Effects of Continuous Moderate Heat Environment on Serum and Hypothalamic Brain-Gut Peptide Content, Cecal Volatile Fatty Acid Content, and Microbial Diversity in Broiler Chickens

Authors: Chang Shuangshuang, Li Xiumei, Zhang Minhong, Shi Yuxiang, Feng Jinghai

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Abstract

This experiment aimed to investigate the effects of sustained moderately hot environment on serum and hypothalamic brain-gut peptide contents, cecal volatile fatty acid contents, and microbiota diversity in broiler chickens. A single-factor design was adopted. One hundred and twenty healthy 22-day-old Arbor Acres (AA) broiler chickens with similar body weight were selected and randomly divided into 2 groups, with 6 replicates per group and 10 birds per replicate (half male and half female). The six groups of broiler chickens were transferred to an environmental control chamber with ambient temperature of 21 °C and relative humidity of 60% for a 7-day adaptation period. The formal experiment commenced when the birds were 29 days old. The experimental group was exposed to an ambient temperature of 31 °C, while the control group was maintained at 21 °C; both groups were kept at 60% relative humidity. These experimental conditions were maintained until the end of the trial, lasting a total of 14 days. The results showed: 1) On day 7 of the experiment, serum 5-hydroxytryptamine (5-HT) and vasoactive intestinal peptide (VIP) contents in the 31 °C group were significantly lower than those in the 21 °C group ($P<0.05$), whereas serum substance P (SP) content showed no significant difference from the 21 °C group ($P>0.05$); on day 14 of the experiment, there were no significant differences in serum 5-HT, VIP, and SP contents between the two groups ($P>0.05$). 2) On day 7 of the experiment, there were no significant differences in hypothalamic 5-HT, VIP, and SP contents between the two groups of broiler chickens ($P>0.05$); on day 14 of the experiment, hypothalamic 5-HT content in the 31 °C group was significantly lower than that in the 21 °C group ($P<0.05$), while hypothalamic VIP and SP contents showed no significant differences from the 21 °C group ($P>0.05$). 3) On day 14 of the experiment, cecal isobutyric acid content in the

31 °C group was significantly higher than that in the 21 °C group (P<0.05); on days 7 and 14 of the experiment, sustained moderately hot environment had no significant effects on cecal acetic acid, propionic acid, butyric acid, valeric acid, and isovaleric acid contents in broiler chickens (P>0.05). 4) On both day 7 and day 14 of the experiment, cecal bacterial band numbers and diversity indices in the 31 °C group were lower than those in the 21 °C group. 5) Sustained moderately hot environment was unfavorable for the colonization of Clostridium termitidis and Bacteroides vulgatus in the cecum of broiler chickens. It can be concluded that sustained moderately hot environment caused significant decreases in serum 5-HT and VIP contents on day 7 of the experiment, significantly increased cecal isobutyric acid content on day 14 of the experiment, and also reduced cecal microbiota diversity, altered microbiota structure, and inhibited the growth of Clostridium termitidis and Bacteroides vulgatus (common bacteroides) in broiler chickens.

Full Text

Effects of Constant Moderate Temperature on Brain Gut Peptide Contents in Serum and Hypothalamus, Cecal Volatile Fatty Acid Contents and Microflora Diversity of Broilers

CHANG Shuangshuang[1],[2], LI Xiumei[2], ZHANG Minhong[2]*, SHI Yuxiang[1], FENG Jinghai[2]

[1]College of Agriculture, Hebei University of Engineering, Handan 056021, China
[2]State Key Laboratory of Animal Nutrition, Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing 100193, China

Abstract

This experiment was conducted to investigate the effects of constant moderate temperature on brain gut peptide contents in serum and hypothalamus, cecal volatile fatty acid contents, and microflora diversity of broilers. Using a single-factor design, 120 healthy 22-day-old Arbor Acres (AA) broilers with similar body weight were randomly divided into two groups, each consisting of six replicates with ten birds per replicate (equal numbers of males and females). All broilers were transferred to environmental chambers maintained at 21°C and 60% relative humidity for a 7-day acclimation period. At 29 days of age, the formal experiment began: the experimental group was exposed to 31°C while the control group remained at 21°C, both at 60% relative humidity, for a total duration of 14 days.

The results showed: 1) On day 7, serum 5-hydroxytryptamine (5-HT) and vasoactive intestinal peptide (VIP) contents in the 31°C group were significantly lower than those in the 21°C group (P<0.05), while serum substance P (SP) content showed no significant difference (P>0.05). On day 14, no significant

differences were observed between groups for serum 5-HT, VIP, or SP contents (P>0.05). 2) On day 7, hypothalamic 5-HT, VIP, and SP contents did not differ significantly between groups (P>0.05). On day 14, hypothalamic 5-HT content in the 31°C group was significantly lower than in the 21°C group (P<0.05), while VIP and SP contents remained comparable (P>0.05). 3) On day 14, cecal isobutyric acid content was significantly higher in the 31°C group (P<0.05). Throughout days 7 and 14, constant moderate temperature had no significant effect on cecal acetic, propionic, butyric, pentanoic, or isopentanoic acid contents (P>0.05). 4) On both days 7 and 14, the number of bacterial bands and diversity indices of cecal microflora were lower in the 31°C group. 5) Constant moderate temperature was not conducive to colonization by *Clostridium termitidis* and *Bacteroides vulgatus*. In conclusion, constant moderate temperature significantly reduced serum 5-HT and VIP contents on day 7, significantly increased cecal isobutyric acid content on day 14, decreased cecal microflora diversity, altered microflora structure, and inhibited the growth of *Clostridium termitidis* and *Bacteroides vulgatus*.

Keywords: constant moderate temperature; brain gut peptide; volatile fatty acids; microflora diversity; broilers

Introduction

The rapid development of the poultry industry combined with global climate warming has made heat stress a persistent concern in broiler production. Our preliminary research found that constant moderate heat (26°C and 30°C) affected broiler resting behavior and significantly elevated core body temperature at 30°C. Additionally, constant moderate heat (26°C and 31°C) influenced glucose and lipid metabolism and avian uncoupling protein (avUCP) mRNA expression, reducing growth performance, with varying degrees of impact depending on temperature level. Compared with 21°C, constant moderate heat (26°C and 31°C) also decreased cecal microflora diversity. These findings demonstrate that constant moderate temperature negatively affects broiler physiology, behavior, metabolism, intestinal microflora, and production performance.

Brain gut peptides, distributed in both gastrointestinal and nervous systems, extensively regulate gastrointestinal activity peripherally and centrally. 5-hydroxytryptamine (5-HT), vasoactive intestinal peptide (VIP), and substance P (SP) have been identified as brain gut peptides associated with gastrointestinal activity. Volatile fatty acids (VFAs), also known as short-chain fatty acids (SCFAs), promote brain gut peptide secretion, inhibit Gram-negative bacteria growth, and encourage beneficial bacteria such as *Bifidobacterium* and *Lactobacillus*. However, research on the effects of constant moderate temperature on brain gut peptide and VFA contents in broilers remains unreported. Therefore, this study investigated the effects of constant moderate temperature on brain gut peptide contents in serum and hypothalamus, cecal VFA contents, and microflora diversity to provide scientific evidence for healthy broiler production.

Materials and Methods

1.1 Experimental Animals and Management

The experiment employed a single-factor design. One hundred twenty healthy 22-day-old Arbor Acres (AA) broilers with similar body weight were randomly divided into two groups, each comprising six replicates with ten birds per replicate (equal numbers of males and females). All broilers were transferred to environmental chambers maintained at 21°C and 60% relative humidity for a 7-day acclimation period. At 29 days of age, the formal experiment commenced: the experimental group was housed at 31°C while the control group remained at 21°C, both at 60% relative humidity, for a total duration of 14 days. The experiment was conducted in environmental chambers at the State Key Laboratory of Animal Nutrition, with automatic temperature and humidity control (precision ±1°C, ±7%), no air movement, and 24-hour lighting.

1.2 Basal Diet

The experiment utilized a corn-soybean meal basal diet formulated as powdered complete feed according to NRC (1994) nutritional requirements. The composition and nutrient levels of the basal diet are presented in Table 1 .

1.3 Husbandry Management

Broilers were raised in single-tier floor cages developed by our laboratory, with free access to feed and water, and received routine immunizations.

1.4 Sample Collection and Measurements

1.4.1 Blood Collection and Analysis On days 7 and 14, six broilers per group (equal numbers of males and females, one bird per replicate) were randomly selected for blood collection via wing vein. Blood samples were allowed to clot for 2 hours before centrifugation at 3,000 rpm for 10 minutes at low temperature. Serum was harvested and stored at -80°C until analysis. Serum 5-HT, VIP, and SP contents were determined by enzyme-linked immunosorbent assay (ELISA).

1.4.2 Hypothalamus Collection and Analysis On days 7 and 14, six broilers per group (equal numbers of males and females, one bird per replicate) were randomly selected, euthanized, and the hypothalamus was dissected, placed in sterile centrifuge tubes, snap-frozen in liquid nitrogen, and stored at -80°C. Hypothalamic 5-HT, VIP, and SP contents were measured by ELISA.

1.4.3 Cecal Content Collection and Analysis Sample Collection: On days 7 and 14, six broilers per group (equal numbers of males and females, one bird per replicate) were euthanized and disinfected. The abdominal cavity was opened, the intestine was isolated, the ileocecal junction was ligated, and the

cecum was rapidly transferred to a laminar flow hood. The cecal wall was cut open with sterile scissors, and contents were collected. Six samples from the same group were immediately mixed, placed in sterile centrifuge tubes, snap-frozen in liquid nitrogen, and stored at -80°C.

Volatile Fatty Acid Concentration Determination: Approximately 2 g of cecal content was accurately weighed into a centrifuge tube, and 5 mL of ultrapure water was added. The mixture was vortexed for 3-5 minutes and centrifuged at 5,000×g for 10 minutes. One milliliter of supernatant was transferred to a plastic ampoule, mixed with 0.2 mL of 25% metaphosphoric acid, capped, shaken vigorously, and placed in an ice-water bath for 30 minutes. After centrifugation at 10,000×g for 10 minutes, the supernatant was collected for determination of acetic, propionic, butyric, isobutyric, isovaleric, and valeric acid contents.

Genomic DNA Extraction: Genomic DNA was extracted using the FastDNA™ SPIN Kit for Soil.

Bacterial 16S rDNA Fragment PCR Amplification: Using extracted genomic DNA as template, bacterial universal primers GC-338F and 518R were used to amplify 16S rDNA hypervariable region sequences. Primer information is listed in Table 2 . PCR was performed using a T-gradient thermocycler (Biometra) with the following reaction mixture (50 L): 10×PCR buffer 5 L, dNTP mixture (2.5 mmol/L) 3.2 L, Ex Taq (5 U/ L) 0.4 L, GC-338F (20 mol/L) 1 L, 518R (20 mol/L) 1 L, template DNA 50 ng, and ddH O to 50 L. The PCR program consisted of: 94°C for 5 min; 30 cycles of 94°C for 1 min, 55°C for 45 s, 72°C for 1 min; and final extension at 72°C for 10 min. PCR products were purified using the OMEGA DNA Gel Extraction Kit.

Denaturing Gradient Gel Electrophoresis (DGGE) Analysis: Ten microliters of PCR product were analyzed by DGGE using 7% polyacrylamide gel with a 35-55% denaturing gradient in 1×TAE buffer at 150 V, 60°C for 5 hours. After electrophoresis, gels were silver-stained through the following steps: 1) fixation in fixative (50 mL ethanol, 2.5 mL glacial acetic acid, diluted to 500 mL with distilled water) for 15 min; 2) Milli-Q water rinse for 20 s and 2 min; 3) staining in silver nitrate solution (1 g AgNO , 0.75 mL 37% formaldehyde, diluted to 500 mL) for 15 min; 4) Milli-Q water rinse for 20 s and 2 min; 5) development in developer (7.5 g NaOH, 2.5 mL 37% formaldehyde, diluted to 500 mL) for 5-7 min; 6) termination in stop solution (50 mL ethanol, 2.5 mL glacial acetic acid, diluted to 500 mL). Gels were photographed using a Gel-Doc2000 system (Bio-Rad).

Sequence Determination of Dominant DGGE Bands: DGGE bands were excised, re-amplified using primers 338F/518R, purified, ligated into pMD18-T vector, transformed into DH5 competent cells, and sequenced. Sequencing results were compared with GenBank sequences to identify bacterial types. Three clones were sequenced per band.

1.5 Statistical Analysis

Data were analyzed by t-test using SAS 9.2 statistical software. Results are expressed as mean ± standard deviation, with significance set at $P<0.05$. DGGE profile diversity was analyzed using Quantity One software.

Results

2.1 Effects of Constant Moderate Temperature on Serum Brain Gut Peptide Contents

As shown in Table 3 , on day 7, serum 5-HT and VIP contents in the 31°C group were significantly lower than those in the 21°C group ($P<0.05$), while serum SP content showed no significant difference ($P>0.05$). On day 14, no significant differences were observed between groups for serum 5-HT, VIP, or SP contents ($P>0.05$).

2.2 Effects of Constant Moderate Temperature on Hypothalamic Brain Gut Peptide Contents

Table 4 shows that on day 7, hypothalamic 5-HT, VIP, and SP contents did not differ significantly between groups ($P>0.05$). On day 14, hypothalamic 5-HT content in the 31°C group was significantly lower than in the 21°C group ($P<0.05$), while VIP and SP contents remained comparable between groups ($P>0.05$).

2.3 Effects of Constant Moderate Temperature on Cecal Volatile Fatty Acid Contents

Table 5 demonstrates that on day 14, cecal isobutyric acid content was significantly higher in the 31°C group ($P<0.05$). On days 7 and 14, constant moderate temperature had no significant effect on cecal acetic, propionic, butyric, pentanoic, or isopentanoic acid contents ($P>0.05$), though these values were relatively lower in the 31°C group compared to the 21°C group.

2.4 Analysis of Cecal Microflora Diversity

2.4.1 DGGE Profile Analysis Comparison of cecal microflora DGGE profiles (Figure 1 [Figure 1: see original paper]) revealed that on day 7, the 21°C group exhibited more bacterial bands than the 31°C group. On day 14, both groups showed increased band numbers, but the 21°C group still displayed more bands than the 31°C group. Table 6 shows similarity coefficients of 51.5% between groups on day 7 and 44.6% on day 14, with the similarity coefficient within the 31°C group decreasing from day 7 to day 14. These results indicate reduced cecal microflora diversity in the 31°C group.

2.4.2 Microflora Diversity Indices Table 7 shows that microflora diversity varied with ambient temperature. On day 7, Shannon and Simpson indices were

2.45 and 0.91, respectively, in the 21°C group, compared to 2.12 and 0.87 in the 31°C group. On day 14, these values were 2.78 and 0.93 for the 21°C group versus 2.57 and 0.92 for the 31°C group. Richness was consistently lower in the 31°C group throughout the experiment, demonstrating that constant moderate temperature reduced both diversity and richness of cecal microflora.

2.4.3 Specific and Common Microflora Analysis Two specific bands and four common bands were excised from the 16S rDNA V3 region DGGE profiles. As shown in Figure 1 and Table 8 , *Holdemanella biformis* (band 3), *Bacteroides uniformis* (band 4), *Eisenbergiella massiliensis* (band 4), and *Ruminococcus faecis* (band 6) were detected in both groups at both time points, representing common microflora. However, *Clostridium termitidis* (band 1) and *Bacteroides vulgatus* (band 2) were not detected in the 31°C group. All six identified bacteria belonged to Firmicutes and Bacteroidetes phyla, with >90% similarity to GenBank sequences.

Discussion

3.1 Effects of Constant Moderate Temperature on Serum and Hypothalamic Brain Gut Peptide Contents

Brain gut peptides directly affect the central nervous system. Blood-borne brain gut peptides serve as important chemical signals transmitting information from the gastrointestinal tract to the brain. These signals can directly enter the brain through the area postrema to act on the dorsal vagal complex, thereby influencing vagal efferent function and participating in regulation of gastrointestinal motility, appetite, and feeding behavior. 5-HT, also known as serotonin, is a neurotransmitter and important intestinal regulator primarily produced by enteroendocrine cells, mediating intestinal motility and sensation while stimulating intestinal secretion. Studies report that early-life microbiota deficiency elevates plasma tryptophan levels, and *Bifidobacterium* can influence tryptophan metabolism. Oral administration of *Bifidobacterium infantis* has been shown to increase plasma neurotransmitter dopamine and 5-HT levels in rats. In this study, serum 5-HT content decreased significantly on day 7, and hypothalamic 5-HT content decreased on day 14 in the 31°C group, suggesting that constant moderate temperature-induced 5-HT reduction may trigger intestinal dysbiosis.

VIP is a neurotransmitter of the non-cholinergic non-adrenergic inhibitory system that exerts inhibitory regulation on gastrointestinal activity, causing relaxation of gastrointestinal circular smooth muscle. VIP-secreting neurons stimulate pancreatic and intestinal fluid secretion, protect intestinal mucosa, and regulate gastrointestinal absorption. VIP also improves intestinal microcirculation and internal environment, provides nutrients and oxygen to intestinal epithelial cells, and removes harmful substances such as excess oxygen free radicals, thereby reducing lipopolysaccharide-induced mucosal damage and promoting colonization of beneficial bacteria to normalize microflora composition. This

study found decreased serum VIP content on day 7 in the 31°C group, suggesting that constant moderate temperature may alter the cecal internal environment and subsequently change microflora structure.

SP, a tachykinin, increases gastrointestinal motility, strongly promotes smooth muscle contraction, enhances colonic mass peristalsis, and participates in inflammatory and immune responses. This study found no significant effect of constant moderate temperature on serum or hypothalamic SP content.

3.2 Effects of Constant Moderate Temperature on Cecal Volatile Fatty Acid Contents

The animal intestine, particularly the large intestine, contains substantial VFAs including acetic, propionic, and butyric acids, primarily produced by microbial fermentation of carbohydrates (oligosaccharides, organic acids, etc.). These are metabolic end-products of bacterial fermentation and degradation of polysaccharides and unabsorbed oligosaccharides, providing essential environments and nutrients for intestinal bacterial growth and reproduction. VFAs are recognized by free fatty acid receptors (FFAR2 and FFAR3) on enteroendocrine cells (EECs) distributed throughout the intestine. Activation of FFAR2 and FFAR3 on EECs can promote brain gut peptide secretion. Additionally, VFAs can reduce colonic pH, control harmful enzyme activity, inhibit non-acid-tolerant bacteria, precipitate bile salts, reduce serum cholesterol, and inhibit Gram-negative bacteria while promoting beneficial bacteria such as *Bifidobacterium* and *Lactobacillus*.

This study showed that constant moderate temperature had no significant effect on cecal acetic, propionic, butyric, pentanoic, or isopentanoic acid contents, though these tended to be lower in the 31°C group, possibly due to decreased carbohydrate fermentation capacity following bacterial inhibition. Isobutyric acid has been reported to increase fibrolytic bacterial numbers and total VFA content. This study found significantly increased isobutyric acid content under constant moderate temperature, with decreased *Ruminococcus faecis* abundance, warranting further investigation.

3.3 Effects of Constant Moderate Temperature on Cecal Microflora Diversity

Under normal conditions, intestinal microflora forms an important non-specific defense barrier that interacts with the host to maintain dynamic equilibrium of the intestinal microecosystem, antagonizing certain pathogenic microorganisms and protecting host health. However, stress (including environmental factors) can disrupt this balance, potentially generating numerous pathogenic bacteria that compromise health and production performance. DGGE profile and diversity analysis demonstrated that constant moderate temperature reduced cecal bacterial band numbers and inter-bacterial similarity, indicating that constant moderate temperature affects cecal microflora diversity and disrupts microflora balance.

Previous studies have shown that the broiler cecum contains complex microbial communities, predominantly Firmicutes, followed by Proteobacteria, Bacteroidetes, and Actinobacteria. Elevated temperature during poultry development alters intestinal microflora structure. Broilers exposed to 34-38°C heat stress show decreased abundance of *Bacteroides*, *Faecalibacterium*, *Oscillospira*, *Clostridium*, *Phascolarctobacterium*, *Sutterella*, and *Dorea*, while *Lachnospiraceae*, *Ruminococcaceae*, *Anaeroplasma*, *Anaerotruncus*, *Blautia*, *Eubacterium*, and *Butyricimonas* increase. This study demonstrates that constant moderate temperature inhibited *Clostridium termitidis* and *Bacteroides vulgatus* growth and reduced *Holdemanella biformis* and *Ruminococcus faecis* abundance. *Clostridium termitidis* belongs to Firmicutes, the dominant phylum in hindgut microflora (60-70% abundance), with most clostridial groups producing butyric acid that serves as a primary nutrient for intestinal epithelial cell regeneration and repair, inhibits putrefactive bacteria, and promotes beneficial bacteria growth. *Bacteroides vulgatus* belongs to Bacteroidetes, the most abundant group of Gram-negative intestinal bacteria that can degrade host-indigestible polysaccharides, hydrolyze and ferment exogenous fibers, metabolize bile acids and steroids, participate in antagonistic reactions, and enhance host innate immunity. The mechanism of *Holdemanella biformis* in broiler intestine requires further investigation.

In summary, constant moderate temperature altered serum 5-HT and VIP contents, affected cecal VFA contents, and changed cecal microflora diversity and structure. However, the precise relationships among serum brain gut peptides, cecal VFA contents, and microflora diversity require further investigation.

Conclusion

1. Compared with the 21°C group, the 31°C group showed significantly decreased serum 5-HT and VIP contents on day 7 and significantly increased cecal isobutyric acid content on day 14.
2. Compared with the 21°C group, the 31°C group exhibited reduced cecal microflora diversity and altered microflora structure. Constant moderate temperature inhibited *Clostridium termitidis* and *Bacteroides vulgatus* growth and reduced *Holdemanella biformis* and *Ruminococcus faecis* abundance.

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Note: Figure translations are in progress. See original paper for figures.