 am for 6 h (**ACS6**), 12 h (**ACS12**), 24 h (**ACS24**).

Broilers in each group were housed in metal cages (180 × 60 × 80 cm), managed and immunized strictly in accordance with the approved guidelines. Birds had free access to water and a commercial diet (Baishicheng Animal Husbandry, Harbin, China) which was divided as early diet (from d 1 until d 21; 19.00% of crude protein [**CP**], 12.80 MJ/kg of metabolizable energy [**ME**]) and late diet (from d 22 until d 50; 17.50% of CP, 13.20 MJ/kg of ME). A continuous lighting program with incandescent light was used to illuminate poultry houses for 23 h in the first 3 d, and then for 12 h a day, from 7 am to 19 pm. All broilers were vaccinated according to the routine immunization program against the Newcastle disease virus (**NDV**) and avian influenza (**AI**) subtype H9 with inactivated vaccine by subcutaneous injection.

Sample Collection

One specimen from each replicate group was collected at 8 am on d 22 (**D22**), 36 (**D36**), 43 (**D43**), 49 (**D49**), and on d 50 after 6 h (ACS6), 12 h (ACS12), 24 h (ACS24) after acute cold stress. Birds were slaughtered by cervical dislocation. Thymus tissues were immediately placed into RNase-free Eppendorf tubes (Takara Biomedical Technology, Dalian, China), frozen in liquid nitrogen, and stored at −80°C. All Blood samples were collected in heparin-coated tubes and centrifuged at 2,000 × g at 4 for 15 min to separate plasma and then were stored at −80°C until further analysis.

RNA Extraction and Gene Expression Analysis

Thymus tissue was mixed with liquid nitrogen, and total RNA was isolated using RNAiso Plus kit (Takara Biomedical Technology, Dalian, China) following the manufacturer's instructions. Quality of extracted RNA was detected using ultra-microspectrophotometer GeneQuant 1300/100 (Boston, MA). Finally, complementary DNA (**cDNA**) was reverse-transcribed using the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). The experiment was carried out following the manufacturer's instructions using RNAse-free equipment. Obtained cDNA was stored at −80°C and then diluted before being used for qRT-PCR.

Primer sequences used in the experiments are shown in Table 1, and were designed with Primer Premier version 5.0 (PREMIER Biosoft International, Palo Alto, CA) and synthesized by Biotechnology Corporation (Sangon, Shanghai, China). qPCR amplifications were performed in a LightCycler 480 II Real-Time PCR System (Roche, Basel, Switzerland). A 10-μL reaction contained: 1 μL of diluted cDNA, 0.3 μL of each primer, 5 μL of THUNDERBIRD SYBR qPCR Mix (Toyobo) and 3.4 μL of PCR-grade water. PCR amplification conditions followed a 3-step method: degeneration at 90°C for 60 s; followed by 40 cycles of amplification at 95°C for 15 s, and annealing at 60°C. Melting curve analysis was employed in each PCR run. The PCR reactions were performed in triplicate, and the threshold cycle (**Ct**) value used in subsequent calculations was the mean of the values from three reactions. β-actin was used as the internal reference gene. Relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

Table 1. The primers used in the experiment.

Gene	Reference sequence	Primer sequences (5'-3')
TLR-1	NM_001081709	Forward:AGTCCATCTTTGTGTTGTCGCC
		Reverse: ATTGGCTCCAGCAAGATCAGG
TLR-2	XM_001232192	Forward: GATTGTGGACAACATCATTGACTC
		Reverse: AGAGCTGCTTTCAAGTTTTCCC
TLR-3	NM_001011691	Forward: TCAGTACATTTGTAACACCCCGCC
		Reverse: GGCGTCATAATCAAACACTCC
TLR-4	NM_001030693.1	Forward: AGTCTGAAATTGCTGAGCTCAAAT
		Reverse: GCGACGTTAAGCCATGGAAG
TLR-5	NM_001024586	Forward: CCTTGTGCTTTGAGGAACGAGA
		Reverse: CACCCATCTTTGAGAAACTGCC
TLR-7	NM_001011688	Forward: TTCTGGCCACAGATGTGACC
		Reverse: CCTTCAACTTGGCAGTGCAG
TLR-15	NM_001037835	Forward:GTTCTCTCTCCCAGTTTTGTAAATAGC
		Reverse:GTGGTTCATTGGTTGTTTTTAGGAC
TLR-21	NM_001030558	Forward: TGCCCCTCCCACTGCTGTCCACT
		Reverse: AAAGGTGCCTTGACATCCT
IL-2	NM_204153.1	Forward: CTGTATTTCGGTAGCAATG
		Reverse: ACTCCTGGGTCTCAGTTG
IL-4	NM_001007079.1	Forward: GTGCCCACGCTGTGCTTAC
		Reverse: AGGAAACCTCTCCCTGGATGTC
IL-6	NM_204628.1	Forward: AAATCCCTCCTCGCCAATCT
		Reverse: AGCTGACTCTGACTAGGAAACTGT
IL-17	AJ493595.1	Forward: GCCATTCCAGGTGCGTGAACTC
		Reverse: CGGCGGAGGACGAGGATCTC
IFN γ	NM_205149.1	Forward: GAACTGGACAGGGAGAAATGAGA
		Reverse: ACGCCATCAGGAAGGTTGTT
IFN α	XM_004937097.1	Forward: GGACATGGCTCCCACACTAC
		Reverse: GGCTGCTGAGGATTTTGAAGA
IFN β	NM_001024836.1	Forward: CACCACCACCTTCTCCT
		Reverse: TGTGCGGTCAATCCAGT
AvBD-2	NM_204992	Forward: GGTTGTCTTCGCCCCGGCGGGA
		Reverse: TTATGCATTCCAAGGCCATTTG
AvBD-4	NM_001001610	Forward: TCATCGTGCTCCTCTTTGTG
		Reverse: AATACTTGGGACGGCATAGC
AvBD-5	NM_001001608	Forward: GCTGTCCCTTGCTCGAGGATT
		Reverse: GGAATACCATCGGCTCCGGC
AvBD-7	NM_001001194	Forward: ACCTGCTGCTGTCTGTCCTC
		Reverse: TGCACAGCAAGAGCCTATT
AvBD-8	NM_001001781	Forward: TTCTCCTCACTGTGCTCCAA
		Reverse: AAGGCTCTGGTATGGAGGTG
AvBD-10	NM_001001609	Forward: GGCTCAGCAGACCCACTTTTCC
		Reverse: CTGCGCCGGAATCTTGGCAC
β-actin	NM_205518.1	Forward: CACCACAGCCGAGAGAGAAAT
		Reverse: TGACCATCAGGGAGTTCATAGC

Determination of Serum Levels of ACTH and CORT

Serum levels of ACTH and CORT were determined using a commercial ELISA kit for chicken (Auwiesen, Beijing, China) following the manufacturer's instructions. Serum was 5-fold diluted for ACTH content determination and 10-fold diluted for CORT content determination with assay buffer. The detection range of CORT and ACTH was 2.81 to 180 and 2.81 to 120 ng/mL respectively and the assay sensitivity for both was 1.688 ng/mL.

Statistical Analysis

Data were analyzed using SPSS version 21.0 (IBM Corp, Armonk, NY). After assessing data normal distribution with Kolmogorov-Smirnov test, to analyze and compare the effects of different age and duration of cold stimulation, mRNA expression of tls (*TLR-1, TRL-2, TRL-3, TRL-4, TRL-5, TRL-7, TRL-15*, and *TRL-21*), *AvBDs* (*AvBD-2, AvBD-4, AvBD-5, AvBD-7, AvBD-8, AvBD-10*), and cytokines (*IL-2, IL-4, IL-6, IL-8, IL-17, IFN-α, IFN-β*, and *IFN-γ*) genes in the thymus were analyzed by two-factor and multiple comparisons with Duncan's test. All results are expressed by mean ± standard deviation. Differences were considered significant when P values were < 0.05.

RESULTS

Effect of Intermittent Cold Stimulation on mRNA Levels of tls

mRNA levels of *TLR-1, TRL-2, TRL-3, TRL-4, TRL-5, TRL-7, TRL-15*, and *TRL-21* in the thymus of broilers are shown in Figure 2. At D22 and D36 during IMCS, most CS groups have demonstrated the lowest values of the mRNA expression levels of tls ($P < 0.05$) except a significant increase of expression levels of *TLR-3* and *TLR-21* were observed in CS4, CS5, CS6 at D36 ($P < 0.05$). At D43 and D49 after IMCS, most CS groups have the same change trend of tls mRNA expression level with age as those in CC group ($P > 0.05$), except *TLR-3* in CS4, *TLR-3*, and *TLR-4* of CS6 ($P < 0.05$). mRNA expression levels of *TLR-15* in CS3, *TLR-1, TLR-2, TLR-7*, and *TLR-15* in CS5, *TLR-2, TLR-4, TLR-7*, and *TLR-15* in CS6 were observably lower than those in CC group ($P < 0.05$). Collectively, these results suggested that IMCS could downregulate expression of mRNAs encoding for tls to re-establish homeostasis. In particular, the CS5 cold stimulation scheme could restore homeostasis steadily most efficiently.

Effect of Intermittent Cold Stimulation on mRNA Levels of AvBDs

Expression levels of *AvBD-2, AvBD-4, AvBD-5, AvBD-7, AvBD-8, AvBD-10* in the thymus of broilers are shown in Figure 3. At D22 and D36 during IMCS, mRNA expression of *AvBD*s in CS4 and CS5 groups decreased significantly ($P < 0.05$). In addition to the *AvBD-2* of CS4, CS5, and CS6 groups, the expression level of *AvBD-8* of CS4 was significantly increased at D36 ($P < 0.05$). At D43 and D49 after IMCS, mRNA expression of *AvBD-7* in CS groups was significantly lower than that in CC group ($P < 0.05$), but the change trend with age was the same as CC group ($P > 0.05$).

Effect of Intermittent Cold Stimulation on Expression Levels of Cytokines

mRNA levels of cytokines (*IL-2, IL-4, IL-6, IL-8, IL-17, IFN-α, IFN-β*, and *IFN-γ*) in the thymus of broilers are shown in Figure 4. At D22 and D36 during IMCS, mRNA expression levels of *IL-4, IFN-α, IFN-β*, and *IFN-γ* in all CS groups at D22 were significantly lower than those in CC group ($P <0.05$), while the expression levels of *IL-2* at D22 (except CS4) and D36 were significantly higher ($P < 0.05$). At D36, the mRNA expression

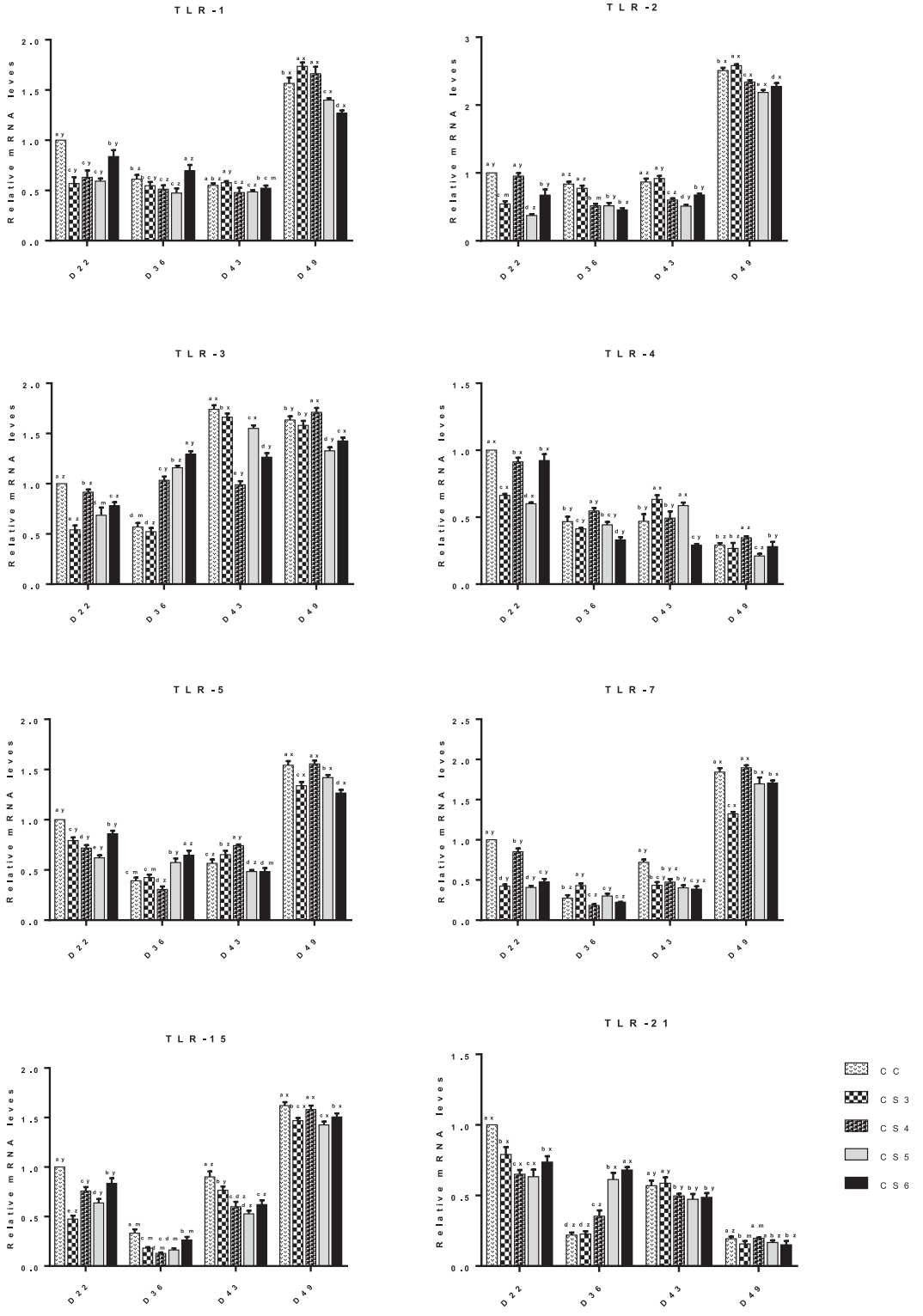

Figure 2. mRNA levels of TLR-1, TLR-2, TLR-3, TLR-4, TLR-5, TLR-7, TLR-15 and TLR-21 in the thymus of broilers. Different letters indicate significant differences ($P < 0.05$) between treatment groups (a, b, c) and days of age (x, y, z).

levels of *IL-2, IL-4, IL-6, IL-8, IL-17*, and *IFN-α* in CS5 group were significantly higher than those in CC group ($P < 0.05$).

Serum Levels of CORT and ACTH After ACS

Serum levels of CORT and ACTH after ACS are shown in Figure 5. At ACS6, most CS groups have not change significantly of the serum ACTH and CORT ($P > 0.05$), except a significant decrease ACTH was observed in CS5 and CS6 at A6 ($P < 0.05$). At ACS12, serum ACTH in all CS groups (except CS4) have not change significantly ($P > 0.05$), but serum CORT in all CS groups observed a significant decrease ($P < 0.05$). At ACS24, ACTH in CS3, CS4, and CS5 group, CORT in CS3 and CS5 group have significant reduction ($P < 0.05$). By comparing the contents of serum ACTH and

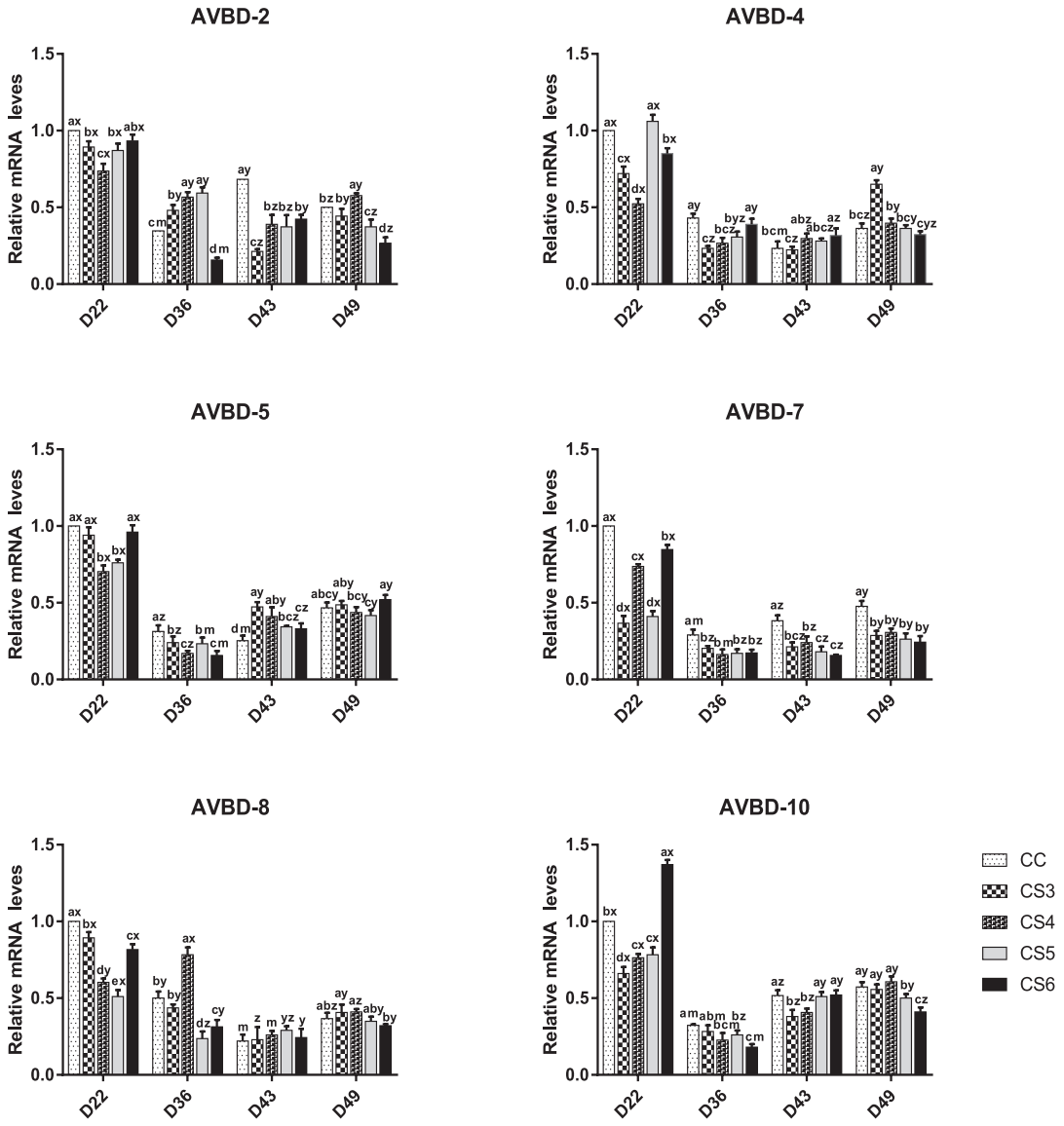

Figure 3. mRNA levels of AvBD-2, AvBD-4, AvBD-5, AvBD-7, AvBD-8, and AvBD-10 in the thymus of broilers. Different letters indicate significant differences ($P < 0.05$) between treatment groups (a, b, c) and days of age (x, y, z).

CORT at ACS6, ACS12, and ACS24, only the CS5 group showed no significant change with the time of acute cold stress ($P > 0.05$).

DISCUSSION

Negative effects of low temperature pressure on poultry production (Hangalapura et al., 2004; Zhao et al., 2013; Tsiouris et al., 2015). Previous studies have shown that intermittent cold stimulation training in early growth response can improve immunity and cold resistance in late growth growth response (Wei et al., 2018; Su et al., 2019; Liu et al., 2020). We still need more results from different organizations in order to evaluate cold stimulus programs comprehensively. In a previous study conducted by our group, intermittent cold stimulation training at early stages of development improved immune capacity and cold resistance of intestinal tract, bursa of Fabricius, and spleen of broilers (Su et al., 2017; Liu et al., 2020; Li et al., 2020; Xue et al., 2021). In the present study, early intermittent cold stimulation training was shown to regulate development and immune function of thymus gland of broilers by regulating the expression of tls, cytokines, and AvBDS genes, which resulted in good maintenance ability during convalescence. Moreover, changes in ACTH and CORT levels in ACS confirmed that cold acclimation improved cold resistance ability of broilers.

Previous studies reported that temperature variation affected mRNA expression of several genes related to the response against pathogen infection, thus impacting immune capacity (Paul et al., 2015; Vandana et al., 2018). During infection, tls stimulate and promote immune response by recognizing a variety of PAMPs (Takeda et al., 2003; Kawai and Akira, 2010). Acute heat stress increased immune capacity by upregulating TLR-4 mRNA expression levels in liver and spleen of broilers (Mohammed et al., 2019). After exposure to high temperature, the significantly decreased expression

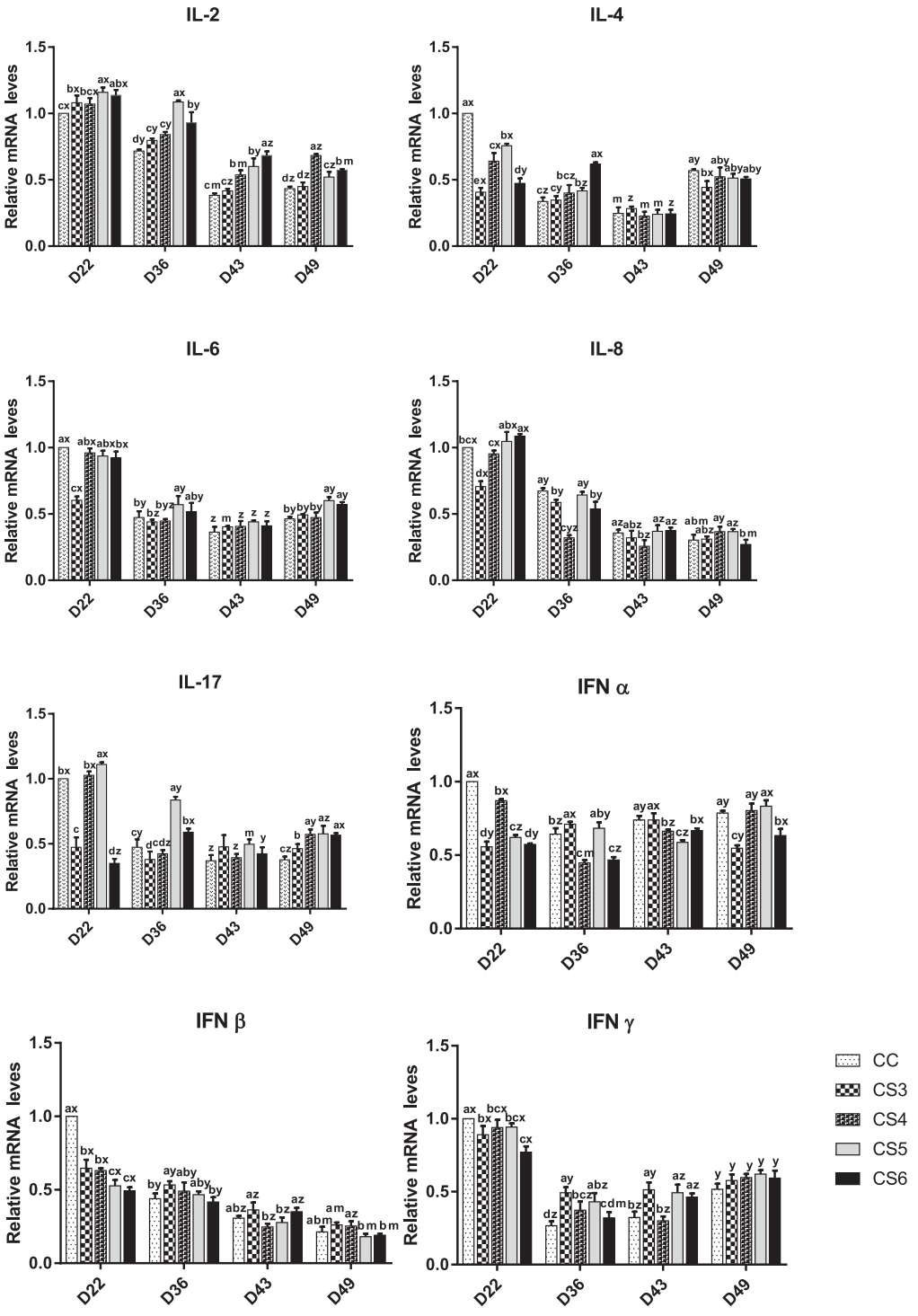

Figure 4. mRNA levels of cytokines IL-2, IL-4, IL-6, IL-8, IL-17, IFN-α, IFN-β, and IFN-γ in the thymus of broilers. Different letters indicate significant differences ($P < 0.05$) between treatment groups (a, b, c) and days of age (x, y, z).

of TLR-2 and TLR-4 mRNAs in monocytes of peripheral blood in bama pig, as well as TLR-1, TLR-4, and TLR-5 in mesenteric lymph nodes of goats, resulted in marked immunosuppression (Vandana et al., 2018; Huang et al., 2021). In addition, Li et al. (2020) found mRNA expression of TLR-4 in duodenum of broilers significantly increased after IMCS 3h, but significantly decreased it at 6 h. Liu et al. (2020) found that mRNA expression level of TLR-4 of bursa in broiler significantly reduce after IMCS 3 h, 4 h, 5 h and 6 h. The above findings all indicate that TLR-4 is different among different species and tissues, and is closely related to the adaptive regulation of homeostasis in the body. In our experimental results, *TLR-4* mRNA in different CS groups were different, we suggested that was important to improve immunity. At D22, *TLR-4* in all CS groups decreased, which was beneficial to reduce the expression of pro-inflammatory factors and limit inflammatory injury. In CS3 group at D36 and D43, *TLR-4* mRNA in CS4 group at D49 and in CS5 group at D43 increased significantly ($P < 0.05$), which were beneficial to promote the chemotaxis of immune cells and enhance immune capacity. At D49,

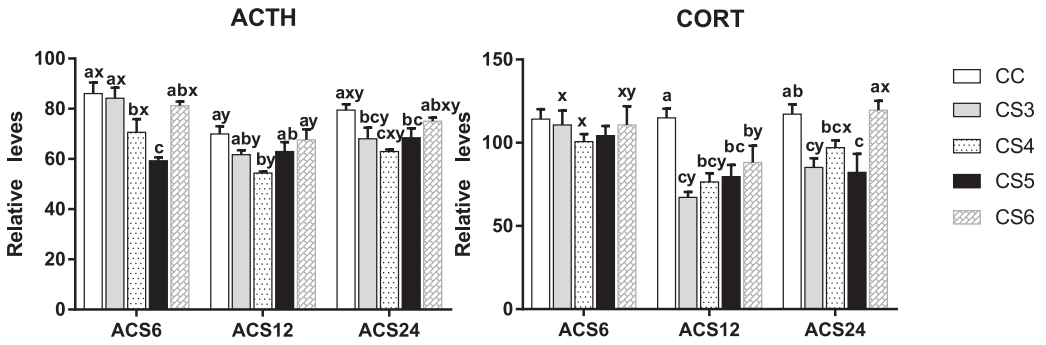

Figure 5. Contents of serum CORT and ACTH after acute cold stress (ACS) of broilers. Different letters indicate significant differences ($P < 0.05$) between treatment groups (a, b, c) and hours of ACS (x, y, z).

TLR-4 mRNA only in CS5 group had a decrease expression, which may indicate that MICS for 5h improved the response threshold of *TLR-4* and establishes adaptability in the early stage. Figure 2 depicts expression levels of tls in the thymus gland of broilers. At the beginning and at the end of IMCS training, expression levels of tls in CS groups were generally lower than those in CC group, and mRNA expression levels of *TLR-1, TLR-2, TLR-4, TLR-5, TLR-7*, and *TLR-21* in the CS5 group at D22 were the lowest in all CS group ($P < 0.05$). Collectively, these results suggest that mild cold stimulation at early stages of development (D22) did not result in higher infection rates compared with the control group, and the organism could save organic matter consumption by reducing expression of tls genes. In particular, after 14 days of recovery, expression levels of tls in the CS5 group were significantly lower than those in the CC group, and the expression of *TLR-2, TLR-3, TLR-4, TLR-5*, and *TLR-15* genes in the CS5 group at D49 reached the lowest value ($P < 0.05$). Comparing differences in immune capacity, the scheme applied to the CS5 group could be considered the most energetic efficient and stable cold adaptation scheme. tls recognize and neutralize a variety of pathogens. TLR-2 recognizes lipoproteins from Gram-positive bacteria, whereas TLR-4 recognizes predominantly lipopolysaccharides (**LPS**) of Gram-negative bacteria (Wheaton et al., 2007; Zahringer et al., 2008), and expression of inflammatory cytokines is then induced (St Paul et al., 2011, 2012). TLR-3, TLR-7, TLR-15, and TLR-21 initiate and modulate host responses to viral infection, while TLR-15 and TLR-21 are also important in response to bacterial infection (Alexopoulou et al., 2001; Kawai and Akira, 2010; Brownlie and Allan, 2011). In the present study, the expression of tls genes in the thymus of broilers, especially considering birds submitted to IMCS, showed a decreasing trend and then an upward trend in the early stages of development in all groups; expression of tls genes in the CS5 group did not consistently show the lowest levels in ICMS late and recovery stages, indicating that broilers might activate different immune responses after 14 days of cold stimulation. Expression levels of *TLR-3* and *TLR-21* at D36 were significantly increased in CS4, CS5, and CS6 groups ($P < 0.05$), while expression levels of effector molecules IFN-α and IFN-β did not significantly increase compared with CC group ($P > 0.05$), suggesting no increased risk of disease. These result suggested thymus immune function was improved as well as the ability to withstand viral and fungal infection under low temperature stress, especially in longer cold stimulation training.

The ability of thymus to fight against infectious disease threats in response to low temperature stress was enhanced in CS4, CS5 and CS6 groups compared with CC group at D43. On d 7 of recovery (D43), expression levels of *TLR-4* and *TLR-5* were significantly higher in CS group compared to CC group ($P < 0.05$), and differences were not consistent with expression levels at D36. Expression levels of *IL-6, IL-8*, and *IL-17* did not significantly increase ($P > 0.05$), suggesting that increased resistance against bacterial infection might be prioritized by the organism in early recovery from cold stimulation.

Cytokines play an important role in inflammatory response and immune cell differentiation (Hangalapura et al., 2003; Reese et al., 2006; Coondoo, 2011; Wei et al., 2018). Among them, IL-2, IL-8, IL-17, and IFN-γ are important pro-inflammatory factors, while IL-4 has anti-inflammatory effects, and IL-6 has been reported to have both pro-inflammatory and anti-inflammatory effects (O'Shea and Murray, 2008; Martha et al., 2014). It was found that cold stimulation can increase the expression of pro-inflammatory factor genes (Hangalapura et al., 2006). Similar results were also found in our study in D22 and D36 of IMCS. At D22, only *IL-2* mRNA levels were significantly increased in the CS groups ($P < 0.05$). At 36, *IL-2, IL-4, IL-17*, and *IFN-γ* mRNA levels in CS groups were significantly increased ($P < 0.05$), *IL-6* mRNA levels changed not significantly ($P > 0.05$). We suggested that, at the early stage of IMCS (D22), *IL-2* may improve the non-specific immunity by increasing the proliferation of NK cells, so as to relieve the pressure of thymus specific immunity in chicks. After virus activation, NK cells secrete IFN-γ to resist infection (Sconocchia et al., 1999; Deaglio et al., 2002). However, the results of our experiment showed that *IFN-γ* in CS groups was significantly reduced at D22, which once again proved that IMCS program did not cause virus infection but improved its ability to resist virus. The difference of cytokines in late IMCS (D36) CS group was different from that in D22 group.

We believed that, after 21 days of IMCS training, the body could enhance the differentiation of Th0 cells through cytokines to improve specific immunity. IL-2 and IFN-γ induce Th1 maturation and participate in cellular immunity (Zhao et al., 2020). IL-4 can induce Th0 cells to differentiate into Th2 cells, participate in humoral immunity, promote the production of immunoglobulin and remove parasites in intestinal mucosa (Davenport and Tipping, 2003; Jin et al., 2017). IL-6 promotes the differentiation of Th0 into Th17 cells which secrete IL-17 to participate in inflammatory responses and respond to infection (Li et al., 2017). In addition, IL-17 can stimulate epithelial cells to secrete defensins, recruit and activate neutrophils, and participate in innate immunity (Park and Lee, 2010; Ma et al., 2019). Based on these studies above, the result that mRNA expression levels of *IL-2, IL-4, IL-6, IL-8, IL-17*, and *IFN-α* all reached the highest values in the CS5 group at D36 suggested that IMCS for 5 h may be the optimal scheme for thymus immunity.

AvBDs play a crucial role in innate immune defense upon stimulation by exogenous factors, including bacteria, virus, LPS, inflammatory cytokines, among others. (Cuperus et al., 2013; Yang et al., 2018; Yu et al., 2018). In a previous study, expression levels of *AvBDs* in spleen, thymus and bursa of Fabricius of broilers increased upon microbial infection and inflammatory stimulation (Hong et al., 2012; Hancock and Scott, 2000). Su et al. (2019) reported that cold stimulation and ACS at 10°C significantly increased relative expression of *AvBD7* in the trachea of broilers. Moreover, expression levels of *AvBD10* were significantly increased 34 d after chronic cold stimulation (Su et al., 2019). In the present study, expression levels of most AvBDs genes in CS groups were lower compared to those in the CC group during IMCS training, which diverged from findings of Su et al.(2019); observed differences can be attributed to varying organ functions and experimental conditions. In another study, expression levels of *AvBD-1, AvBD-2, AvBD-4, AvBD-6*, and *IL-8* were upregulated in the cecum during embryo development three days before birth, suggesting that innate immunity is developed at an early stage of development. In response to infection, IL-8 recruits AvBD-2-positive cells, thus showing synchronous changes. In the present experiment, asynchronous changes of *IL-8* and *AvBD-2* expression in CS groups revealed that mild cold stimulation training did not lead to an increase in infection rates. At early stages of IMCS (D22), expression of *AvBDs* genes initially decreased and then increased with prolonged cold stimulation time, suggesting that the organism can protect the structure and function of thymus gland by improving nonspecific immunity. At D22, expression levels of tls genes in CS groups were lower than those in CC group; expression levels of *AvBD-2* and *AvBD-5* in the CS6 group were comparable to those in CC group, but expression level of *AvBD-10* in the CS6 group was significantly higher than that in CC group. Thus, it can be hypothesized that the organism preferentially initiates nonspecific immunity in response to environmental stressors.

As an important center for maintaining homeostasis, the HPA axis produces ACTH and CORT indicating stress level (Imaki et al., 1995; Feng et al., 2021). Studies have shown that low temperature stimulation can significantly increase the serum CORT content of piglets and mice, and the degree of CORT increase varies with different temperature and stress duration (Sasaki et al., 1990; Xu et al., 2019; Liu et al., 2021). This is because, under stress conditions, ACTH and CORT secretion increase, resulting in increased blood sugar and lipid concentrations, thereby activating gluconeogenesis and enhancing tolerance to external stress stimuli (Simons et al., 2017). However, if CORT levels are too high, adverse reactions such as immunosuppression can occur (Gonzalezjurado et al., 2009). Collectively, the results presented herein revealed that ACTH and CORT levels in broilers submitted to cold stimulation training were significantly lower than those in birds that did not undergo cold stimulation training and ACS. A similar study using mice found that mice which had undergone chronic cold stress (4 h at 4°C for 21 d) had lower ACTH levels after ACS in adulthood compared with mice that did not undergo chronic cold stress during early stages of development (Bhatnagar and Meaney, 1995). Thus, it can be speculated that broilers developed cold adaptation after cold stimulation training and had improved ability to withstand severe environmental changes in late stages of development, thereby reducing the impact of adverse environments on the organism, with birds included in the CS5 group showing that strongest resistance to acute cold stimulation.

CONCLUSIONS

In conclusion, by changing the expression levels of TLRs, cytokines and AvBDs encoding genes, early IMCS can regulate the development and function of thymus gland, maintain homeostasis, and improve the cold resistance of broilers at late growth stage. The optimal cold adaptation scheme is 3°C lower than the conventional feeding temperature for 5 h. The results discussed herein provide a theoretical basis for the study of the adaptive mechanism of IMCS in broilers, which might enable applying cold adaptation scheme in poultry production. Future cytological experiments will be conducted to further explore the molecular mechanisms established by cold adaptation.

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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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