

Effects of Increasing Stocking Density on the Performance and Ileal Microbiota of Broilers

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This study was conducted to investigate the effects of increasing stocking density under suitable environmental conditions on the performance and ileal microbiota of broilers. A total of 108 Arbor Acres male broilers (28 days old) were allocated to a normal stocking density (NSD, normal stocking density; 31 kg/m²) and a maximum allowed stocking density group (MSD, maximum stocking density; 39 kg/m²). All birds were reared at a constant temperature of 21°C. At 42 days of age, bacterial DNA was extracted from ileal content, and the V3–4 hypervariable region of 16S rRNA was amplified. Increasing stocking density had no significant effect on average daily gain, average daily feed intake, and feed conversion ratio ($P > 0.05$). The alpha and beta diversities of the ileal microbiomes did not differ significantly between the NSD and MSD groups; however, increasing stocking density altered the composition of ileal microbiota. The relative abundance of Lactobacillales, including *Lactobacillus*, *Enterococcus*, and *Streptococcus*, significantly decreased in MSD broilers, compared with NSD broilers. The present results suggest that even under suitable environmental conditions, an increase in stocking density to a level of 39 kg/m² may disturb the composition of ileal microbiota in broilers. Further studies are needed to determine the reasons and the potential consequences for animal health and physiology.

Key words: broiler chicken, community structure, ileal microbiota, pyrosequencing, stocking density

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Introduction

The modern broiler industry frequently opts for high stocking densities because higher profits can be obtained through increasing the numbers of birds per unit space. However, higher stocking density (exceeding 34–38 kg/m² at the end of the growing period) has negative impacts on welfare, health, and performance of broilers and exacerbates mortality related to heat stress (reviewed by Estevez 2007). The gut microbiota plays a key role regarding health and growth of the host (Forder *et al.*, 2007; Niba *et al.*, 2009; Yin *et al.*, 2010). Several researchers have found that a high stocking density (46–50 kg/m²) decreased the abundance of *Lactobacillus* in the cecum (Zhang *et al.*, 2013) and small intestine (Cengiz *et al.*, 2015), and it increased the abundance

of *Escherichia coli* in the cecum (Zhang *et al.*, 2013). Using temporal temperature gradient gel electrophoresis, Guardia *et al.* (2011) found that high stocking density (43 kg/m²) affected the community structure of the crop and cecal microbiota of three-weeks-old broilers, suggesting that the effects of a high stocking density on the microbiota may compromise health and performance of birds.

The adverse effects of high stocking density are relevant regarding temperature, humidity, and ventilation rate within the shed (Dawkins *et al.*, 2004; Jones *et al.*, 2005). In Europe, the normal allowed stocking density is 33 kg/m², and a higher stocking density (up to 39 kg/m²) may be authorized if the producer addresses additional criteria, such as temperature, humidity, and NH₃ and CO₂ concentrations within the shed (European Commission 2007). In China, the recommended stocking density is 30 kg/m² (Standardization Administration of China 2005), and a higher stocking density (up to 39 kg/m²) is frequently used by some broiler production enterprises in spring or autumn. Under suitable environmental conditions, increasing stocking density from 33 to 39 kg/m² may have no marked effects on the health and performance of broilers. However, the effects of increasing stocking density on the gut microbiota of broilers are still unknown. The aim of this study was to provide more details about the variation in the

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gut microbiome in broilers reared at 39 kg/m² using high-throughput sequencing.

Materials and Methods

This study was approved by the Animal Welfare and Ethics Committee of the Institute of Animal Science, Chinese Academy of Agricultural Sciences (approval number: IAS 2021-114). The birds were managed according to the Arbor Acres broiler management guide and in compliance with the Guidelines for Experimental Animals established by the Ministry of Science and Technology (Beijing, China). One-day-old male Arbor Acres broiler chicks were obtained from a commercial hatchery and were reared in controlled climate chambers using a standard corn-soybean meal diet (Table 1). At 28 days of age, 108 broiler chicks with similar body weight were selected and allocated to a normal stocking density group (NSD) and a maximum allowed stocking density group (MSD), with six single-deck replicate cages (0.8×0.8 m, plastic net floor) per group. Eight birds were placed in each of the six cages in the NSD treatment (12.5 birds/m², 31 kg/m², assuming a final body weight [BW, body weight] of 2.5 kg at the end of the growing period), and 10 birds were used per cage in the MSD treatment (15.6 birds/m², 39 kg/m²). The controlled climate chambers were maintained at a constant temperature of 21±1°C and 60±10% relative humidity during a 14-days trial period.

Sample Collection

At 42 days of age, six randomly selected birds from each group were euthanized by cervical dislocation. The terminal ileum (3–4 cm to the ileocecal junction) content of each bird was aseptically collected in sterile vials, frozen in liquid

nitrogen, and stored at –80°C until used for the sequencing of microbial genes. The other birds in each cage were weighed after 12 h of fasting, and feed consumption was recorded on a cage basis. The average daily gain, average daily feed intake, and feed conversion ratio during the trial period (from 29 to 42 days of age) were calculated.

DNA Extraction

Total bacterial DNA was extracted from frozen ileum content using the E.Z.N.A.[®] Stool DNA Kit (Omega Bio-Tek, Norcross, USA), according to the manufacturer's instructions. DNA integrity was assessed visually using 1.0% (w/v) agarose gel (containing ethidium bromide) electrophoresis.

16S rDNA Gene Sequencing

The microbial 16S rDNA was amplified using a KAPA HiFi Hotstart PCR kit, with universal primers U341F (5'-ACTCCTACGGGAGGCAGCAG-3') and U806R (5'-GGA-CTACHVGGGTWTCTAAT-3') targeting the V3–4 region. The PCR products were analyzed by 2.0% agarose gel electrophoresis and were recovered using an AxyPrep DNA Gel Recovery Kit (Axygen Biosciences, Union City, CA, USA). The 16S rRNA gene high-throughput sequencing was performed by the Realbio Genomics Institute (Shanghai, China) using the Illumina HiSeq PE250 platform (Illumina, San Diego, USA). The sequence data reported in this study have been deposited in the NCBI SRA database under accession no. PRJNA560155.

Bioinformatic Analyses

Raw fastq files were quality-filtered using Trimmomatic and merged using FLASH software. USEARCH (version 7.1, <http://drive5.com/uparse/>) was used to filter chimeras, and the remaining sequences were clustered to generate

Table 1. Composition and nutrient content of the basal diet

Items	Starter (day 1–21)	Finisher (day 22–42)
Ingredients, %		
Corn	53.22	56.29
Soybean meal	38.50	35.52
Soybean oil	4.10	4.50
Limestone	1.15	1.00
Dicalcium phosphate	2.01	1.78
DL-Methionine	0.22	0.11
Sodium chloride	0.30	0.30
Vitamin-mineral premix ^a	0.50	0.50
Calculated composition, %		
ME (MJ kg ⁻¹)	12.46	12.74
CP	21.44	20.07
Calcium	1.00	0.90
Nonphytate phosphorus	0.45	0.40
Lysine	1.17	1.00
Methionine	0.56	0.42
Methionine + Cystine	0.91	0.78

^aPremix provided the following nutrients per kilogram of diet: vitamin A, 12,500 IU; vitamin D3, 3,750 IU; vitamin E, 20 IU; vitamin K3, 2.5 mg; vitamin B1, 2.5 mg; vitamin B2, 8.0 mg; vitamin B6, 2.5 mg; vitamin B12, 0.015 mg; pantothenic acid calcium, 12.5 mg; nicotinic acid, 32.5 mg; folic acid, 1.25 mg; biotin, 0.125 mg; choline, 1,000 mg; Zn(ZnSO₄·7H₂O), 60 mg; Fe(FeSO₄·7H₂O), 80 mg; Cu(CuSO₄·5H₂O), 8 mg; Mn(MnSO₄·H₂O), 110 mg; I(KI), 0.35 mg; Se(Na₂SeO₃), 0.15 mg

operational taxonomic units (OTUs) at a 97% similarity level. A representative sequence of each OTU, operational taxonomic unit was assigned to a taxonomic level using the RDP Classifier (version 2.11, <http://sourceforge.net/projects/rdp-classifier/>) against the Silva (SSU132) 16S rRNA database (<http://www.arb-silva.de>) using a confidence threshold of 70%. To compare samples with different sequencing depths, each sample was rarefied to the same number of reads (31,611 sequences). Alpha diversity indices, including observed species, chao1, simpson even, and Simpson diversity, were calculated. To assess beta diversity, the Bray–Curtis distance was determined using the QIIME pipeline (version 1.9.1, <http://qiime.org/tutorials/tutorial.html>).

Statistical Analysis

The cage was considered the experimental unit ($n=6$ cages per treatment), and data on growth performance were analyzed using Student's t-test in SAS 9.4 software (SAS Institute, Cary, NC, USA). Differences were considered significant at $P<0.05$.

The Mann–Whitney test was performed to analyze differences in alpha diversity and mean relative abundance of dominant bacterial taxa between the two groups. Principal coordinate analysis (PCoA, principal coordinate analysis) and analysis of similarity (ANOSIM, analysis of similarity) tests were conducted based on the Bray–Curtis distance. The linear discriminant analysis (LDA, linear discriminant analysis) effect size (LEfSe, LDA effect size) method was used to compare the differential abundances of bacteria among groups at different taxonomic levels (threshold ≥ 2).

Results

Effects on Performance

The effects of increasing stocking density on growth performance of broilers are shown in Table 2. Compared with the NSD group, the final body weight, average daily gain, average daily feed intake, and feed conversion ratio of broilers were not significantly different between the two groups ($P>0.05$).

Effects on Alpha Diversity

Increasing stocking density had no significant effect on the alpha diversity of broiler ileal microbiota. Species richness estimators (observed species, Chao 1), evenness index (Simpson even), and diversity index (Simpson) were not

significantly different between NSD and MSD (Table 3).

Effects on Beta Diversity

PCoA revealed that NSD did not form a distinct cluster that was clearly separated from the MSD based on the Bray–Curtis distance (Fig. 1A). The ANOSIM test statistic (R) was -0.009 for the Bray–Curtis distance ($P=0.406$; Fig. 1B).

Comparison of Microbial Composition

The dominant phyla (relative abundance $>1\%$) in the NSD and MSD samples were Firmicutes (85.65% and 90.90%), Proteobacteria (8.48% and 3.17%), Cyanobacteria (0.76% and 5.17%), and Bacteroidetes (5.05% and 0.72%, respectively; Fig. 2A). The relative abundances of these dominant phyla were not significantly different between the NSD and MSD groups. At the order level, the ileal microbiota of the broilers in the NSD and MSD groups were dominated by Clostridiales (72.63% and 87.66%), Lactobacillales (12.91% and 3.20%), Enterobacteriales (7.31% and 1.10%), norank-c-Cyanobacteria (0.76% and 5.16%), and Bacteroidales (5.05% and 2.18%, respectively; Fig. 2B). The relative abundance of Lactobacillales in the MSD samples was significantly lower than that in the NSD samples ($P=0.02$); the other predominant orders were not significantly different between the two groups. At the genus level, the ileal microbiota of broilers in the NSD and MSD groups were dominated by unclassified-f-*Peptostreptococcaceae* (42.61% and 62.32%), *Romboutsia* (25.07% and 21.53%), *Lactibacillus* (10.46% and 3.04%), *Escherichia-Shigella* (7.21% and 1.06%), norank-c-*Cyanobacteria* (0.76% and 5.16%), and *Bacteroides* (5.02% and 0.54%, Fig. 2C). The relative abundances of *Lactibacillus* and *Escherichia-Shigella* in the MSD samples were significantly lower than those in the NSD samples ($P<0.05$), whereas the other predominant genera were not significantly different between the two groups.

We employed LEfSe to identify the specific taxa in the ileal microbiome that were significantly associated with increasing stocking density. The LEfSe results (Fig. 2D) showed that the lineages Bacilli- Lactobacillales- Lactobacillaceae- Lactobacillus, - Enterococcaceae- *Enterococcus*, and - Streptococcaceae- *Streptococcus*, were notably lower in the MSD samples than in the NSD samples. The lineages Negativicutes, Selenomonadales, Acidaminococcaceae, *Phascolarctobacterium*, and Clostridiaceae- *Candidatus-Arthromitus* in MSD samples were also lower than those in NSD samples.

Table 2. Effects of increasing stocking density on growth performance of broilers

Measurements	Treatment ^a		P-value
	NSD	MSD	
Initial BW, g/bird	1325.0 ± 13.5	1328.3 ± 10.5	0.647
Final BW, g/bird	2546.7 ± 38.9	2567.6 ± 53.9	0.460
Weight gain, g/day/bird	87.26 ± 2.99	88.52 ± 3.65	0.530
Feed intake, g/day/bird	175.55 ± 4.78	178.68 ± 3.00	0.204
FCR	2.01 ± 0.05	2.02 ± 0.06	0.636

^a Broilers were reared at normal stocking density (NSD; 31 kg/m²) and maximum allowed stocking density (MSD; 39 kg/m²); six replicates per treatment were used

Table 3. Effects of increasing stocking density on alpha diversity of the ileal microbiota in broilers

Items		NSD ^a	MSD ^a	P-value
Richness estimators	Observed Species	70.67±6.19	66.00±40.66	0.38
	Chao1	83.64±8.81	92.15±37.90	0.99
Evenness index	Simpsoneven	0.04±0.02	0.03±0.01	0.57
Diversity index	Simpson	0.57±0.20	0.29±0.27	0.09

^aNSD, normal stocking density (31 kg/m²); MSD, maximum allowed stocking density (39 kg/m²).

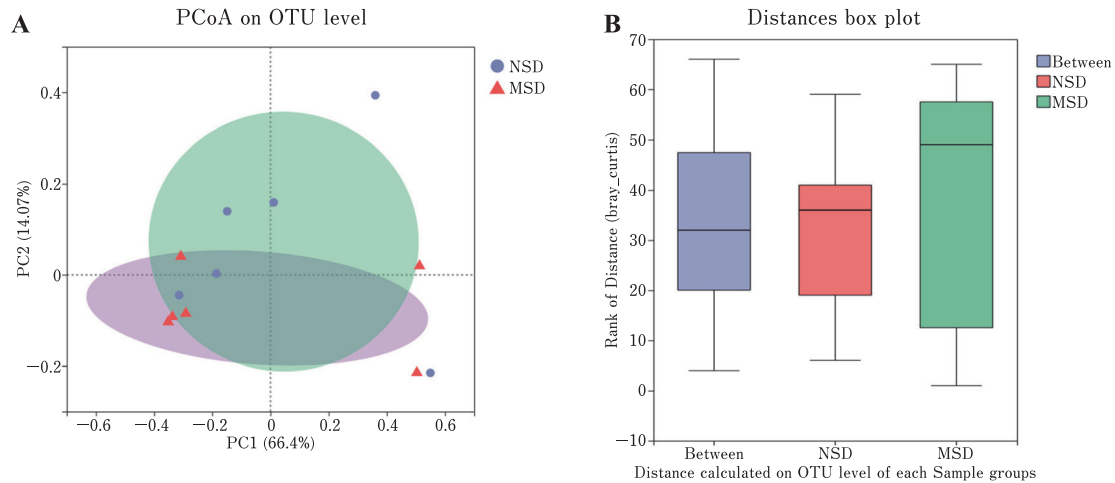


Fig. 1. Effects of increasing stocking density on beta diversity of the ileal microbiota in broilers. A: Principal coordinate analysis (PCoA) based on the Bray-Curtis distance for each individual broiler at normal stocking density (NSD) and maximum allowed stocking density (MSD). B: Analysis of similarity (ANOSIM) based on the Bray-Curtis distance for similarity within the NSD and MSD treatments, and among all samples.

Discussion

High stocking density (exceeding 34–38 kg/m²) has negative impacts on the welfare, health, and performance of broilers (Estevez 2007). The adverse effects of high stocking density are relevant to the environment within the shed (Dawkins *et al.*, 2004; Jones *et al.*, 2005). Dawkins *et al.* (2004) found that at a range of densities from 30 to 46 kg/m², broiler health and welfare was to a great extent determined by the quality of the environment provided by producers. The allowed stocking density is increased from 33 to 39 kg/m² if temperature, humidity, and air quality within the shed are strictly controlled (European Commission 2007). The present results confirmed that under suitable environmental conditions, increasing stocking density from 31 to 39 kg/m² had no significant effect on the performance of broilers.

High stocking density (46–50 kg/m²) influenced the community structure of the gut microbiota of three-weeks-old broilers, but did not significantly affect the bacterial community in the ileum at six weeks of age (Guardia *et al.*, 2011). The results of the present study show that under suitable environmental conditions, increasing stocking density to 39 kg/m² had no significant effects on the community

structure of six-weeks-old broiler ileal microbiota. High stocking density decreased the counts of *Lactobacillus* in the cecum (Zhang *et al.*, 2013) and small intestine (Cengiz *et al.*, 2015), and increased the counts of *Escherichia coli* in the cecum (Zhang *et al.*, 2013). The results of the present study show that increasing stocking density decreased the relative abundance of *Lactobacillus* and *Escherichia/Shigella* in ileal samples.

The ileum is the main site of nutrient absorption, which harbors large (10⁸–10⁹ cells g⁻¹) bacterial populations (Apajalahti *et al.*, 2004). The microbial composition in the ileum of broilers affects intestinal function and nutrient absorption (Stanley *et al.*, 2014). *Lactobacillus* is the dominant genus in the ileum of broilers (Choi *et al.*, 2014; Oakley *et al.*, 2014; Xiao *et al.*, 2016). Most species of *Lactobacillus* confer health benefits to the human and animal gut and have a long history of safe use as probiotic strains (Heeney *et al.*, 2018). *Lactobacillus* species can improve intestinal morphology and barrier integrity, protect the host against enteric pathogens, and alleviate inflammation and intestinal impairment (Li *et al.*, 2018). The decrease in *Lactobacillus* in broilers stocked at 39 kg/m² indicated that increasing stocking density may have a detrimental impact on the health of broilers. However,

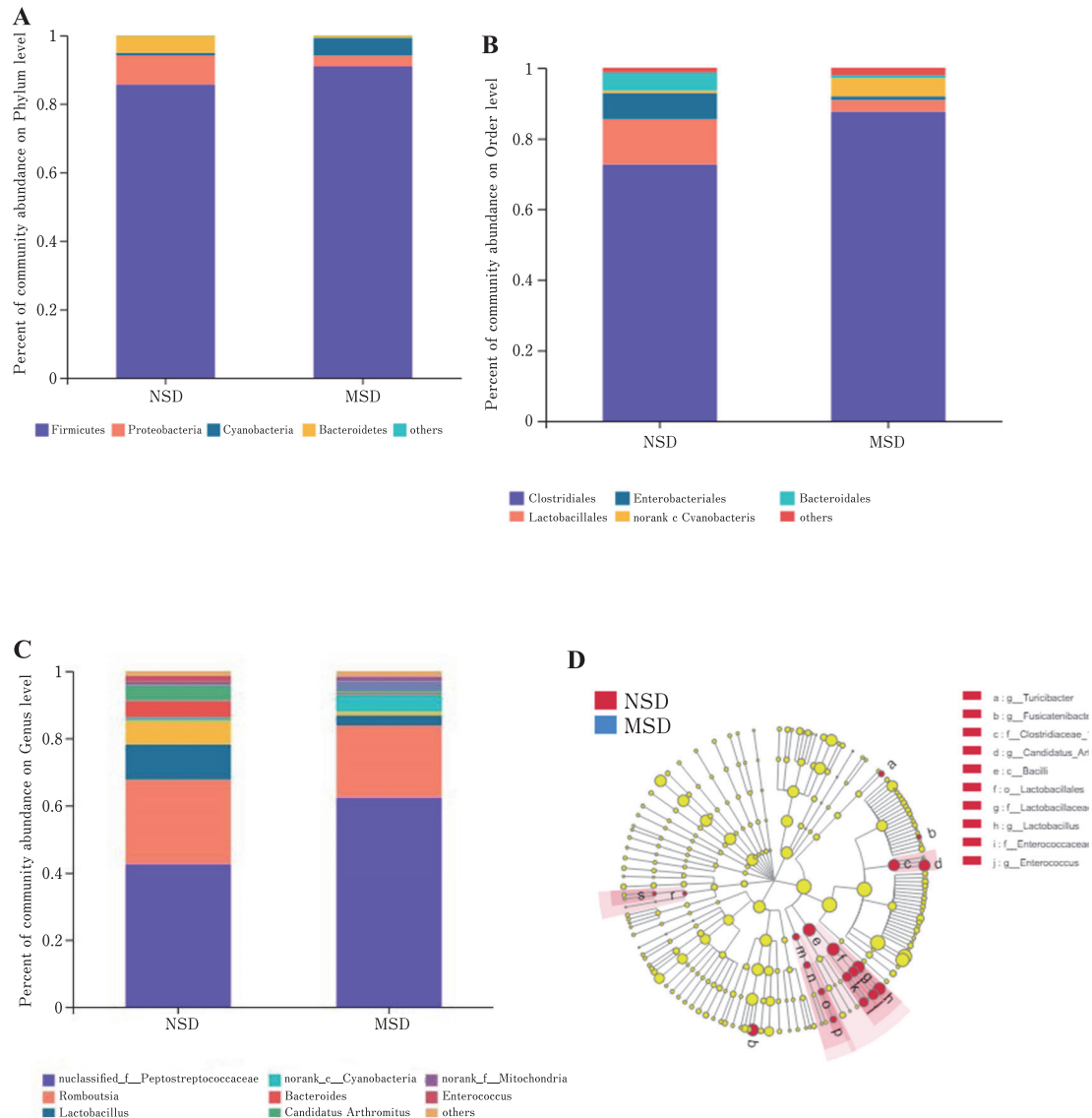


Fig. 2. Effects of increasing stocking density on the microbiota composition in broilers ileum. The relative abundance of the major phyla (>1%, A), orders (>2%, B) and genus (>1%, C) in the ileal content at normal stocking density (NSD) and the maximum allowed stocking density (MSD). D: Differences in bacterial taxa among groups determined by linear discriminative analysis effect size (LEfSe). Circle color indicates change in abundance: yellow, no change; red, decreased in MSD; and blue, elevated in MSD. An LEfSe score of ≥ 2 was considered significant.

the relative abundance of *Escherichia/Shigella* in broilers stocked at 39 kg/m² also decreased. *Escherichia/Shigella* taxa were given a different genus name to distinguish pathogenic forms from the non-pathogenic *E. coli* (Lan and Reeves, 2002). Therefore, the consequences of increasing stocking density to 39 kg/m² altering the composition of the ileal microbiota requires further study.

The effect of high stocking density on the gut microbiota may be due to changes in the litter bacterial composition (Guardia *et al.*, 2011). High stocking density increases litter

moisture and microbial load (Jayalakshmi *et al.*, 2009). Litter is ingested by chickens and affects the composition of the digestive microbiota (Torok *et al.*, 2009). However, it is unlikely that this occurred in the present study because the birds were reared on a plastic net floor and rarely were in contact with manure or litter. High stocking density decreases the feed intake of broilers (Bessei 2006; Estevez 2007), which may influence the gut microbiota of broilers. Xing *et al.* (2019) found that the changes in the cecal microbiota of laying hens during heat stress are mainly associated with

reduced feed intake. However, it is unlikely that this occurred in the present study because increasing stocking density from 31 to 39 kg/m² had no significant effects on the feed intake of broilers. High stocking density may limit the movement of broilers. Inactivity may lead to gut microbial dysbiosis in humans (Monda *et al.*, 2017; Sket *et al.*, 2018). We did not observe the movement of broilers reared at different stocking densities. Therefore, it remains unclear how increased stocking density altered the composition of ileal microbiota of broilers, and the consequences regarding animal health and physiology require further research.

In conclusion, under suitable environmental conditions, increasing stocking density from 31 to 39 kg/m² had no significant effects on the performance of broilers, but altered the composition of the ileal microbiota.

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Conflict of Interest

The authors have no conflict of interest to declare.

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