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Caecal microbiome and metabolites associated with different growth performances of broilers

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ABSTRACT

We investigated changes in the caecal microbial composition and metabolic compounds of broiler chickens weighing approximately 0.8-1.5 kg. Arbor Acres (AA) broilers (n =186) were divided into four groups (A-D) according to body weight on day 35. The results showed that there were significant differences in the average daily feed intake (ADFI), average daily gain (ADG) and feed-to-gain ratio (F:G) of chickens ($P < 0.05$). The abundance of 11 genera were found to be significantly different in the four groups ($P < 0.05$). The broilers with poor performance had increased levels of D-mannose, hexadecanoic acid, cholesterol, L-valine, L-leucine, glutamic acid, glucopyranose, α-D-allopyranose and phosphoric acid ($P < 0.05$) in the cecum. Microbial compositions were different in the ceca of broilers with different growth performances and higher growth performance correlated with changes in metabolic pathways related to energy, amino acids and others.

Key words: Broilers, Growth performance, Metabolites, Microbiome.

INTRODUCTION

Broiler performance has been found to be influenced by a range of factors such as breed, sex, diet and growth environment (Korver, *et al*. 2004; Brake *et al*. 2003; Ali *et al*. 2018; Ghosh *et al*. 2012). However, even for broilers of the same breed, sex and age that are raised in controlled environments with the same diet and water, the growth performance of individuals is different. Some studies have investigated the effects of the environment (Butler *et al*. 2016), age, sex (Lumpkins *et al*. 2008), supplement addition (Biswas *et al*. 2018) and differential feed conversion ration (Stanley *et al*. 2012) on the gut microbiota of chickens. In poultry, the cecum is the most studied gastrointestinal tract because it is an ideal habitat for the microbiome and caecal microbiota have the ability to digest feeds rich in cellulose, starch and resistant polysaccharides (Clench and Mathias 1995).

In this study, we report changes in the cecal microbiome and metabolites in broilers with different growth performances using high-throughput sequencing and gas chromatography mass spectrometry (GC/MS). Our findings may be applicable not only to the livestock industry but also to human health.

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MATERIALS AND METHODS

A total of 200 one-day-old Arbor Acres broilers were obtained from a local commercial hatchery (Jilin Deda Company, Changchun, China). The broilers were placed on a plastic net (6 m×10 m) and each broiler was weighed with the stomachs empty on day 35. One hundred eighty-six AA broilers were selected and divided into four groups according to body weight, with the chickens of each group differing by 0.2 kg: group A (1.5≃1.7 kg), group B (1.3≃1.5 kg), group C (1.0≃1.3 kg) and group D (0.8≃1.0 kg). Each group had 46, 53, 76 and 11 broilers, respectively. Groups A and B had 6 replicate pens with approximately equal to 8 to 9 broilers per pen and group C had 6 replicate pens with 12≃13 broilers per pen and the replicates were placed in cages (1.5 m×1 m×0.75 m). Group D had six replicates with 1≃2 broilers that were placed in small cages (0.3 m×0.3 m×0.75m). The diet and water were offered *ad libitum* and the broilers were fed a standard corn/soybean meal ration without antimicrobial additives which met the 1994 National Research Council (NRC 1994, Table 1) standards.

Table 1: Ingredient and chemical composition of the basal diets.

Ingredients	1-21 d	22-42 d	Nutritive value	1-21 d	22-42 d
Corn	52.07	55.57	Metabolizable energy, MJ/kg	12.75	12.72
Soybean	35.50	34.00	Crude Protein, %	20.72	19.61
Fish meal	5.00	3.60	Calcium, %	0.91	0.87
Soybean oil	4.00	3.5	Total Phosphorus, %	0.68	0.58
Phosphate	1.00	0.70	Available Phosphorus, %	0.45	0.36
Limestone	1.00	1.20	Lysine, %	1.35	1.25
Salt	0.30	0.30	Methionine, %	0.46	0.43
Lysine	0.04	0.04			
Methionine	0.09	0.09			
Additive	1.00	1.00			
Total	100	100			

Supplied per kilogram of diet: vitamin A, 5,000 IU; vitamin D3, 1500 IU; vitamin E, 15 IU; menadione, 0.8 mg; vitamin B12, 0.01mg; folic acid, 0.5mg; nicotinic acid, 50 mg; biotin, 0.1 mg; pantothenic acid, 8 mg; pyridoxin, 2.2 mg; riboflavin, 4.4 mg; thiamine mononitrate, 1.6 mg. Mineral premix contained per kilogram of diet: Fe, 80g; Cu, 6 mg; Mn, 100 mg; Zn, 80 mg; I, 0.4 mg; Se, 0.2 mg.
The nutritive values of Crude protein, Metabolizable energy, calcium, Avail P and Lysine were the calculated values.

During the experimental period from days 35 to 42, feed intake was recorded daily for each pen. On day 42, broilers were weighed with the empty stomachs by the pen in the morning to detect the ADFI, ADG and F:G. Two broilers per replicate pen were sacrificed on day 42 and one side of the caecum (digesta) was tied using string and stored at -20°C for DNA extraction and the remaining part was stored at -80°C until GC/MS analysis.

DNA of the cecum was isolated using the Fast DNA Spin Kit for Soil (MP Biotechnology, USA) in accordance with the manufacturer's protocol. The DNA samples were sent to Biomarker Technologies Co, Ltd (China) for PCR amplification and high-throughput sequencing with the Illumina Hiseq method.

The cecal digesta samples were dried by a freeze drier (MIKRO-22R, Germany) for 24 h and 0.05 g of dried samples were mixed with 100 µl (20 mg/ml) methoxyamine hydrochloride and pyridine and vigorously vortexed and mixed for 30 s in 1.5 ml tubes. Then the samples were heated in a water bath at 37°C for 90 min, followed by 200 µl bis (trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) being added and heated at 70°C for 60 min. The tubes were taken out and left to stand for 10 min at room temperature and subsequently the samples were centrifuged at 13,000×*g* at 4°C for 5 min. Then 50 µl of the supernatant of each sample was transferred to a GC vial.

Each sample (1 µl) was injected into an Agilent 7890/5975C system equipped with a fused silica capillary column (30.0 m×0.25 mm i.d.) with a 0.25-µm HP-5MS stationary phase (Agilent, Shanghai, China). Helium was used as the carrier at a constant flow rate of 1.0 mL/min. The temperature of the column was maintained at 70°C for 4 min, increased to 100°C at a rate of 4°C/min and maintained for 5 min, increased to 150°C at a rate of 10°C/min and maintained for 10 min, then increased to 240°C at a rate of 5°C/min and maintained for 8 min and finally increased to 270°C at a rate of 10°C/min and maintained for 5 min. The temperature of injection, quadrupole and ion source were 280, 230 and 150°C, respectively. The mass spectra were acquired from 20-800 *m/z*.

Data of growth performance and cecal microbiota (at the genus level) were analyzed using the SPSS 17.0 and differences between means were assessed using Duncan's multiple-range test. The results are presented as means ± standard deviation. The metabolic profile data were processed by SIMCA 13.0 software and orthogonal partial least squares-discriminate analysis (OPLS-DA) and principal component analysis (PCA) were employed to process the cecum metabolomic data. Both the variable importance in the projection (VIP>1) values and Student's *t* test ($P<0.05$) were compared to find the metabolites among the groups.

RESULTS AND DISCUSSION

The ADFI of group D was significantly lower than those of groups A, B and C ($P < 0.05$, Table 2). The ADG of the groups A, B, C and D on day 42 increased with increasing weight on day 35 and there were significant differences among the groups ($P < 0.05$). Compared with group A, the F:G was significantly increased by 14.86%, 42.86% and 72.00% in groups B, C and D, respectively. It can be concluded that the ADFI, ADG and feed conversion ratio were lowest in group D with the poorest growth performance.

In Table 2, there were big differences in the growth performances of broilers even though they were the same breed and were fed the same diet under the same conditions. Broilers with high body weight showed higher feed conversion ratios than those with lower body weights. Similar results were reported by Stanley *et al*. (2012), who demonstrated that the composition or proportion of cecal microbiota and metabolites are different among different growth performance broilers.

All groups of different weight broilers harbored diverse lineages of bacterial phyla. A total of 10 phyla were detected

Table 2: The difference of growth performance of broilers from day 35 to 42 in different groups.

Item	A (1.5k~1.7kg)	B (1.3kg~1.5kg)	C (1.0kg~1.3kg)	D (0.8kg~1.0kg)
ADFI/g	154.75±21.31[a]	151.99±15.50[a]	158.56±18.20[a]	126.89±8.38[b]
ADG/g	88.03±8.56[a]	75.39±4.50[b]	63.18±4.07[c]	42.16±3.67[d]
F:G	1.75±0.09[a]	2.01±0.09[b]	2.50±0.17[c]	3.01±0.44[d]

in all groups: *Bacteroides*, *Firmicutes*, *Tenericutes, Verrucomicrobia, Proteobacteria, Actinobacteria, Synergistetes, Cyanobacteria* and *Lentisphaerae* (Fig 1). The predominate phyla were *Firmicutes* and *Bacteroidetes,* followed by *Proteobacteria* and *Tenericutes* in groups A, B and C. However, *Verrucomicrobia* was the third most abundant phylum behind phyla *Firmicutes* and *Bacteroidetes* in group D. The proportions of phyla *Bacteroidetes* in group C and *Firmicutes* in group D were lower than those in other groups. Only trace amounts of *Fusobacteria* were detected in groups C and D.

At the genus level, a total of 89 genera were detected among the four groups and the abundances of genera in the top 10 are listed in Fig 2. Sequences that could not be classified into any known genus were named as unknown. The predominate genus was *Alistipes* followed by *Barensiella* and *Bacteroides*. Significant relative abundances of genera in the four groups of different weight broilers are listed in Table 3. Genus *Parabacteroides* in groups B and D was significantly higher than that in groups A and C. Genus *Coprobacter* was higher in groups A and B; however, genus *Barnesiella* was the most abundant when compared with the other groups (P<0.05). The proportions of genera *Streptococcus* and *Blautia* in group A were larger than in groups B, C and D, respectively (P < 0.05). The abundances of genera *Incertae Sedis* and *Subdoligranulum* in group C were the highest and were significantly higher than those in group A (P < 0.05) and genus *Anaerofilum* in group C was higher than in the other groups. Genera *Ruminococcus* in group A, *Escherichia-Shigella* in group D and *Streptococcus* in group C were significantly higher than those in group B (P < 0.05).

Bacteroidetes and *Firmicutes* were found to be the predominate phyla among all the tested samples and made up more than 90% of the cecal microbiota. *Bacteroidetes* and *Firmicutes* are known to utilize complex carbohydrates and produce short-chain fatty acids, which provide energy and regulate metabolism (Benítez-Paez *et al*. 2016; Pieper *et al*. 2012). In Fig 1, the abundances of phyla *Bacteroidetes* in group C and *Firmicutes* in group D were the lowest when compared with other groups. *Fusobacteria* were detected in groups C and D and this phylum is widely pathogenic to other vertebrates (Gupta and Sethy 2014). It is likely that high concentrations of phyla *Bacteroidetes* and *Firmicutes* in the cecum contributed to increased broiler weight, but phylum *Fusobacteria* had the opposite effect. Human study has shown that the ratio of *Bacteroidetes* to *Firmicutes* is correlated with weight (Ley *et al*. 2005). However, there was no significant correlation between weight and the *Bacteroidetes*/*Firmicutes* ratio in the present study, even though the broilers showed significantly different growth performances. And there was no significant difference in abundances among groups at the phylum level, while the compositions of *Bacteroidetes, Firmicutes*, *Proteobacteria,* and *Actinobacteria* at the genus level were altered in different

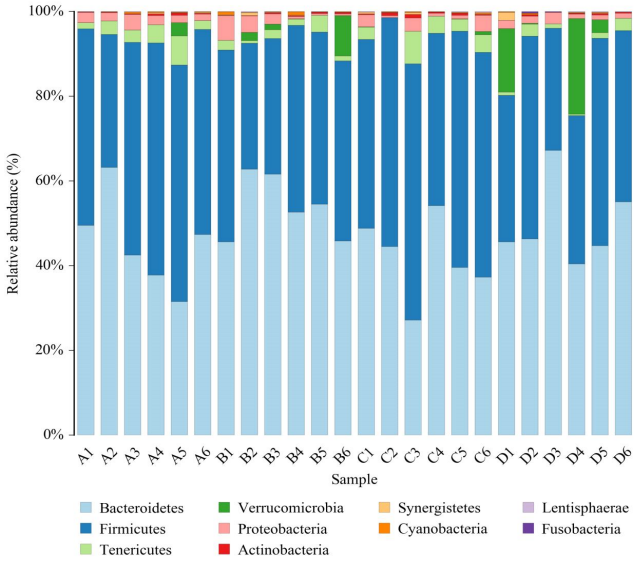


Fig 1: Relative abundance of microbial communities at phylum level of broilers.

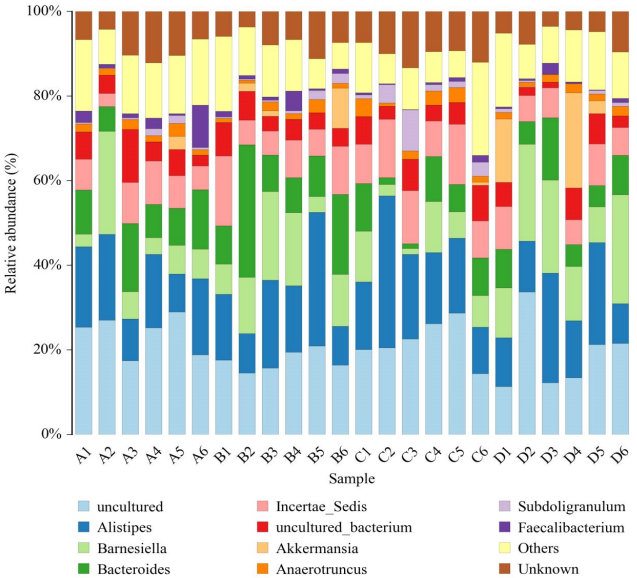


Fig 2: Relative abundance of microbial community at genus level of broilers.

groups. Not all proportions of the same genus changed consis- tently with increasing or decreasing of weights of broilers.

Principle component analysis (PCA) score plots of the four groups of cecum samples are shown in Fig 3. Each dot stands for a sample and the sample C-6 was removed since it was outside of the 95% confidence interval. Based on the PCA results and the OPLS-DA plots of the cecum metabolomic data, there were clear separations between groups A and C, groups A and D, groups B and C, groups B

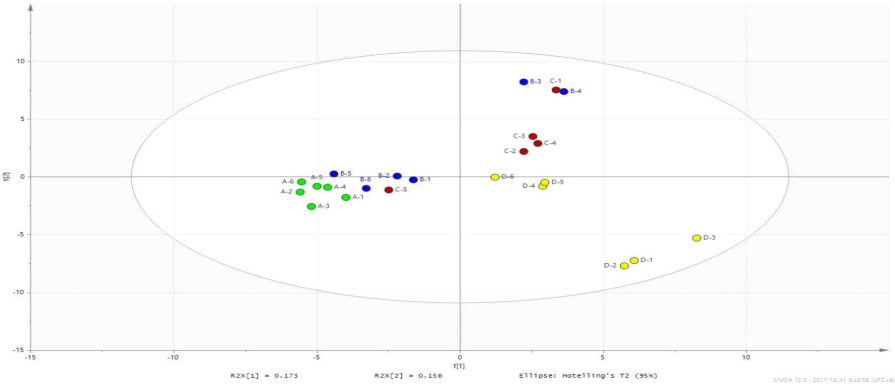


Fig 3: PCA score plots of different groups and each dot stands for a cecal sample of four groups.

Table 3: Relative abundance of genera level that were significantly affected of the four groups.

phylum	genus	Group A	Group B	Group C	Group D
Bacteroidetes	*Parabacteroides*	0.55±0.18^{a}	1.39±0.29^{b}	0.25±0.14^{a}	1.95±0.81^{b}
	Coprobacter	0.94±0.28^{b}	1.10±0.40^{b}	0.28±0.07^{a}	0.92±0.42^{ab}
	Barnesiella	8.61±3.23^{a}	12.50±2.59^{ab}	7.00±1.84^{a}	17.30±2.91^{b}
Firmicutes	*Streptococcus*	0.95±0.42^{b}	0.07±0.03^{a}	0.27±0.10^{ab}	0.36±0.24^{ab}
	Blautia	0.67±0.13^{c}	0.49±0.10^{bc}	0.24±0.07^{a}	0.31±0.06^{b}
	Incertae_Sedis	7.31±1.08^{a}	9.12±1.73^{ab}	11.20±1.09^{b}	7.63±0.76^{ab}
	Anaerofilum	0.08±0.02^{a}	0.09±0.03^{a}	0.23±0.06^{b}	0.08±0.02^{a}
	Ruminococcus	1.14±0.35^{b}	0.30±0.07^{a}	0.40±0.16^{ab}	0.38±0.10^{ab}
	Subdoligranulum	0.82±0.29^{a}	1.02±0.39^{ab}	3.54±1.35^{b}	0.55±0.17^{ab}
Proteobacteria	*Escherichia-Shigella*	0.12±0.05^{b}	0.01±0.01^{a}	0.12±0.08^{ab}	0.28±0.15^{b}
Actinobacteria	*Eggerthella*	0.19±0.04^{ab}	0.14±0.03^{a}	0.30±0.08^{b}	0.16±0.05^{ab}

Table 4: Different cecum metabolites among the different groups.

Metabolic pathway	metabolites	groups	VIP	*P*-value	FC
Carbohydrate metabolism	D-mannose	A vs C	4.47	0.03	2.99
		C vs D	5.24	0.05	0.39
Lipid metabolism	Hexadecanoic acid	A vs D	2.94	0.01	1.77
		B vs D	2.62	0.03	1.42
	Cholesterol	A vs D	1.34	0.04	1.95
		B vs D	1.83	<0.01	2.23
Amino acid metabolism	L-valine	A vs C	4.47	0.03	2.99
		A vs D	1.28	<0.01	2.49
	L-leucine	A vs C	1.29	0.01	2.59
		A vs D	1.39	<0.01	2.78
	Glutamic acid	A vs C	1.89	0.03	1.69
Other	Glucopyranose	A vs C	1.59	0.01	9.12
	α-D-allopyranose	A vs C	1.41	0.02	3.99
		B vs C	1.99	<0.01	7.84
	Phosphoric acid	A vs D	1.18	<0.01	5.15
	Butanedioic acid	B vs D	1.21	<0.01	3.89
		C vs D	1.19	<0.01	2.76

FC: fold change in the matabolite concentration (later group/former group).

and D and groups C and D, respectively, which were related to four KEGG pathways: carbohydrate metabolism, lipid metabolism, amino acid metabolism and other metabolic pathway.

A total of 10 metabolic candidates were identified among the four different groups and are shown in Table 4. For carbohydrate metabolism, the concentration of D-mannose in group C increased by 2.99-fold compared with group A ($P = 0.05$). The concentrations of hexadecanoic acid and cholesterol, which are related to lipid metabolism, were increased by 1.42- to 2.23-fold in group D compared with groups A and B ($P < 0.05$). The concentrations of L-leucine and L-valine in groups C and D were significantly increased, respectively, compared with group A ($P < 0.05$). The concentrations of glutamic acid, glucopyranose and α-D-allopyranose in group C were higher than those in group A, and α-D-allopyranose increased by 7.87-fold compared with group B ($P < 0.05$). The concentration of phosphoric acid in group D was significantly higher than that in group A ($P < 0.01$). The concentration of butanedioic acid in group D was increased by 3.89-fold and 2.76-fold compared with groups B and C, respectively ($P < 0.01$).

In the current study, metabolomic profiling was used to investigate the impact of growth performance on broiler cecum metabolites. As a result, energy, amino acids and other metabolic factors were significantly changed with changing weight. The increased D-mannose in the cecum of group D may have been caused by disturbed carbohydrate metabolism so that it was not absorbed by the host. Lipid metabolism is closely associated with the growth performance (Zhao *et al*. 2007). Besides carbohydrate and lipid pathways, amino acid metabolism pathways, such as for L-valine and L-leucine, were implicated. Valine has been proposed to be the 4th limiting amino acid (Tavernari *et al*. 2013) and Ferreira *et al*. (2016) reported that the body weight gain of broilers increased with increasing digestible valine intake. The present results showed that 10 significantly changed metabolites were more enriched in the lower growth performance groups (groups C and D) than in the best growth performance group (group A), which is consistent with the findings of Zhou *et al*. (2016), who reported that most colonic compounds of carbohydrate metabolism, lipid metabolism and amino acid metabolism are more enriched in a low protein diet group (lower growth performance) when compared with a normal protein diet group (higher growth performance). The current results indicated that broilers with poor performance might have restricted energy, amino acids and other metabolic pathways in the cecum, which might influence the absorption of dietary nutrition.

CONCLUSION

We find that chickens with higher growth performance contain microorganisms and metabolites that contribute to more efficient performance, while those with lower growth performance have negatively influenced nutrition digestibility.

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