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Cecal microbiome divergence of broiler chickens by sex and body weight

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The divergence of gut bacterial community on broiler chickens has been reported as potentially possible keys to enhancing nutrient absorption, immune systems, and increasing poultry health and performance. Thus, we compared cecal bacterial communities and functional predictions by sex and body weight regarding the association between cecal microbiota and chicken growth performance. In this study, a total of 12 male and 12 female 1-day-old broiler chickens were raised for 35 days in 2 separate cages. Chickens were divided into 3 subgroups depending on body weight (low, medium, and high) by each sex. We compared chicken cecal microbiota compositions and its predictive functions by sex and body weight difference. We found that bacterial 16S rRNA genes were classified as 3 major phyla (Bacteroidetes, Firmicutes, and Proteobacteria), accounting for > 98% of the total bacterial community. The profiling of different bacterial taxa and predictive metagenome functions derived from 16S rRNA genes were performed over chicken sex and bodyweight. Male chickens were related to the enrichment of *Bacteroides* while female chickens were to the enrichment of *Clostridium* and *Shigella*. Male chickens with high body weight were associated with the enrichment of *Faecalibacterium* and *Shuttleworthia*. Carbohydrate and lipid metabolisms were suggested as candidate functions for weight gain in the males. This suggests that the variation of cecal bacterial communities and their functions by sex and body weight may be associated with the differences in the growth potentials of broiler chickens.

Keywords: body weight, broiler chicken, cecal bacterial community, metagenome function, sex, 16S rRNA sequencing

Introduction

The gastrointestinal tract (GIT) of animals harbors a broad and complex array of microorganisms (a.k.a., microbiome) that plays a vital role in animal health, nutrition, physiology, and subsequent productive performance (Lan *et al.*, 2005; Wei *et al.*, 2013). A variety of factors has been known to influence the GIT microbiome of animals, including genotype (Turnbaugh *et al.*, 2006), nutrient composition in feeds (Torok *et al.*, 2013), feed additives (Singh *et al.*, 2013), and age (Niu *et al.*, 2015). Therefore, advanced knowledge on GIT microbiome with profitable factors are essential to improve animal health and productive performance.

The chicken is the most common livestock in the world and serves as an important animal model for other animal health and production (Burt, 2007). As observed in other mammals, male chickens show a faster growth rate than female chickens probably due to sexual differences in growth and development. However, this sexual variation in the growth rate may also be associated with differences in the GIT microbiome between sexes because the GIT microbiome has a considerable effect on nutrient digestion, absorption, and metabolism in animal's body (Turnbaugh *et al.*, 2006; Rinttilä and Apajalahti, 2013), and it is also highly associated with host immune systems and health status in animals (Lan *et al.*, 2005; Kogut, 2013). However, information regarding how the GIT microbiome varies between sexes in poultry is very scarce. Also, it is recognized that alteration in the GIT microbiome is closely linked with body weight (BW) of animals including pigs (Guo *et al.*, 2008), chickens (Rinttilä and Apajalahti, 2013), and humans (Ley *et al.*, 2006). However, the interaction between sex and BW of chickens for the GIT microbiome has not been elucidated.

Development of DNA sequencing has extended the knowledge of microbial composition, structure, and diversity. Because of its ability to process sequence reads of uncultivated microbes, the next generation sequencing (NGS) enables the provision of much microbial genomic information sampled from the environment, human, and animals. Amplicon sequencing targeting 16S rRNA gene has been widely applied to explore bacterial structure, composition, and diversity (Sharpton, 2014). This developed sequencing method has allowed understanding the diversity and functions of GIT microbiota in livestock animals (Choi *et al.*, 2015).

The objectives of the current experiment, therefore, were to investigate the cecal microbiome of broiler chickens differing in sex and BW via the NGS technique.

Materials and Methods

Animals and sample peparation

A total of 12 male and 12 female ROSS 308 broiler chickens at hatching were obtained from a local commercial hatchery (Yangji hatchery, Pyeongtaek, Republic of Korea) and were raised in wire-floored battery cages (76 × 78 × 45 cm, width × length × height) placed in an environmentally controlled room. Male and female birds were housed separately in the

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cage. The initial BW of birds was 40.2 ± 3.8 g. A commercial-type broiler diet was fed to birds *ad libitum* for 35 days. Water was available at all times. The room temperature was maintained at 30°C during the first week of the experiment and then gradually decreased to 24°C at the end of the experiment. A 24 h lighting schedule was used. No birds were dead during the experiment. At the conclusion of the experiment, all birds were weighed and categorized sequentially into 3 BW groups within each sex (n = 4; female with low BW = FL, female with medium BW = FM, female with high BW = FH, male with low BW = ML, male with medium BW = MM, and male with high BW = BH). Feeds were not provided to chickens for 12 h. All birds were euthanized by cervical dislocation; then the cecum was ligated at both sides and removed from the GIT. The contents were aseptically collected into an Eppendorf tube. The cecal contents were immediately frozen at -80°C until analysis. The protocol for this experiment was reviewed and approved by the Institutional Animal Care and Use Committee at Chung-Ang University.

Cecal bacterial DNA extraction and sequencing

Approximately each 0.5 g of cecal content aliquots centrifuged at 16,000 rpm for 5 min were used for DNA extraction with QIAamp Fast DNA Stool Mini kit (Qiagen), following manufacturer's protocols. Extracted bacterial DNA was amplified with primers (forward: 5′-CCAGCAGCYGC GGTAAN-3′ and reverse: 5′-CCGTCAATTCNTTTRAGT-3′) targeting V4-V5 hypervariable region of bacterial 16S rRNA genes. Amplicons were generated as following; denaturation at 95°C for 3 min, 25 cycles of amplification including denaturation (95°C, 30 sec), annealing (55°C, 30 sec), and elongation (72°C, 30 sec), and final elongation at 72°C for 5 min. The final products were subjected to be sequenced using Illumina MiSeq platform at Macrogen Inc.. The 16S rRNA gene sequences were submitted to the NCBI's Sequence Read Archie (SRA) with the accession number SRP105986.

Table 1. Body weight distribution of broiler chickens at the end of the experiment

Sex	Body weight	PD	Chao1
Female	Low	27.80	865
	Medium	28.20	1,116
	High	25.85	825
Male	Low	25.28	777
	Medium	25.15	706
	High	24.20	678
Pooled SEM (n = 4)		2.439	150.4
Sex	Female	27.28	935
	Male	24.88	720
Pooled SEM (n = 12)		1.408	86.8
Body weight	Low	26.54	821
	Medium	26.68	911
	High	25.03	751
Pooled SEM (n = 8)		1.725	106.3
P - value for main effects			
Sex		0.24	< 0.10
Body weight		0.76	0.58
Sex × Body weight		0.96	0.53

Body weight data analysis

Data for BW within each sex (n = 4) and between sexes (n = 12) were analyzed using the GLM procedure of SAS (SAS Institute). When BW was significantly different ($P < 0.05$), means were compared using Duncan's multiple range comparison procedures of SAS. Significance and the tendency for statistical tests were set at $P < 0.05$ and $0.05 \leq P \leq 0.10$, respectively.

Microbial community analysis

16S rRNA data and its richness were explored via Quantitative Insights Into Microbial Ecology 1.9.0 software (QIIME; http://qiime.org). Illumina adapters and primers were removed from raw sequences. Trimmed forward and reverse sequences were combined (Eren *et al.*, 2013). These sequences were clustered into 97% similarity operational taxonomic units (OTUs) with UCLUST (Edgar, 2010). Taxonomic assignment of reference OTU sequences was performed by UCLUST Consensus TaxonAssigner (Bokulich *et al.*, 2015) against Greengenes database (McDonald *et al.*, 2012) with 0.5 confidence threshold, and was taxonomically identified as up to species level. For the analysis of bacterial richness, sequences were rarefied with the steps of 3,000 to compare the number of OTUs picked from the same number of sequences. Rarefied OTUs were used for the measurement of bacterial richness using the total length of phylogenetic branches (Phylogenetic Diversity [PD]) (Faith and Baker, 2006), and relative proportions of rare sequences (Chao1) (Neufeld and Mohn, 2005). Unweighted UniFrac distance (Lozupone and Knight, 2005; Chang *et al.*, 2011) was used for the comparison of bacterial communities depending on chicken sex or BW. Based on both phylogenetic and sample information, redundancy analysis (RDA) with clustered OTUs were performed to compare bacterial community structures in R statistical software version 3.3.0 (R Developement Core Team, 2015). Application of sex and BW to Hellinger-transformed OTUs were performed, when analyzing RDA. To assess whether both sex and BW groups had significantly been separated, we used a non-parametric statistical test, analysis of similarity (ANOSIM). The significance of differences between groups was determined through permutations (n = 999) using *vegan* package in R statistical software.

Profiling of predictive metagenome functions from bacterial communities

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) provided predictive metagenome functions from bacterial 16S rRNA gene data (Langille *et al.*, 2013). Complete known genomes and query genomes were required for the prediction of metagenome functions. The newest Greengenes reference database was downloaded (download available at http://greengenes.secondgenome.com) and used for closed-reference OTU assignment. Closed-reference OTUs were picked at 97% similarity against Greengenes and were normalized by calculation of 16S rRNA gene copy number abundance. Then, metagenome functions of bacterial communities were predicted and categorized with Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Due to the diversity of metabolic path-

Table 2. Relative abundance of top 7 bacterial taxa in chicken ceca

Phylum [%][1]	Class [%]	Order [%]	Family [%]	Genus [%]
Bacteroidetes [58.66 ± 2.22]	Bacteroidia [58.64 ± 2.32]	Bacteroidales [58.64 ± 2.22]	Bacteroidaceae [51.24 ± 2.36]	*Bacteroides* [51.24 ± 2.46]
Firmicutes [37.80 ± 1.92]	Clostridia [37.06 ± 1.94]	Clostridiales [36.94 ± 1.85]	Lachnospiraceae [9.57 ± 0.70]	*Ruminococcus*[2] [2.09 ± 0.47]
				Shuttleworthia [0.87 ± 0.14]
			Ruminococcaceae [18.38 ± 1.17]	*Oscillospira* [6.72 ± 0.54]
				Faecalibacterium [4.46 ± 0.60]
				Ruminococcus [2.08 ± 0.13]
Proteobacteria [1.65 ± 0.47]	Gammaproteobacteria [1.63 ± 0.49]	Enterobacteriales [1.63 ± 0.47]	Enterobacteriaceae [1.63 ± 0.49]	*Escherichia* [1.55 ± 0.47]

[1] Mean percentages and standard error (S.E.) of bacterial abundance observed from 24 samples.
[2] Genus *Ruminococcus* belonging to Lachnospiraceae family is required to be reclassified because some *Ruminococcus* have been reclassified to a new genus, *Blautia*.

ways found in predicted metagenome functions, we narrowed pathways by selecting carbohydrate, energy, endocrine system, lipid, and glycan metabolism about nutrient metabolisms.

Identification of bacterial features that discriminate groups

Linear Discriminant Analysis Effect Size (LEfSe) explored the bacterial features that differentiate bacterial communities of sex or BW groups in the ceca of broiler chickens (Segata *et al.*, 2011). All bacterial quantitative taxa and functional prediction estimations were applied to the calculation of effect size on sex and BW. The threshold on logarithmic Linear Discriminant Analysis (LDA) score were set as 2.0 (default). Kruskal-Wallis rank-sum statistical test was conducted during the LDA score calculation to assess whether the measurements that discriminate groups were significant. Statistically different bacterial taxa and metabolic pathways were used for the analyses of the relationships between bacterial communities and sex or BW.

Results and Discussion

Analysis of microbial community in chicken ceca

Bacterial communities in the ceca of broiler chickens were profiled using hypervariable regions (V4-V5) of bacterial 16S rRNA genes from all 24 broiler chickens. A total of 1,567,757 raw reads from Illumina MiSeq platform were merged into 320,976 reads with an average of taxonomic units (OTUs) using UCLUST algorithm (Edgar, 2010). A total of 8 phyla, 21 classes, 32 orders, 55 families, 104 genera, and 155 species were assigned via UCLUST Consensus TaxonAssigner that uses Greengenes database (Bokulich *et al.*, 2015). Three major phylum-level phyla (Bacteroidetes, Firmicutes, and Proteobacteria) dominated cecal bacterial community more than 98% (Table 2). Of these phyla, the most dominant phylum was Bacteroidetes with an average of 58.66 ± 2.22%, followed by Firmicutes (37.80 ± 1.92%), and Proteobacteria (1.65 ± 0.47%). The other phyla such as Tenericutes, Acidobacteria, Actinobacteria, and Verrucomicrobia also were classified, but these bacteria were present at relatively very low abundance (less than 1.5%). At the genus level, we found that genus *Bacteroides* belonged to Bacteroidetes was the most predominant cecal bacterium. Not only several Firmicutes such as genera *Ruminococcus*, *Oscillospira*, and *Faecalibacterium* but also genus *Escherichia*, one of Proteobacteria, also were observed (more than 0.3%).

Our results revealed that the abundance of each phylum was different from previous studies (Wise and Siragusa, 2007; Wei *et al.*, 2013; Choi *et al.*, 2014). We found that chicken ceca were mainly occupied by *Bacteroidetes*, whereas Torok *et al.* (2011), Wei *et al.* (2013), and Choi *et al.* (2014) reported *Firmicutes* were the most predominant microbes in the ceca of chickens. The reason for these variations among experiments may be associated with the fact that establishment of the GIT microbiome is influenced by the types of diets (Voreades *et al.*, 2014). The relationship between GIT microbiome and the efficacy of energy harvest from food components has been investigated for several years. De Filippo *et al.* (2010), Tremaroli and Bäckhed (2012) reported the relationship between diet type and human GIT microbiome, suggesting that diet type has a significant impact on the shaping

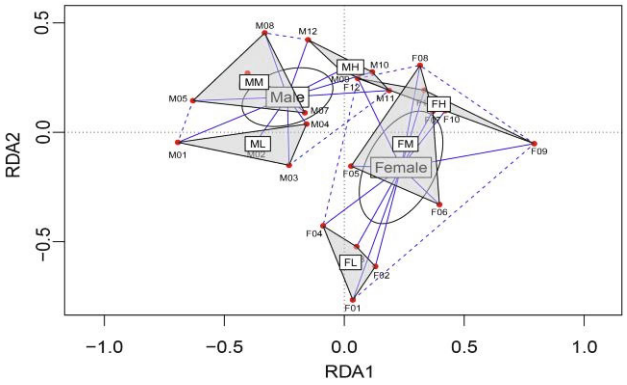

Fig. 1. **Redundancy analysis of the microbial association by sex and BW.** Circles refer to the dispersion of bacterial community of each sex group and solid lines represent the connection of samples from the centroid of each bacterial communities group. The dotted lines connect each of samples on the edges. The associations of bacterial community-sex ($P < 0.01$) and bacterial community-BW ($P < 0.01$) are statistically significant tested by analysis of similarity (ANOSIM) (permutations = 999).

Table 3. Bacterial community differences between male and female broiler chickens using Linear Discriminant Analysis Effect Size (LEfSe)

Group[1]	Phylum	Class	Order	Family	Genus	Species	LDA score (log_{10})[2]	*P*-value[3]
M	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*		4.71	< 0.05
	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	*Blautia*	*producta*	2.61	< 0.01
F	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	*Alistipes*		2.84	< 0.05
	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	*Alistipes*	*massiliensis*	2.67	< 0.05
	Bacteroidetes	Bacteroidia	Bacteroidales	Unclassified	Unclassified		2.88	< 0.01
	Firmicutes						4.55	< 0.05
	Firmicutes	Clostridia					4.55	0.04
	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	*Clostridium*		2.86	< 0.05
	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	*Clostridium*	*citroniae*	2.77	0.04
	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	*Clostridium*	Unclassified	2.65	0.02
	Firmicutes	Clostridia	Clostridiales	Mogibacteriaceae			3.09	0.01
	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	*Clostridium*		3.56	0.02
	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	*Clostridium*	*maritimum*	3.45	0.02
	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	*Holdemania*		3.46	0.01
	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Shigella*		3.12	0.01
	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Shigella*	*sonnei*	2.42	0.01
	Proteobacteria	Gammaproteobacteria	Pseudomonadales				4.36	0.01
	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae			4.33	0.01

[1] Abbreviation of each group (Male; M, Female; F)
[2] Logarithmic (base 10) Linear Discriminant Analysis (LDA) scores of each differentially abundant bacterium.
[3] Kruskal-Wallis sum-rank test for the detection of the features with significantly differential abundance between groups.

of GIT microbiome. Pourabedin and Zhao (2015) also reported that feed components affected the GIT microbiome, in particular for the lower part of GIT. Specifically, *Bacteroidetes* and *Firmicutes* are strongly associated with diet types for human GIT microbiome. For example, high fiber and low-fat diets are reported to increase the number of *Bacteroidetes*, whereas low fiber and high-fat diets are reported to increase the number of *Firmicutes* in not only humans but also chicken GIT microbiome. Short chain fatty acids (SCFAs) including acetate, propionate, and butyrate generated by GIT microbiota have been related to BW changes (Clarke *et al.*, 2012). Increased amounts of SCFAs, especially for acetate, are found in overweight individuals. Conversely, mannanoligosaccharides (MOS) in diets are reported to alter cecal bacterial communities with increased number of *Bacteriodetes* (Conterno *et al.*, 2011).

Variation of bacterial communities by chicken sexes and body weight

Redundancy analysis (RDA) showed that bacterial communities of broiler chickens were well-separated depending on their sex (Fig. 1). Both female and male broiler chickens were also well-separated depending on their BW (low, medium, and high). Analysis of similarity (ANOSIM) represented that the division of bacterial communities by sex was statistically significant ($P < 0.01$) as well as BW ($P < 0.01$). This result shows that bacterial communities in chicken ceca vary for sex and their performance.

Several bacterial taxa were found to discriminate different sex broiler chicken groups via LEfSe (Segata *et al.*, 2011). Female broiler chickens were linked to the increased relative abundance of Bacteroidetes, Firmicutes, and Proteobacteria (Table 3). In particular, genera *Alistipes*, *Holdemania* (Bacter-

Table 4. Bacterial community differences of body weights in female broiler chickens using Linear Discriminant Analysis Effect Size (LEfSe)

Group[1]	Phylum	Class	Order	Family	Genus	Species	LDA score (log_{10})[2]	*P*-value[3]
L	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Oscillospira*		4.45	0.02
	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Anaerofilum*		3.47	0.02
	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Clostridium*	*hungatei*	3.12	0.04
	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	*cc_115*		3.23	0.04
	Firmicutes	Unclassified					3.14	0.04
	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*Rhizobium*	Unclassified	3.12	0.04
M	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	*Alistipes*		3.30	0.02
	Bacteroidetes	Bacteroidia	Bacteroidales	Unclassified			3.24	0.03
	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	*vaginalis*	3.43	0.02
	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Unclassified		3.33	0.03
	Firmicutes	Clostridia	Clostridiales	Ruminococcaceaeh	*Sporobacter*	*termitidis*	3.18	0.02
	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Shigella*	*sonnei*	3.24	0.03
H	Tenericutes						3.82	0.02

[1] Abbreviation of each group (Low; L, Medium; M, High; H)
[2] Logarithmic (base 10) Linear Discriminant Analysis (LDA) scores of each differentially abundant bacterium.
[3] Kruskal-Wallis sum-rank test for the detection of the features with significantly differential abundance between groups.

Table 5. Bacterial community differences based on body weights in male broiler chickens using Linear Discriminant Analysis Effect Size (LEfSe)

Group[1]	Phylum	Class	Order	Family	Genus	Species	LDA score (log_{10})[2]	*P*-value[3]
L	Firmicutes	Clostridia	Clostridiales	Ruminococcus	*Clostridium*	*methylpentosum*	2.10	0.02
H	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	*Shuttleworthia*		3.41	0.04
	Firmicutes	Clostridia	Clostridiales	Ruminococcus	*Faecalibacterium*	Unclassified	2.58	0.03
	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	Unclassified		2.79	0.04
	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Cronobacter*		2.87	0.03

[1] Abbreviation of each group (Low; L, Medium; M, High; H)
[2] Logarithmic (base 10) Linear Discriminant Analysis (LDA) scores of each differentially abundant bacterium.
[3] Kruskal-Wallis sum-rank test for the detection of the features with significantly differential abundance between groups.

oidetes), and *Clostridium* (Firmicutes) were enriched. There also were *Shigella* and Moraxellaceae affiliated to Proteobacteria associated to increased relative abundance in female broiler chickens. Similarly, male broiler chickens were linked to the enriched relative abundance of Bacteroidetes and Firmicutes, but only two genus level taxa (*Bacteroides* and *Blautia*) were detected as enriched bacteria that discriminate male and female groups. At the species level, increased abundance of *Alistipes massiliensis, Clostridium citroniae*, *Clostridium maritimum*, and *Shigella sonnei* showed strong correlations with female chickens, whereas that of *Blautia producta* was intensely related to male chickens. Lumpkins *et al.* (2008) and Zhao *et al.* (2013) proposed that the sex be the important factor affecting GIT bacterial communities, representing different bacterial ecosystems between male and female chickens. However, little is known why male and female chickens have different GIT bacterial ecosystems. We hypothesized that bacterial differences in the ceca between female and male chickens are related to different biological processes such as sex hormone secretion (Schumacher *et al.*, 2014). Despite limited knowledge of the relationship between GIT microbiome and sex in the ceca of chickens, we found a statistically significant difference of bacterial taxa between sexes. In the current experiment, many anaerobic Firmicutes (e.g. genera *Oscillospira*) and Tenericutes were observed in female chickens, whereas many Bacteroidetes, particularly genus *Bacteroides* were observed in male chickens. These bacteria are known to be related to the ability to degrade indigestible fiber in the GIT. Thus, it is suggested that these bacterial taxa profiled from chicken ceca regulate biological processes within the GIT that discriminate female and male chickens such as degrading feed components.

Cecal microbiome by BW in the female and male broiler chickens

Within each sex, different bacterial taxa were identified depending on BW. In female broiler chickens, we observed Bacteroidetes, Firmicutes, Proteobacteria, and Tenericutes with increased relative abundance, but broiler chickens with high BW had only Tenericutes dominantly (Table 4). Several Firmicutes members including *Oscillospira*, *Anaerofilum*, *Clostridium hungatei*, and *cc_115*, and unclassified *Rhizobium* affiliated to Proteobacteria were significantly linked to broiler chickens with low BW. Conversely, the enrichment of *Alistipes* (Bacteroidetes), *Lactobacillus*, unclassified Lachnospiraceae, *Sporobacter* (Firmicutes), and *Shigella* (Proteobacteria) were detected in broiler chickens with medium BW. At the species levels, mostly observed bacteria in broiler chicken with low BW were classified as *Clostridium hungatei*. With respect to broiler chickens with medium BW, relative abundances of *Lactobacillus vaginalis*, *Sporobacter termitidis*, and *Shigella sonnei* were enriched, compared with other BW groups.

LEfSe showed that a total of five bacterial taxa determined the discrimination among BW groups in male broiler chickens (Table 5). Increased bacterial abundance of *Clostridium* belonged to Firmicutes was linked to male broiler chickens with low BW. In contrast, the prevalence of *Shuttleworthia*, *Faecalibacterium*, and unclassified Erysipelotrichaceae (Firmicutes), and *Cronobacter* (Proteobacteria) was significantly higher in male broiler chickens with high BW. At species level, bacteria identified as *Clostridium methylpentosum* became enriched in the chicken ceca with low BW, whereas increased abundance of unclassified *Faecalibacterium* that

Table 6. Predictive metagenome functions that discriminate between sex groups

Group[1]	Predicted biological process[2]		LDA score (log_{10})	*P*-value[3]
F	Endocrine system	Progesterone-mediated oocyte maturation	2.67	0.01
	Energy metabolism	Methane metabolism	2.96	< 0.05
	Glycan biosynthesis and metabolism	Peptidoglycan biosynthesis	2.95	0.04
	Lipid metabolism	Glycerophospholipid metabolism	3.01	0.03
		Lipid biosynthesis proteins	2.34	0.04
M	Carbohydrate metabolism	Carbohydrate metabolism	2.48	0.02
		Fructose and mannose metabolism	2.68	< 0.05
		Galactose metabolism	2.93	0.04
	Lipid metabolism	Arachidonic acid metabolism	2.44	0.02

[1] Abbreviation of each group (Male; M, Female; F)
[2] Predicted Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways from 16S rRNA genes via Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt). Carbohydrate metabolism, energy metabolism, lipid metabolism, and glycan biosynthesis and metabolism were chosen to compare differences between groups using LEfSe algorithm.
[3] Kruskal-Wallis sum-rank test for the detection of the features with significantly differential abundance between groups.

Table 7. Predicted metagenome function differences based on male broiler chicken body weights

Group[1]	Predicted biological process[2]		LDA score (log_{10})	*P*-value[3]
M	Glycan biosynthesis and metabolism	N-Glycan biosynthesis	2.49	< 0.05
	Lipid metabolism	Arachidonic acid metabolism	2.57	< 0.05
		Steroid hormone biosynthesis	2.62	0.04
H	Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism	3.03	0.02
	Lipid metabolism	Biosynthesis of unsaturated fatty acids	2.55	0.04

[1] Abbreviation of each group (Low; L, Medium; M, High; H)
[2] Predicted Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways from 16S rRNA genes via Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt). Carbohydrate metabolism, energy metabolism, lipid metabolism, and glycan biosynthesis and metabolism were chosen to compare differences between groups using LEfSe algorithm.
[3] Kruskal-Wallis sum-rank test for the detection of the features with significantly differential abundance between groups.

was related to broiler chickens with high BW was identified. LEfSe could not identify any bacterial taxa related to broiler chickens with medium BW. These results suggest that differences in cecal bacterial communities may be associated with the productive performance of chickens, due to different metabolic pathways of each bacterium. Conterno *et al.* (2011), Clarke *et al.* (2012), and Okeke *et al.* (2014) investigated the effect of BW on gut microbiota, and they revealed increased numbers of Firmicutes and decreased numbers of Bacteroidetes in obese individuals. Many Firmicutes were related to degrading indigestible feed, such that a host can uptake more nutrients and subsequently gain more weight. Our results showed that Firmicutes were widely spread regardless of chicken BW. Although Firmicutes are involved in the fermentation of indigestible feeds, they seem to have a less impact on weight gain at least in female chickens. Despite less association between weight gain and Firmicutes in female chickens, several of them including *Shuttleworthia* and *Cronobacter* have a significant effect on the BW in male chickens. It is speculated that these microbes contribute to weight gain (Cho *et al.*, 2012; Fei and Zhao, 2013).

Predictive metagenome functions in broiler chickens

Predicted KEGG pathways from 16S rRNA gene sequences were profiled via PICRUSt to compare between sexes and among BW groups within each sex. Fifty five weight-gain-related metabolic pathways affiliated to carbohydrate, energy, endocrine system, lipid, and glycan metabolism were selected from a total of 328 predicted KEGG pathways. These biological pathways were applied to LEfSe for identifying discriminatory factors between female and male chickens. Functional prediction revealed that energy metabolism, glycan biosynthesis/metabolism, and lipid metabolism were different by sex (Table 6). For example, methane metabolism (energy metabolism), peptidoglycan biosynthesis (glycan biosynthesis and metabolism), glycerophospholipid metabolism, and lipid biosynthesis proteins (lipid metabolism) may be related to female broiler chickens. Conversely, arachidonic acid metabolism (lipid metabolism), as well as carbohydrate metabolism including fructose, mannose, and galactose metabolisms may be related to male broiler chickens. BW based metagenome function predictions explored that carbohydrate, energy, lipid, and glycan metabolism, and endocrine system may be potential factors of weight gain (Table 7). In female broiler chickens, we did not profile any KEGG pathways that discriminate BW groups, but glycan biosynthesis and metabolism, lipid metabolism, and carbohydrate metabolism were profiled in male broiler chickens. Singla *et al.* (2010) and Nikiforova *et al.* (2014) highlighted the effects of obesity on metabolic reactions such as carbohydrate and lipid metabolism. Both metabolisms were associated with weight gain by increasing glyoxylate level in blood linking hyperglycemia (Nikiforova *et al.*, 2014) and basal lipolysis in adipose tissue (Singla *et al.*, 2010). Thus, these metabolic pathways may be a candidate for further researches in weight gain and growth performance of broiler chickens.

Conclusion

Bacterial communities in broiler chicken ceca were statistically significantly different by sex and BW. Differential bacterial taxa and biological processes (e.g. carbohydrate, energy, and lipid metabolism) by inferred functional analysis will likely extend our knowledge on chicken GIT microbiome as well as improve chicken health and productivity.

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