Effects of compound organic acid calcium on growth performance, hepatic antioxidation and intestinal barrier of male broilers under heat stress

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Objective: The aim of this study was to evaluate the effects of compound organic acid calcium (COAC) on growth performance, hepatic antioxidant status and intestinal barrier of male broilers under high ambient temperature (32.7°C).

Methods: Nine hundred healthy one-d-old Cobb-500 male broiler chicks were randomly assigned into three groups with six replicates of 50 birds each. A basal diet supplemented with 0% (control), 0.4% and 0.8% COAC, respectively were fed to birds for 6 weeks. All treatments were under high ambient indoor temperature of 32.7°C, and had a constant calcium and available phosphorus ratio.

Results: The results showed that, compared with control, the average daily gain of broilers in 0.4% and 0.8% was significantly increased and the ratio of feed to gain in 0.4% and 0.8% was significantly decreased at 1 to 21, 22 to 42 and 1 to 42 days of age (p<0.05). Compared with control, 0.8% COAC slightly decreased (p = 0.093) the content of malondialdehyde in liver at 42 days of age while 0.4% COAC significantly decreased (p<0.05) the activity of alkaline phosphatase. Furthermore, 0.4% COAC significantly enhanced the intestinal barrier function via increasing jejunal and ileal occln transcription, promoting jejunal mucin 2 transcription at 42 days of age (p<0.05), and decreasing jejunal toll-like receptor 2 (TLR-2) and ileal TLR-15, inducible nitric oxide synthase compared with control group (p<0.05). Whereas, no significant differences on the transcription of interleukin-1β in jejunum and ileum were observed among three treatments (p>0.05). Overall, heat stress caused by high natural environment temperature may induce the damage to hepatic antioxidation and intestinal barrier.

Conclusion: Dietary inclusion of COAC can improve the tolerance of broilers to thermal environment through the modification of antioxidative parameters in liver and the mRNA expression of genes in intestinal barrier, resulting in an optimal inclusion level of 0.4%.

Keywords: Growth Performance; Compound Organic Acid Calcium; Antioxidation; Intestinal Barrier; Heat Stress; Broiler

INTRODUCTION

Heat stress caused by high ambient temperature and relative humidity is one of the most severe issues in commercial poultry production due to its serious damage on performance, immunity and antioxidation status of broilers [1], gene expression [2], and body temperature at hatching [3], culminating in economic losses. An unreliable housing environment, especially with the additional challenge by the rising temperature due to global warming, could lead to a disruption of the boilers’ body temperature control with dysfunction in the respiratory system and circulation [4]. There are numerous poultry factories in regions across China that suffer from hot summers with at least 30°C for a few hours every day, which have been experiencing technical limitations in attempting make an economic profit. Broilers...
in these factories would be more vulnerable to heat increments compared to younger birds due to their increased body mass and development of feather layers as they grow [3]. In order to solve those increasing problems caused by hot climate, different methods have been carried out [5]. Excluding lowering stocking density, dietary supplementation of probiotics, certain acids, organic acids, etc. can be effective methods. As reported, *Bacillus licheniformis* might be useful for ameliorating the adverse influence of heat on the egg production and gut health of laying hens [6], *Lactobacillus* improved the average daily gain (ADG) of broilers [7], methionine hydroxy analogue alleviated major damage of broilers through the action on some genes related to thioredoxin complex activity [8], and organic acid improved microbiological quality of chicken meat [9]. Numerous studies have reported that dietary organic acid or organic acid salts can improve the growth performance, immune status and gut health of broilers [10, 11]. However, few data are available for their effects on broilers under heat stress [12].

Therefore, the main goal of the present study was to investigate the effects of commercial compound organic acid calcium (COAC) on hepatic antioxidant status and intestinal barrier in male broilers under high ambient temperature.

**MATERIALS AND METHODS**

**Animals and management**

The experiment was conducted in summer. The experimental use of animals and related procedures were performed in accordance with the Chinese Guidelines for Animal Welfare and approved by the Institutional Animal Care and Use Committee of Zhejiang University (Hangzhou, China).

Nine hundred healthy one-d-old Cobb-500 male broiler chicks with an average initial body weight of 45.60±0.58 g were obtained from a commercial hatchery, and randomly distributed into three treatment groups with 6 replicate pens containing 50 birds each. Dietary treatments, with a constant calcium and available phosphorus ratio, were arranged as follows: The control group received the basal diet and the treatment groups received the same basal diet supplemented with 0.4% and 0.8% COAC, respectively. The COAC, provided by Hangzhou Guogu Biological Technology Co., Ltd (Zhejiang, China), is a compound acidifier blend of calcium formate, calcium citrate and calcium lactate with 7:2:1 ratio, and a total calcium content at 26.45%. The experimental diets (starter diets from 1 to 21 d; grower-finisher diets from 22 to 42 d) were formulated to meet the NRC [13] nutrient recommendations for broilers. Ingredients and chemical composition of the diets are presented in Table 1.

Chicks were handled carefully to avoid any pain or injury and placed in a room with adjoining floor pens (50 birds/pen, 0.28 m×0.26 m floor area/bird), which were separated by wire fencing. The room was cleaned and sterilized thoroughly with formaldehyde and potassium permanganate solution (KMnO₄ + formaldehyde solution) 15 days prior to the arrival of the birds. Birds had ad libitum access to diets (in powder form) and water at a constant mean indoor temperature of 32.7°C. The relative humidity was kept from 55% to 65% (Table 2), and the light is nature lighting. Broilers were raised using common management practice for broiler chicks.

**Sample collection and analytical determination**

At 21 d and 42 d, after a 12 h feed withdrawn, one bird from each replicate was randomly chosen and killed by cervical dislocation. Then, the liver tissue was collected immediately according to the method of Bai et al [14]. The middle-jejunum and ileum segments were obtained by the method of Adil et al [11]. All samples were quickly snap-frozen in liquid nitrogen and stored at –80°C for further analysis.

**Growth performance**

The broilers were weighed at 08:00 am on an empty stomach in terms of replicate at 21 d and 42 d respectively. The amount of feed and feed remaining were recorded for each replicate. The ADG, the average daily feed intake (ADFI), the ratio of feed to gain (F/G) were calculated.

**Liver biochemical and antioxidant indexes**

Liver samples were homogenized with ice-cold physiologic saline with the ratio of 1:9, then centrifuged at 2,500 r/min for 15 min under 4°C. The supernatant were collected for the analysis of glutamic oxalacetic transaminase (GOT), aspartate amino transferase (GPT), alkaline phosphatase (AKP), the total antioxidant capacity (T-AOC), total superoxide dismutase (T-SOD), and glutathione peroxidase (GSH-Px) activities, total protein (TP), malondialdehyde (MDA) contents. The kits were provided by Nanjing Jiancheng Biological Engineering Institute (Nanjing, China).

**RNA extraction and quantitative real-time polymerase chain reaction**

The mid-jejunum and ileum segments were carefully dissected and rinsed with sterilized saline. Jejunal and ileal mucosa were gently scraped off. All the samples were placed in liquid nitrogen immediately and stored at –80°C till further analysis. Total RNA was extracted using RNAiso Plus method (TaKaRa, Dalian, China). Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using M-MLV reverse transcriptase (TaKaRa, China). Transcriptional changes were then identified by quantitative polymerase chain reaction (PCR), which was performed using the Premix Ex TaqTM with SYBR Green (TaKaRa, China) and the ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA). The thermocycle protocol lasted for

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30 s at 95°C, followed by 40 cycles of 5 s denaturation at 95°C, 34 s annealing/extension at 60°C, and then a final melting curve analysis to monitor purity of the PCR product. Primer sequences are presented in Table 3. The 2−ΔΔCt method was used to estimate mRNA abundance. ΔCt is Ct, target – Ct, reference and ΔΔCt is ΔCt, treatment – ΔCt, control. Relative gene expression levels were normalized to those of eukaryotic reference gene β-actin [15].

Statistical analysis
After being organized by Microsoft Excel 2003 (Microsoft Corp., Redmond, WA, USA), the data were analyzed by one-way analysis of variance of the SPSS 20.0 (SPSS Inc., Chicago, IL, USA) followed by a Duncan’s multiple range test [16]. The results were expressed in terms of the means plus pooled standard error of means. Significance was declared at p<0.05 and statistical tendencies noted at 0.05<p<0.10.

RESULTS

Growth performance
Effects of dietary compound organic acid calcium on growth performance of broilers are shown in Table 4. At 1 to 21 d, the ADG in 0.4% COAC and 0.8% COAC were increased by 7.22% and 6.13% (p<0.05) respectively, F/G were decreased by 3.47% and 4.05% (p<0.05) respectively compared with control. At 22 to 42 d, the ADG in 0.4% COAC and 0.8% COAC were improved by 7.84% and 7.86% (p<0.05) respectively, F/G were reduced by 5.24% and 4.76% (p<0.05) respectively, At 1 to 42 d, the ADG in 0.4% COAC and 0.8% COAC were increased by 7.60% and 7.23% (p<0.05) respectively, F/G were decreased by 5.08% and 4.27% (p<0.05) respectively. No remarkable differences (p>0.05) were ob-
Liver biochemical and antioxidant status

Effects of treatments on liver biochemical and antioxidant status in broilers at d 21 and d 42 under seasonal heat stress are presented in Table 5. No statistical differences were observed in the indicators of GOT, GPT, TP, and AKP (d 21) in liver (p>0.05). However, the activity of AKP was significantly lower in 0.4% COAC compared to 0.8% COAC and control at 42 day of age (p<0.05). Although no significant differences were observed in the activities of T-AOC, T-SOD, GSH-Px, and the content of MDA (d 21) in liver of broilers at two stages (p>0.05), the content of MDA was slightly lower in 0.4% and 0.8% COAC at 42 days of age compared to control group (p = 0.093).

The expression of genes related to intestinal physical barrier in jejunum of broilers

Changes in the mRNA expression of mucin 2 (MUC-2), occludin (Ocln), claudin 1 (Cldn1), claudin 3 (Cldn3) and transforming growth factor-beta β2 (TGF-β2) in jejunum of broilers are shown in Figure 1. Compared with control group, at d 21, jejunal Cldn3 and TGF-β2 mRNA expressions of birds fed 0.8% COAC were significantly decreased (p<0.05), while no remarkable differences (p>0.05) were observed on Ocln, Cldn1 transcriptions among all groups (p>0.05). At d 42, 0.4% COAC significantly increased (p<0.05) the expression of genes related to intestinal physical barrier in jejunum of broilers.

Table 4. Gene names and primer sequences

| Gene   | Primer sequence 5’-3’            |
|--------|----------------------------------|
| MUC-2  | F: GCCTGCCCAGGAATCAAG            |
|        | R: CGACAGCTTTGCGCAT              |
| Ocln   | F: GAGGCCCAGACTACCAAAGCAGA      |
|        | R: GTCTAAGTGGAAAGGCTTGG          |
| Cldn1  | F: TGCCAGCTATGGTGATGG            |
|        | R: AACCCTGTAAGAAAGGTACTAG        |
| Cldn3  | F: AATGCGCCATCTGCAAC             |
|        | R: GTTCTCCGCAAGACTCTCC          |
| TLR2   | F: TGTTCCTGTCAACACTCACTTCT      |
|        | R: AGTGGAGTGGTCCACTCAGT         |
| β-actin| F: TTGTGCAAGGCGGTTC              |
|        | R: TGTCTTCTGGCCATACCA            |
| TLR4   | F: GAATGACAGGAGACTCTT            |
|        | R: ACATGGAACCTCTGCAAC           |
| TLR15  | F: CTTGTCGTCTGGTGCTAA            |
|        | R: ATGTGACTGTAGTGTAG             |
| IL-1β  | F: CGACATACACAGAAGCTTT          |
|        | R: GTCAAGGGCGTAAAGATGA           |
| INOS   | F: TACTCTTGGCGCATCTC            |
|        | R: GCATAGATCAGTCACCTT            |
| TGF-β2 | F: TCTGGAGACGGCAGTGA             |
|        | R: AACTCAGTCTCCGCTTCCG          |

F, forward; R, reverse; MUC-2, mucin 2; Ocln, occluding; Cldn, claudin; TLR, toll-like receptor; IL, interleukin; INOS, inducible nitric oxide synthase; TGF-β2, transforming growth factor-beta 2.

Table 4. Effects of dietary compound organic acid calcium on growth performance of broilers

| Items              | 21 days of age | SEM | p-value |
|--------------------|---------------|-----|---------|
|                    | Control       | 0.4% COAC | 0.8% COAC |
| Body weight (g)    |               |       |         |
| 1 day of age       | 45.80         | 45.19  | 45.40   |
| 21 days of age     | 563.45        | 600.15 | 594.71  |
| 42 days of age     | 1,498.89      | 1,608.86| 1,603.45|
| 1 to 21 days of age| 600.15        | 600.15 | 600.15  |
| ADFI (g)           | 42.68         | 44.02  | 43.43   |
| ADG (g)            | 24.65         | 26.43  | 26.16   |
| F/G                | 1.73          | 1.67   | 1.66    |
| 22 to 42 days of age|              |       |         |
| ADFI (g)           | 93.43         | 95.59  | 95.98   |
| ADG (g)            | 44.54         | 48.03  | 48.04   |
| F/G                | 2.10          | 1.99   | 2.00    |
| 1 to 42 days of age|              |       |         |
| ADFI (g)           | 67.99         | 69.77  | 69.71   |
| ADG (g)            | 34.60         | 37.23  | 37.10   |
| F/G                | 1.97          | 1.87   | 1.88    |

Values reported as means (n = 6).
COAC, compound organic acid calcium; SEM, standard error of means for 6 broilers each; ADFI, the average daily feed intake; ADG, the average daily gain; F/G, the ratio of feed gain.

1) Control = basal diet without any feed additive; 0.4% COAC = basal diet + 0.4% compound organic acid calcium; 0.8% COAC = basal diet + 0.8% compound organic acid calcium.

Means in the same row with different superscripts differ statistically (p<0.05).
and TGF-β2 expressions, whereas, 0.8% COAC reduced (p<0.05) the cldn1 and TGF-β2 expressions.

The expression of genes related to intestinal physical barrier in ileum of broilers

Effect of dietary COAC on the mRNA expressions of MUC-2, ocn, cldn1, cldn3, and TGF-β2 in ileum of broilers are shown in Figure 2. At d 21, the mRNA expressions of ocn and cldn3 in ileum were significantly higher (p<0.05) in birds fed 0.4% COAC than control group. Compared with control and 0.4% COAC, birds in 0.8% COAC had a lower cldn3 expression and a higher MUC-2 expression (p<0.05). At d 42, 0.4% COAC obviously elevated (p<0.05) the mRNA expression of ocnl and cldn1 compared with 0.8% COAC.

The expression of genes related to intestinal immunological barrier in jejunum of broilers

Figure 3 shows the changes in the mRNA expression of toll-like receptor (TLR), nitric oxide synthase (iNOS) and interleukin-1β (IL-1β) in jejunum of broilers. Compared with control group, at d 21, 0.4% COAC significantly decreased (p<0.05) TLR-2 transcription, and 0.8% COAC notably increased (p<0.05) iNOS expression. At d 42, birds fed 0.4% COAC had significantly decreased the mRNA expression of TLR-15 (p<0.05), while no significant differences were observed in other gene expressions among all treatments (p>0.05).

The expression of genes related to intestinal immunological barrier in ileum of broilers

As shown in Figure 4, at d 21, compared with control group, the COAC groups reduced iNOS expressions and 0.4% COAC obviously decreased TLR-15 expression (p<0.05). At d 42, birds fed 0.8% COAC had significantly decreased (p<0.05) mRNA expression of iNOS. No significant differences were observed on the mRNA expressions of TLR-2, TLR-4, and IL-1β among all treatments (p>0.05).

DISCUSSION

The addition of acidifiers or organic acid salts to broiler diets beneficially improves the palatability of the diet while participating in the body’s metabolic response and accelerating the body’s absorption of nutrients [17]. Thereby promoting broiler weight gain and reducing F/G. Some studies reported that dietary supplemented organic acids can significantly increase daily gain and feed conversion in broilers [10], which is agreement with our results. In the present study, there was no significant difference in ADFI observed between experiment groups, which is consistent with studies reported by Sultan et al [18] and Gunal et al [19]. While, the studies by Chowdhury et al [10] and Haque et al [20] showed that the addition of organic acids to the diet increased the feed intake of broilers. And other studies found that dietary supplement organic acids reduced the feed intake of broilers [17,21], which might be related to the type of organic acid, the amount of addition, the feeding environment and the feeding method [18,20,22].

The structure and physiology of the cells can be affected by high temperature, causing impairment of transcription, oxidative metabolism, membrane structure and function [23] and gene expression [2]. Also, heat stress can result in oxidative stress and induce cells to generate small amounts of free radicals or reactive oxygen species (ROS) [5], which may damage biological macro-molecules and tissues if not con-
trolled [24]. However, these damages might be minimized via antioxidant defense systems, including antioxidant enzymes such as SOD, GSP-Px, etc. [25]. The biochemical and antioxidant indices play a vital role in protecting cellular and tissue damage from harmful effects of ROS [25], and are usually used as indicators of the health condition of animals [5]. The activities of GOT and GPT can reflect the functions and integrity of tissues like liver [26]. AKP performs lipid transportation in the intestine, calcification in bone and is present in liver, and usually remains normal or moderately increased in acute viral hepatitis [27], decreased in hypothyroidism and pernicious anaemia [28]. In the present study, COAC did not influence the activities of GOT, GPT, TP, and AKP in liver at d 21. While the AKP activity was significantly decreased in 0.4% COAC, which may be have a protective effect in the liver. MDA, one of the important metabolites of lipid peroxidation, can serve as an indicator of tissue injury. The antioxidant enzymes like T-AOC, T-SOD, and GSH-Px can minimize the

Figure 1. Effects of compound organic acid calcium on MUC-2, occln, cldn1, cldn3 and TGF-β2 mRNA expression in jejunum of broilers. At 21 and 42 days of age, the expression of MUC-2 (A), occln (B), cldn1 (C), cldn3 (D) and TGF-β2 (E) were measured by real-time polymerase chain reaction. MUC-2, mucin 2; Ocln, occluding; Cldn, claudin; TGF-β2, transforming growth factor-beta 2. Different letters (a-c) denote a statistical difference (p<0.05).
oxidative damage by protecting the cell against cellular oxidants and preventing the accumulation of oxidatively injured molecules [29]. Although no significant differences were observed on the activities of T-AOC, T-SOD, and GSH-Px along with the content of MDA (d 21) in liver of broilers, the content of MDA was slightly lower in both 0.4% and 0.8% COAC compared with control group, which is in agreement with the results of Seven et al [29] and Ma et al [30] where heat stress increased the concentration of MDA in liver of both broilers and laying hens.

The integrity of the seal of the paracellular pathway between cells is crucial for the epithelial cell layer to function properly [15], and this seal is achieved by a physical barrier, especially tight junctions [31]. The functional barrier of the intestinal epithelial separates the intestine from the outside world, and requires the formation of tight junctions that allow cells to adhere tightly to each other and control the intestinal permeability [32]. Tight junctions such as ocln, cldn are structural
and functional proteins [15], among which the most abundant mucin is MUC-2, that can create the first line of defense against microbial encroachment [33]. In the present study, the transcript levels of all the tested tight junctions were upregulated by COAC and the effect was more obvious when birds were fed 0.4% COAC. A damaged intestinal epithelial integrity may facilitate the invasion of endotoxins from gut microbes, leading to a local imbalance of anti- and pro-inflammatory molecules in the intestines [34]. COAC improved the intestinal physical barrier and may also participate in enhancing the intestinal immunological barrier function.

In addition, TLRs are a group of evolutionarily conserved membrane receptors broadly expressed on various innate immunity cells and non-immune cells, they function as primary sensors that can initiate innate immune responses via responding to pathogen-associated molecular patterns from

Figure 3. Effects of compound organic acid calcium on TLR, iNOS and IL-1β mRNA expression in jejunum of broilers. At 21 and 42 days of age, the expression of TLR-2 (A), TLR-4 (B), TLR-15 (C), iNOS (D) and IL-1β (E) were measured by real-time polymerase chain reaction. TLR, toll-like receptor; iNOS, inducible nitric oxide synthase; IL-1β, interleukin 1β. Different letters (a, b) denote a statistical difference (p<0.05).
bacteria, viruses, etc. [35]. Moreover, these TLRs such as TLR2 and TLR4 are expressed in pro-inflammatory macrophages and vascular, which would release more TLRs when tissues were injured [36], as iNOS and IL-1β. In the current study, we found that COAC significantly decreased the expressions of TLR-2, TLR-15 and pro-inflammatory cytokine iNOS, which might be related to the stimulation of organic acid calcium on immune related organs [37].

CONCLUSION

Dietary supplementation of compound organic acid calcium can improve the growth performance and alleviate the damages in broilers under heat stress. The effect was demonstrated by increasing the immunological competence, regulating TLR-2, TLR-4, and MUC-2 expressions, resulting in the improvement of broilers tolerance to the damages in liver and...
small intestine. However, only limited studies on the molecular mechanism of dietary organic acid or organic acid salts against the damage of heat stress in broilers have been carried out, which needs further exploration.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with my financial organization regarding the material discussed in the manuscript.

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