Effects of Stocking Density on Cecal Microbiota Diversity, Volatile Fatty Acids, and Serum Brain-Gut Peptides in Broiler Chickens under Continuous Mild Heat Stress: Postprint

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Abstract

This experiment aimed to investigate the effects of stocking density on cecal microbiota diversity, volatile fatty acids, and serum brain-gut peptides in broiler chickens under a continuous heat-stressed environment. A single-factor design was adopted, and 144 healthy 22-day-old Arbor Acres broiler chickens with similar body weight were selected and randomly divided into 3 groups, with 6 replicates per group, and each replicate was housed in a single cage. The broiler chickens from the three groups were transferred into an environmental control chamber with a temperature of 21 °C and a relative humidity of 60% for a 7-day acclimation period. The formal experiment commenced when the chickens were 29 days old. The floor area per cage was 0.64 m^2, and the stocking densities were set at 6 birds per cage (half male and half female, Group), 8 birds per cage (half male and half female, Group), and 10 birds per cage (half male and half female, Group). The environmental temperature was 31 °C with a relative humidity of 60%, and these conditions were maintained until the end of the experiment, lasting a total of 14 days. The results showed that: 1) Under the heat-stressed environment, Group exhibited the highest richness and diversity of cecal microbiota, and the similarity of cecal microbiota between Groups and was lower than that between Groups and ; 2) The stocking density of Group was conducive to the colonization of cecal Bacteroides vulgatus under the heat-stressed environment; 3) Under the heat-stressed environment, stocking density had no significant effect on the content of cecal volatile fatty acids in broiler chickens ($P > 0.05$); 4) On day 7 of the experiment, the serum 5-hydroxytryptamine (5-HT) content in Group was significantly higher than that in Group under the heat-stressed environment ($P < 0.05$), whereas stocking density had no significant effect on the serum vasoactive intestinal peptide (VIP) and substance

P (SP) contents in broiler chickens under the heat-stressed environment (P > 0.05). In summary, stocking density under a heat-stressed environment could alter cecal microbiota diversity and structure as well as serum brain-gut peptide 5-HT content, but did not affect cecal volatile fatty acid content.

Full Text

Effects of Stocking Density on Cecal Microflora Diversity, Volatile Fatty Acids, and Serum Brain-Gut Peptides in Broilers under Moderate Heat Stress

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Abstract

This study investigated the effects of stocking density on cecal microflora diversity, volatile fatty acids, and serum brain-gut peptides in broilers under moderate ambient temperatures. Using a single-factor design, 144 healthy 22-day-old Arbor Acres broilers with similar body weight were randomly divided into three groups, each containing six replicates with single-cage housing. All groups were acclimated for 7 days in an environmental chamber at 21°C and 60% relative humidity. The formal experiment began when the broilers reached 29 days of age. With a single cage area of 0.64 m^2, stocking densities were set at 6 birds per cage (half male and half female, Group), 8 birds per cage (half male and half female, Group), and 10 birds per cage (half male and half female, Group). The environmental conditions were maintained at 31°C and 60% relative humidity throughout the 14-day experimental period. The results showed that: (1) Group exhibited the highest richness and diversity of cecal microflora, with lower similarity between Groups and compared to Groups and ; (2) The stocking density in Group favored colonization of *Bacteroides vulgatus* in the cecum; (3) Stocking density had no significant effect on cecal volatile fatty acid concentrations (P>0.05); and (4) On day 7, serum 5-hydroxytryptamine (5-HT) content in Group was significantly higher than in Group (P<0.05), while stocking density did not significantly affect serum vasoactive intestinal peptide (VIP) or substance P (SP) levels (P>0.05). In conclusion, stocking density under moderate heat stress can alter cecal microflora diversity and structure as well as serum 5-HT content, but does not affect cecal volatile fatty acid concentrations.

Keywords: moderate temperature; stocking density; microflora diversity; volatile fatty acid; brain-gut peptide; broiler

Current research on high-temperature effects in broilers has primarily focused on conditions above 32°C [1]. However, the impact of moderate heat stress below 32°C cannot be overlooked in practical production, as studies have shown that moderate temperatures adversely affect broiler physiology, behavior, nutrient metabolism, intestinal microflora, and growth performance [2-4]. Stocking density is another critical concern in poultry production, as it directly affects broiler activity space [5] and indirectly influences house temperature, growth performance, behavior, cecal microflora, and welfare [6-8]. Research indicates that short-chain fatty acids (SCFA) can lower intestinal pH, thereby promoting the proliferation of beneficial bacteria while inhibiting colonization of specific pathogens [9-11]. Additionally, studies have demonstrated reciprocal interactions between intestinal microflora and brain-gut peptide secretion [12]. For instance, *Bifidobacterium tetragenous* viable tablets can significantly increase VIP levels while decreasing substance P (SP) levels [13]. Brain-gut peptides such as 5-hydroxytryptamine (5-HT), VIP, and SP have been found to be associated with gastrointestinal activity [14]. However, no studies have reported the combined effects of stocking density under moderate heat stress on cecal microflora, volatile fatty acids, and serum brain-gut peptides in broilers. Therefore, this experiment aimed to investigate these effects to provide a theoretical basis for scientific broiler management.

1.1 Experimental Animals and Management

This study employed a single-factor design using 144 healthy 22-day-old Arbor Acres broilers with similar body weight, randomly divided into three groups with six replicates per group, each replicate housed in a single cage. All groups were transferred to an environmental chamber at 21°C and 60% relative humidity for a 7-day acclimation period. The formal experiment commenced when the broilers were 29 days old. With a single cage area of 0.64 m^2, stocking densities were established at 6 birds per cage (half male and half female, Group), 8 birds per cage (half male and half female, Group), and 10 birds per cage (half male and half female, Group). Environmental conditions were maintained at 31°C and 60% relative humidity until the end of the 14-day experiment. The trial was conducted in the environmental chamber of the State Key Laboratory of Animal Nutrition, with automatic temperature and humidity control (precision of ±1°C and ±7%, respectively), no airflow, and 24-hour lighting.

1.2 Experimental Diets

A corn-soybean meal basal diet was used, formulated as a powder according to NRC (1994) nutrient requirements. The composition and nutrient levels of the basal diet are presented in .

1.3 Management Practices

All experimental broilers were raised in single-tier floor cages developed by our laboratory [15], with free access to feed and water and conventional immuniza-

tion.

1.4 Sample Collection and Analysis

1.4.1 Blood Collection and Analysis On days 7 and 14 of the experiment, six broilers were randomly selected from each group (half male and half female, one bird per replicate). Blood was collected via wing vein, allowed to clot for 2 hours, then centrifuged at 3,000 rpm for 10 minutes at low temperature. Serum was harvested and stored at -80°C. Serum 5-HT, VIP, and SP concentrations were determined using enzyme-linked immunosorbent assay (ELISA).

1.4.2 Cecal Content Collection and Analysis 1.4.2.1 Cecal Content Collection
On days 7 and 14, six broilers per group (half male and half female, one bird per replicate) were euthanized by cervical dislocation. After whole-body disinfection, the abdominal cavity was opened, the intestine was isolated, and the ileocecal junction was ligated. The cecum was excised and rapidly transferred to a laminar flow hood, where the intestinal wall was aseptically opened using sterile scissors. Cecal contents were collected, and the six samples from each group were immediately mixed (not mixed for volatile fatty acid analysis), placed in sterile centrifuge tubes, snap-frozen in liquid nitrogen, and stored at -80°C.

1.4.2.2 Volatile Fatty Acid Measurement
Approximately 2 g of cecal digesta was weighed into a centrifuge tube, and 5 mL of ultrapure water was accurately added. The mixture was vortexed for 3-5 minutes and centrifuged at 5,000×g for 10 minutes. The supernatant (1 mL) was transferred to a plastic ampoule, mixed with 0.2 mL of 25% metaphosphoric acid, sealed, shaken, and placed in an ice bath for 30 minutes. After centrifugation at 10,000×g for 10 minutes, the supernatant was collected for determination of acetic, propionic, and butyric acid concentrations.

1.4.2.3 DNA Extraction
Genomic DNA was extracted from samples using the Fast DNA™ SPIN Kit for Soil.

1.4.2.4 Bacterial 16S rDNA Fragment PCR Amplification
Using extracted genomic DNA as template, bacterial universal primers GC-338F and 518R were employed to amplify the hypervariable region of 16S rDNA. Primer information is provided in . The PCR reaction mixture (50 L) contained: 10×PCR buffer 5 L, dNTP Mixture (2.5 mmol/L) 3.2 L, ExTaq (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 minutes; 30 cycles of 94°C for 1 minute, 55°C for 45 seconds, and 72°C for 1 minute; and final extension at 72°C for 10 minutes. PCR products were purified using the OMEGA DNA Gel Extraction Kit. A Biometra T-gradient thermocycler and Bio-Rad Gel-Doc2000 gel imaging system were used.

1.4.2.5 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, run at 150 V and 60°C for 5 hours in 1×TAE buffer. After electrophoresis, silver staining was performed as follows: (1) fixation in 50 mL ethanol and 2.5 mL glacial acetic acid diluted to 500 mL for 15 minutes; (2) two washes with Milli-Q water for 20 seconds and 2 minutes; (3) staining in 1 g silver nitrate and 0.75 mL 37% formaldehyde diluted to 500 mL for 15 minutes; (4) two washes with Milli-Q water for 20 seconds and 2 minutes; (5) development in 7.5 g sodium hydroxide and 2.5 mL 37% formaldehyde diluted to 500 mL for 5-7 minutes; and (6) termination in 50 mL ethanol and 2.5 mL glacial acetic acid diluted to 500 mL.

1.4.2.6 Sequencing of Dominant DGGE Bands

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

1.5 Statistical Analysis

Data were analyzed using SAS 9.2 software for one-way ANOVA, with Duncan' s multiple comparison test. Differences were considered significant at $P<0.05$. DGGE profile diversity was analyzed using Quantity One software.

2.1.1 PCR-DGGE Fingerprint Analysis of Cecal Microflora

As shown in [Figure 1: see original paper] and [Figure 2: see original paper], moderate heat stress and stocking density affected the number of cecal microflora bands in broilers on both days 7 and 14, with Group showing more bands than Groups and . According to , similarity indices among different stocking density groups ranged from 44.3% to 61.0%. The lowest similarity (44.3%) occurred between Groups and , while the highest similarity (61.0%) was observed between Groups and . These results indicate that Group had the highest cecal microflora richness, and the microflora structure differed more between Groups and than between Groups and .

2.1.2 Diversity Analysis of Cecal Microflora Structure

As presented in , stocking density under moderate heat stress influenced cecal microflora structural diversity. On day 7, Shannon and Simpson indices were 2.20 and 0.88 for Group , 2.36 and 0.90 for Group , and 2.12 and 0.87 for Group , respectively. On day 14, these values were 2.59 and 0.92 for Group , 2.70 and 0.93 for Group , and 2.57 and 0.92 for Group . Thus, Group exhibited higher cecal microflora diversity than the other two groups under moderate heat stress.

2.1.3 Analysis of Specific and Common Cecal Microflora

One specific band and four common bands were excised from the 16S rDNA V3 region PCR-DGGE fingerprints. As shown in [Figure 1: see original paper] and , common bands representing *Bacteroides uniformis* (band 2), *Eisenbergiella massiliensis* (band 3), *Clostridium straminisolvens* (band 4), and *Ruminococcus faecis* (band 5) were detected in all groups on both days 7 and 14. However, *Bacteroides vulgatus* (band 1) was detected only in Group . Among the five sequenced bands, most sequences belonged to *Firmicutes* and *Bacteroidetes*, with similarity to GenBank database sequences exceeding 92%.

2.2 Cecal Volatile Fatty Acid Concentrations

As shown in , stocking density under moderate heat stress had no significant effect on cecal volatile fatty acid (acetic, propionic, and butyric acid) concentrations on either day 7 or day 14 ($P>0.05$).

2.3 Serum Brain-Gut Peptide Concentrations

As presented in , on day 7, serum 5-HT content in Group was significantly higher than in Group ($P<0.05$), while stocking density had no significant effect on serum VIP or SP concentrations ($P>0.05$). On day 14, stocking density showed no significant influence on serum 5-HT, VIP, or SP levels ($P>0.05$).

3.1 Effects of Stocking Density on Cecal Microflora Diversity under Moderate Heat Stress

Intestinal microflora constitutes an essential component of the broiler' s internal environment and is beneficial to the host when in balance [16]. Environmental stress during poultry development can alter intestinal microflora structure [17]. Previous studies reported that moderate heat stress (26°C and 31°C) decreased cecal bacterial band numbers and microflora diversity in broilers [4], and that stocking density significantly affected digestive tract microflora at 3 weeks of age, with the most pronounced changes observed in the crop and cecum (R-values of 0.77 and 0.69, respectively, P 0.05). At 6 weeks, stocking density continued to significantly impact crop and cecal microflora (R-values of 0.52 and 0.27, respectively, P 0.05) [18]. The present study demonstrated that Group exhibited the highest cecal microflora richness and similarity under moderate heat stress, suggesting that a stocking density of 8 birds per 0.64 m^2 is conducive to cecal microflora growth.

Research indicates that *Firmicutes* predominates in the broiler cecum, followed by *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* [19-20]. Increased stocking density (above 450 cm^2/bird) has been shown to eliminate beneficial bacteria (*Lactobacillus gastricus* and *Lactobacillus alvi*) in the duodenum of caged laying hens, adversely affecting intestinal microflora balance [21]. This study identified *Bacteroides vulgatus* as a specific microflora component in Group , which was

absent in the other groups. *Bacteroides vulgatus* belongs to *Bacteroidetes*, the most abundant group of Gram-negative bacteria in the intestine that produces enzymes degrading plant cell walls and participates in digestive functions [4].

3.2 Effects of Stocking Density on Cecal Volatile Fatty Acid Concentrations

SCFAs are primary metabolites of intestinal microflora, mainly including acetic, propionic, and butyric acids, produced through anaerobic fermentation of fiber and resistant starch by intestinal bacteria [22]. SCFAs can lower intestinal pH, promoting beneficial bacterial growth while inhibiting pathogen colonization [9-11]. Additionally, intestinal microflora may influence synthesis and release of the important signaling molecule 5-HT via SCFA-mediated effects on enterochromaffin cells [23]. However, this study found no significant differences in cecal volatile fatty acid concentrations among stocking density treatments under moderate heat stress.

3.3 Effects of Stocking Density on Serum Brain-Gut Peptide Concentrations

Brain-gut peptides act directly on the central nervous system, serving as important chemical signals transmitted from the gastrointestinal tract to the brain. These signals can access the brain through the area postrema of the brainstem, affecting vagal efferent function and participating in regulation of gastrointestinal motility, appetite, and feeding behavior [24]. 5-HT, also known as serotonin, is a neurotransmitter and important intestinal regulator produced mainly by enteroendocrine cells, mediating intestinal motility and sensation while directly or indirectly stimulating intestinal secretion. Studies have reported elevated plasma tryptophan levels during early life when microflora is absent [25], and that *Bifidobacterium* can influence tryptophan metabolism, with oral administration of *B. infantis* increasing plasma concentrations of dopamine and 5-HT in rats [26].

VIP is a neurotransmitter of the non-cholinergic non-adrenergic inhibitory system that exerts inhibitory regulation on gastrointestinal activity, causing relaxation of the entire gastrointestinal circular muscle [27]. 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 [28], thereby reducing lipopolysaccharide-induced intestinal mucosal damage and promoting colonization of beneficial bacteria to restore normal microflora balance [29]. SP is a tachykinin that increases gastrointestinal motility, strongly promotes smooth muscle contraction, enhances colonic mass peristalsis, stimulates water and electrolyte secretion from small intestinal and colonic mucosa, dilates gastrointestinal blood vessels, increases vascular permeability, and participates in inflammatory and immune responses [30].

This study found that serum 5-HT content in Group was significantly higher than in Group under moderate heat stress, while stocking density had no significant effect on serum VIP or SP concentrations. These findings may be related to changes in intestinal microflora and warrant further investigation.

In summary, stocking density under moderate heat stress can alter cecal microflora diversity and structure as well as serum 5-HT content, but does not affect cecal volatile fatty acid concentrations. The precise relationships among these parameters require further exploration.

4 Conclusions

1. Under moderate heat stress, Group exhibited higher cecal microflora richness and similarity than Groups and , and this stocking density favored colonization of *Bacteroides vulgatus* in the cecum.
2. Stocking density under moderate heat stress had no significant effect on cecal volatile fatty acid concentrations.
3. Serum 5-HT content in Group was significantly higher than in Group , while stocking density under moderate heat stress had no significant effect on serum VIP or SP concentrations.

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