Cecal microbiota contribute to the development of woody breast myopathy

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ABSTRACT The objective of this study was to characterize the bacterial diversity of cecal microbiota in broilers related to breast phenotype, diet, and genetic strain. Broilers from 2 genetic strains (120 birds/strain) were fed a control diet (15 birds/pen) and an amino acid reduced diet (15 birds/pen, digestible lysine, total sulfur amino acids, and threonine reduced by 20% compared to the control diet). At 8 wk of age, 4 male broilers with normal breast (**NB**, 1 chick per pen) and 4 male broilers with woody breast (**WB**, 1 chick per pen) were selected for each treatment (strain × diet). The DNA of cecal samples was extracted and the 16S rRNA genes were sequenced and analyzed. There were no differences ($P > 0.05$) in the alpha diversity of gut microbiota between 2 phenotypes (NB vs. WB), 2 strains, or 2 diets (control vs. reduced). However, principal coordinate analysis plots (beta diversity) revealed that there were composition differences in samples between the 2 phenotypes ($P = 0.001$) and the 2 diets ($P = 0.024$). The most abundant phyla in all samples were *Firmicutes*, followed by *Bacteroidetes* and *Proteobacteria*. There were differences (false discovery rate, **FDR** < 0.05) in bacterial relative abundance between phenotypes and between diet treatments, but not (FDR > 0.05) between the 2 genetic strains. *Selenomonas bovis* (12.6%) and *Bacteroides plebeius* (12.3%) were the top 2 predominant bacteria in the ceca of WB birds; however, the relative abundances of these 2 bacteria were only 5.1% and 1.2% in NB birds, respectively. Function analysis predicted that the metabolic activities differed (q < 0.05) only between phenotypes. The microbiota of WB birds was characterized as reduced glycolysis and urea cycle but increased tricarboxylic acid (**TCA**) cycles, sugar degradation, and purine and pyrimidine nucleotides biosynthesis. Further studies are needed to investigate if WB incidence could be reduced by regulating gut microbiota and the potential mechanism that leads to decreased WB incidence.

Key words: 16S rRNA sequencing, breast muscle myopathy, microflora, QIIME 2

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INTRODUCTION

Selective breeding for faster growth and increased body and breast weight has resulted in an increased incidence of woody breast (**WB**) meat in the broiler industry. Studies have indicated that the development of WB is dependent on genetics, nutrition, environmental factors, and processing conditions (Bailey et al., 2015; Soglia et al., 2016). Chicken breast myodegradation, such as WB, may appear at as early as 3 wk of age (Baltic et al., 2019). The exact underlying mechanism for WB development is still unknown. In addition, the affected tissue is usually downgraded or discarded, which increases the economic burden to the poultry industry. In severely affected broilers, there are also animal welfare concerns due to potential physical discomfort (Papah et al., 2017).

The gut microbiome is the collective genomes of the micro-organisms inhabiting the gut (Valdes et al., 2018). Gut microbiota contains approximately 600,000 genes, which is 25 times greater than the genes of the host's genome. Therefore, it is considered as an organ of the host. The gastrointestinal (***GI***) tract of broilers has a complex microbiota that interacts with the host to participate in various metabolic processes and plays a crucial role in the host immune system, physiology of the GI tract, health, and productivity (Apajalahti et al., 2004; O'Reilly et al., 2016). Even though the gut microbiota of mature broilers is relatively stable, the taxonomic

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composition of the microbiota is still dynamic and can be affected by genetics, the age of birds, diet, environment, litter management, and the use of antibiotics (Shakouri et al., 2009; Ding et al., 2017). The composition of early embryo microbiota was inherited partially from maternal hens, and the microbial composition and diversity are adjusted by host genetics and environmental factors during different developmental stages (Ding et al., 2017). Wei et al. (2013) reported that *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* were the largest phyla in broiler and turkey intestines, accounting for >90% of all the sequences. The most predominant genera found in chicken gut were *Clostridium, Ruminococcus, Lactobacillus*, and *Bacteroides*. Differential sections of the chicken gut are interconnected. The chicken ceca were the most sampled gut segment based on the annotation of the sequence records, which is not surprising since the ceca retains food for 12 to 20 h and harbors the greatest taxonomic diversity and abundance (Clavijo and Flórez, 2018). The bacterial community in the chicken ceca is especially important in recycling urea, absorbing water, and digesting cellulose, starch, and polysaccharides (Deusch et al., 2015). There are approximately 900 species of bacteria in 100 genera in the ceca of chicken. However, most of them are uncategorized (Apajalahti et al., 2004; Wei et al., 2013). Stanley et al. (2013) used pyrosequencing of the V3 region of the 16S rRNA gene and found that butyrate-producing, cellulose-producing and starch-degrading bacterial communities in the ceca are associated with high performing chickens. These bacteria included *Clostridium islandicum, Ruminococcus* sp., *Bacteroides fragilis*, and *Lactobacillus coleohominis*. Han et al. (2016) amplified and sequenced the V4 region of the 16S rRNA gene using an Illumina MiSeq sequencer to correlate some bacterial groups with the weight of chickens. In the ceca, *Akkermansia, Prevotella* and *Anaerovibrio* negatively affected weight, while *Lactococcus* contributed to weight gain. So far, only one journal paper could be located that reported the differences in microbiota in ileal digesta between WB affected and normal broilers; they found that an unclassified *Lactobacillus* was the predominant genus that was more abundant in the ileum of WB affected broilers (Maharjan et al., 2020). In this study, the objective was to identify differential chicken gut microbiota biomarkers associated with woody chicken breast. The impact of diet and strain were central determinants used as influential parameters on microbiota composition leading to the development of WB.

MATERIALS AND METHODS

Eggs and Broilers

This study was approved by the Institutional Animal Care and Use Committee at Mississippi State University (Approval # IACUC-16-542A). Eggs were procured from 2 strains of commercial breeder hens (1 and 2) that were 30 wk old. All eggs were collected within the same period of time and placed in a single-stage incubator (ChickMaster, USA). Eggs were candled on d 11, and dead and infertile eggs were removed. Eggs were transferred to a hatcher (ChickMaster, USA) on d 18 of incubation. On d 21, a total of 256 (128 birds/strain) newly hatched chicks were transferred to a chicken house at the Mississippi State University Poultry Research Farm. The house was divided into 8 blocks, and 128 chicks of each strain were randomly assigned to 8 pens (16 birds/pen/block, 0.0846 m^2/bird). The birds were fed with either control diets or amino acid (**AA**)-reduced diets in 4 feeding phases: starter (d 0−14), grower (d 14−28), finisher (d 28−41), and withdrawal (d 41−60). The control diets were formulated to meet the highest recommended levels of digestible amino acids (lysine, total sulfur amino acids, and threonine) and other nutrients. The digestible amino acids in the AA-reduced diet were formulated at 20% lower than the recommended levels (Zhang et al., 2020a).

Sample Collection

At 8 wk of age, 32 birds of each treatment (strain × diet) were randomly selected and processed, and the WB myopathy was evaluated 24 h post processing. The percentages of moderate and severe WB were 67% and 84% for strains 1 and 2 fed control diet, respectively; in comparison, the percentages for strains 1 and 2 fed AA-reduced diets were 64% and 35%, respectively. Live male birds were evaluated for WB myopathy by manual palpation. For each strain of birds, 4 birds (n = 1 bird/pen) with NB were selected from blocks 1 to 4 and 4 birds (n = 1 bird/pen) with WB were selected from blocks 5 to 8. After euthanizing with CO_2 gas, the cecal luminal content were squeezed out of the ceca and washed twice with 0.1 M PBS buffer that contained 0.1% tween 20 and 1% β-mercaptoethanol and then frozen at -80°C in an ultralow freezer (5153, Thermo Fisher Scientific, Marietta, OH).

Microbial Community DNA Isolation

Microbial community DNA was extracted from the cecal samples using the QIAamp PowerFecal DNA Kit (Qiagen, Germantown, MD). The quality of the DNA extracts was confirmed using agarose gel (0.8%) electrophoresis, and concentrations were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). The concentration of an aliquot of the extracts was adjusted to 12.5 ng/μL using Tris-EDTA buffer prior to PCR amplification.

PCR

The V3-V4 hypervariable region of the prokaryotic 16S rRNA gene was amplified using a universal primer set (Forward primer [5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′] and reverse primer [5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′]) (Klindworth et al., 2013). For each DNA sample, 1 μL of microbiota DNA template

(12.5 ng/μL) was added to a 24 μL master mix that consisted of 12.5 μL 2x KAPA HiFi HotStart ReadyMix (KAPA Biosystems), 0.5 μL forward and 0.5 μL reverse primers (10 μM), and 10.5 μL nuclease-free water). The PCR mix was subjected to an initial 3-min denaturation step at 95°C. Subsequent cycles included a 30-s denaturation step at 95°C, a 30-s annealing step at 55°C, and a 30-s elongation step at 72°C. After 35 cycles, there was a final 5-min extension at 72°C before a 4°C hold. The PCR products (~550 bp) were confirmed using agarose gel (1%) electrophoresis. The PCR products were purified using AMPure XP beads (Beckman Coulter, Brea, CA) to remove the free primers and primer dimer species.

Construction of 16S rRNA Gene Libraries

The 16S rRNA gene libraries were constructed using the Nextera XT Index Kit (Illumina, San Diego, CA). A standard tagmentation reaction was set up to a final volume of 50 μL, including 25 μL 2x KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA), 5 μL [each] Nextera XT Index Primer, 5 μL DNA and 10 μL nuclease-free water, according to the Nextera protocol. The PCR mix was subjected to an initial 3-min denaturation step at 95°C. Subsequent cycles were consisted of a 30-s denaturation step at 95°C, a 30-s annealing step at 55°C, and a 30-s elongation step at 72°C. After 8 cycles, there was a final 5 min extension at 72°C before a 4°C hold. PCR clean-up was performed following the Nextera protocol using a 0.5:1 ratio of AMPure XP beads (Beckman Coulter, Brea, CA) to PCR reaction products. Reactions were eluted with Illumina elution buffer (10 mM Tris, pH 8.5).

Library Quantification and Size Determination

The 16S rRNA gene libraries were quantified using Qubit assays (Invitrogen, Carlsbad, CA), and the size profiles were analyzed on the Agilent Technologies 2100 Bioanalyzer (Agilent, Palo Alto, CA). The expected size on the Bioanalyzer trace of the final library was ~ 630 bp.

Pooling and Sequencing

The libraries were normalized to 30 nM using Qubit readings and pooled together accordingly. The pooled libraries were diluted to ~10 nM for storage. The 10 nM libraries were denatured with NaOH and diluted with hybridization buffer. The denatured libraries were sequenced using an Illumina MiSeq Next Generation Sequencer (Illumina, San Diego, CA) to generate paired-end 2×300 reads.

Bioinformatics Analysis

Raw 16S rRNA gene sequences were filtered and merged to single-end sequences, and the low-quality regions of sequences were trimmed and removed using the DADA2 plugin in the QIIME2 (quantitative insights into microbial ecology) platform. A feature table generation was conducted using "qiime feature-table tabulate-seqs" and "qiime feature-table summarize" plugins. Alpha and beta diversity analyses were conducted using the "core-metrics-phylogenetic" plugin. Species richness (Chao1 index) and evenness (Shannon index) were estimated for alpha diversity. Bray-Curtis dissimilarity was determined for beta diversity and visualized using Principal Coordinate Analysis (**PCoA**) plots. Group significance tests and visualization of beta diversity parameters were carried out with the "qiime diversity beta-group-significance" plugin and the "qiime emperor plot" plugin. The taxonomic analysis was performed using "qiime feature-classifier" plugin to explore the taxonomic composition of the samples using the greengenes (16S rRNA) reference database (2019.10). The relative abundance of the bacterial community was calculated, and the graph was plotted using R. The predictive functional profiling of microbial communities was performed using **PICRUSt** (phylogenetic investigation of community by reconstruction of unobserved States). The predicted 16S rRNA genes and their functions were aligned to the **KEGG** (Kyoto Encyclopedia of Genes and Genomes) database at level 2. The differences between groups were visualized using **STAMP** (Statistical Analysis of Metagenomic Profiles).

Statistical Analysis

The number of reads in the feature table/operational taxonomic unit (**OTU**) table was normalized based on the total sum method to generate relative abundance. The differences in relative abundance were analyzed using the 2-sided Welch's t-test ($P < 0.05$) followed by an adaptive FDR correction of 0.05 (Benjamini and Hochberg, 2000). All comparisons which were not detected in at least 2/3 of the samples (>=11) were removed within each comparison group to avoid the inclusion of underrepresented bacteria. The Kruskal-Wallis test, a nonparametric statistical test was carried out to compare the alpha diversity of each group at a significance level of 0.05. Permutational multivariate analysis of variance (**PERMANOVA**) tests were used to compare beta diversity between each group at a significance level of 0.05. A 2-sided Welch's t-test ($P < 0.05$) followed by an FDR adjustment of 0.05 (Storey, 2002) was used to determine the statistical significance of predicted functions and pathways. All associations producing an adjusted $q < 0.05$ were considered significant.

RESULTS

Diversity of Cecal Microbiota

Richness and evenness of the species (alpha diversity) present in each sample were assessed by Chao1 and Shannon indices, respectively. Accounting for both estimators, results indicated that there were no differences ($P > 0.05$) in the alpha diversity of gut microbiota

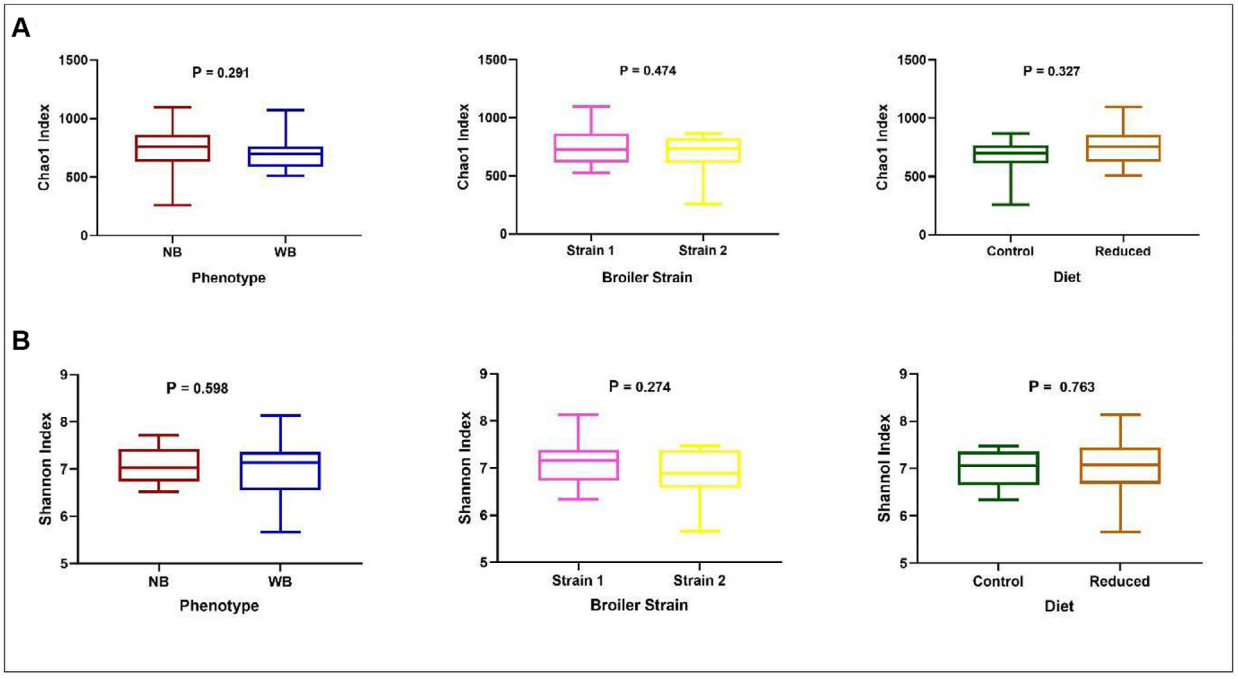

Figure 1. Boxplots for Alpha diversity based on (A) richness (Chao1 index) and (B) evenness (Shannon index) of pooled cecal samples from broilers with normal (NB) and woody breasts (WB) (phenotype), strain 1 and strain 2, and broilers fed with a control (control) and an amino acid reduced (reduced) diets. *P*-values were calculated using the pairwise Kruskal-Wallis test.

between phenotype (NB vs. WB), strain (1 vs. 2) or diet (control vs. reduced) (Figure 1).

Beta diversity was assessed to determine the variation in cecal microbiome between different samples. Based on the PERMANOVA test, the microbial community distribution was different in samples between phenotype ($P = 0.001$) and diet ($P = 0.024$) but not strain ($P = 0.065$). Principal coordinate analysis plots revealed that the samples were clustered mainly on the chicken breast phenotypes (Figure 2) followed by diets. This suggests that the microbiota of each bird with NB was more similar to each other than the microbiota of birds with WB (Figure 3), and the microbiota of WB affected birds was more similar to each other than NB.

Relative Abundance of Cecal Microflora

The most abundant phyla in all samples were *Firmicutes* (66.4%), followed by *Bacteroidetes* (25.4%) and *Proteobacteria* (6.6%) independent of phenotype, strain or diet. Also, there were no differences (FDR > 0.05) in the relative abundance of any phyla between phenotype, strain or diet groups. Since the samples were mainly clustered based on phenotype, the distribution of phyla by grouping samples into NB and WB was plotted (Figure 3) for a better visualization of the bacterial abundance at the phylum level.

At the species level, comparative analysis of NB and WB detected the following bacteria that were more abundant in the WB group: *Bacteroides plebeius* (FDR = 0.0025), *Selenomonas bovis* (FDR = 0.0225), and *Victivallis vadensis* (FDR = 0.0216) (Figure 4). Higher relative abundance in the NB group were observed for *Alistipes putredinis* (FDR = 0.0288), *Butyricicoccus pullicaecorum* (FDR = 0.0096), *Defluviitalea saccharophila* (FDR = 0.0156), and *Lactobacillus hamsteri* (FDR = 0.0167) (Figure 4). In the ceca of WB birds, *S. bovis* (12.6%) and *B. plebeius* (12.3%) were the top 2 predominant bacteria (Figure 4); In contrast, the relative abundances of these 2 bacteria were only 5.1%

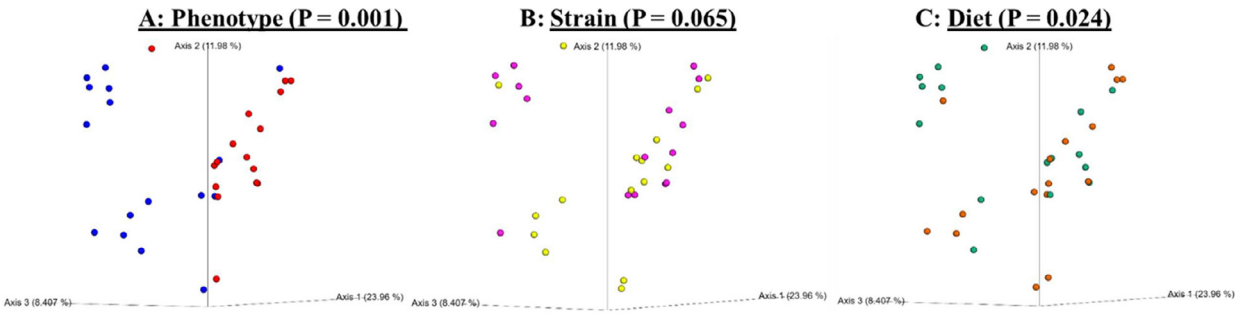

Figure 2. Principal coordinate analysis (PCoA) plots with Bray-Curtis dissimilarity for Beta diversity of (A). Pooled cecal samples from broilers with normal (red) and woody breasts (blue); (B) Pooled cecal samples from broilers strain 1 (pink) and strain 2 (yellow); (C) Pooled cecal samples from broilers fed with a control (green) and an amino acid reduced diet (orange). *P*-values were calculated using PERMANOVA test.

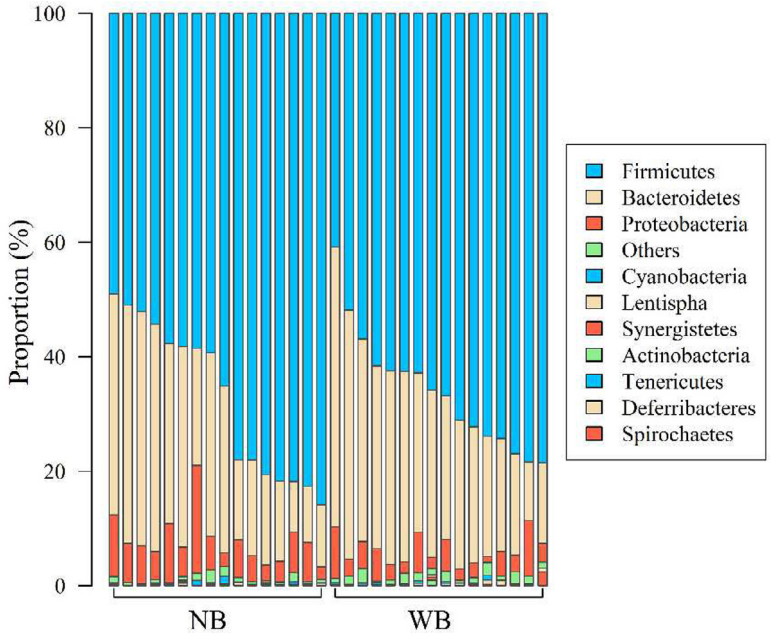

Figure 3. The relative abundance of bacteria population at phylum level in cecal microbiota of normal and woody breast samples.

and 1.2% in normal birds, respectively. *Bacteroides plebeius* belongs to the genus of *Bacteroides*. However, the abundance of the *Bacteroides* genus was not different (FDR > 0.05) between NB and WB groups. Broiler feed comparison results showed that the control diet had a lower relative abundance of *A. indistinctus* (FDR = 0.0004), *A. putredenis* (FDR = 0.0275), *Anaerorhabdus furcosa* (FDR = 0.0275) and *Clostridium ruminatium* (FDR = 0.0259), and a higher relative abundance (FDR = 0.0332) of *Sporobacter termitidis*, compared to the reduced diet (Figure 5).

Prediction of Microflora Functions

PICRUSt2 was utilized to predict the functional profiling from 16S rRNA sequences to describe the metabolic potential of the cecal microbial community in broilers. At KEGG level 2, 35 microbial metabolic

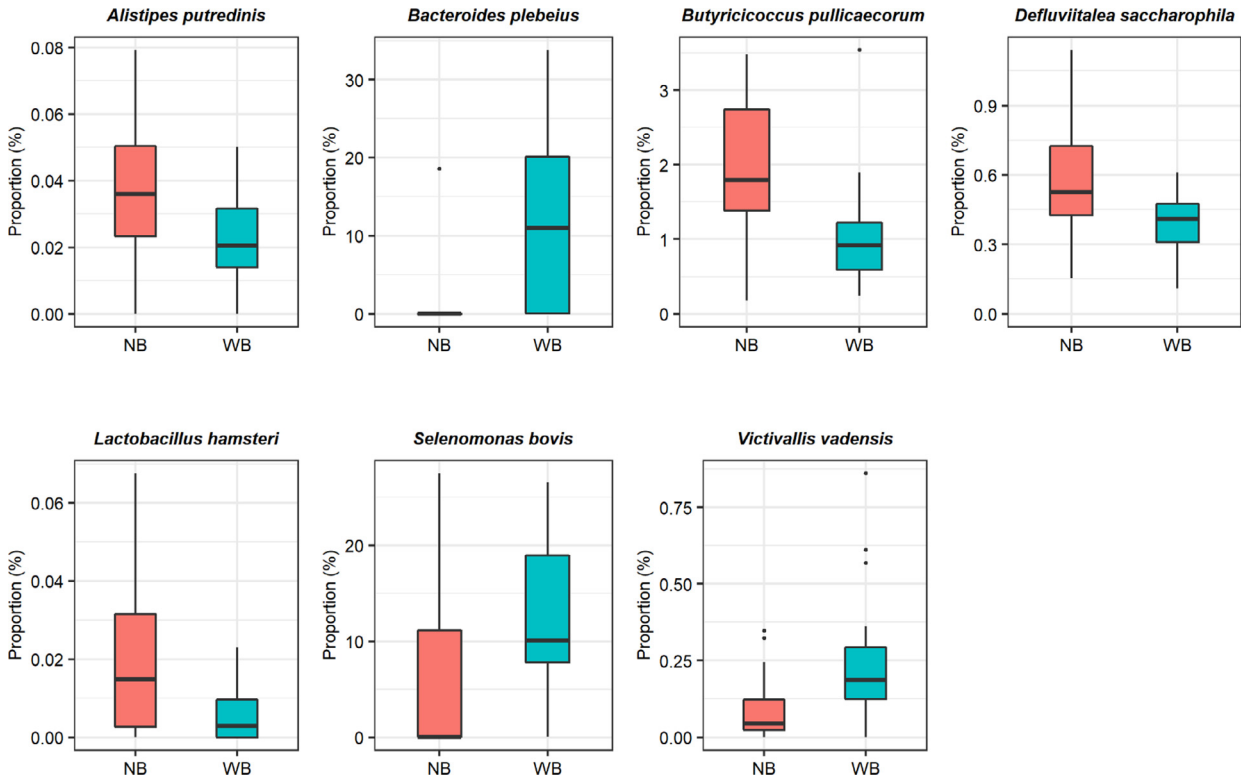

Figure 4. The relative abundance of bacteria population at the species level in cecal microbiota of broilers with normal and woody breast samples (FDR < 0.05).

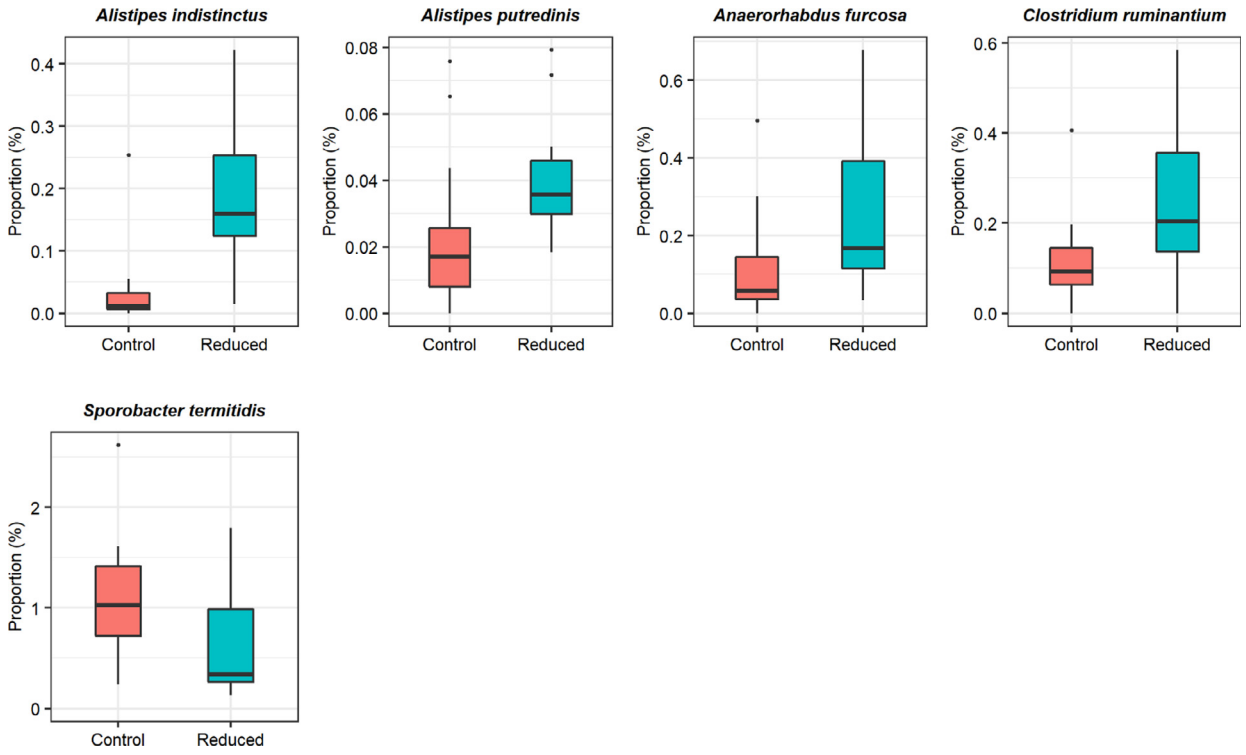

Figure 5. The relative abundance of bacteria population at the species level in cecal microbiota of broilers fed control and amino acid reduced diet (FDR < 0.05).

activities were different (q < 0.05) in the ceca between NB and WB broilers (Figure 6) but none was different (q > 0.05) between broiler strain or diet. The pairwise comparison of microbial pathways abundance reavealed that the altered pathways comprised of glycolysis, the TCA cycle, the urea cycle, and critical metabolic pathways from amino acid, vitamin, and nucleotide metabolism. Microflora from the NB group were enhanced (q < 0.05) in certain energy metabolism pathways, such as glycolysis, homolactic and mixed acid fermentation, and the urea cycle. Pathways such as mannan degradation, fucose, and rhammose degradation were upregulated (q < 0.05) in broilers with the WB myopathy. Nucleotide metabolism pathways related to purine, pyrimidine and their derivatives (7 out of 11) were upregulated (q < 0.05), and 4 pathways were related to adenosine, guanosine and their derivatives were downregulated (q < 0.05) in broilers with the WB myopathy. The following sugar degradation pathways: mannan, fucose and rhamnose pathways, were upregulated (q < 0.05) in broilers with the WB myopathy. Amino acids pathways, L-lysine, L-thronine, and L-methionione were downregulated (q < 0.05) while L-tryptophan, ariginine, and polyamine biosynthesis were upregulated (q < 0.05) in broilers with the WB myopathy. In total, 22 out of 35 pathways were upregulated (q < 0.05) and 13 were downregulated (q <0.05) in broilers with WB myopathy.

DISCUSSION

In our previous studies, it was determined that the WB myopathy of broilers was affected by broiler strain and diet (Zhang et al., 2018). Proteome differences were also observed between NB and WB, which indicated oxidative stress and inflammation of woody birds (Zhang et al., 2020b). The Alterations in gut microbiota have been suggested to play an important role in regulating host metabolism (Martin et al., 2019). Cecal microbiota has recently received more attention with respect to avian growth and metabolism (Wang et al., 2017; Chen et al., 2020) due to the large variety and composition of bacteria in the ceca. According to Yan et al. (2017), the differences in microbiota are the result of interactions with the host, and the interactions shape the host phenotype.

The ceca of broilers are dominated by strictly anaerobic bacteria. *Firmicutes, Bacterioidetes,* and *Proteobacteria* were the most abundant phyla, independent of strain, phenotype, or diet, which is consistent with previous reports (Xiao et al., 2017). These 3 phyla are closely related to bird growth performance according to Torok et al. (2011) who investigated 3 broiler feeding trials and identified performance-linked operational taxonomic units within both the ilea and ceca. In this study, however, some bacteria were not assigned to a known taxonomy, especially at the genus and species levels. In general, more than 60% of these bacteria belong to the order of *Clostridiales*, a known group of bacteria that can utilize complex plant-derived carbohydrates and produce butyrate. The most abundant family was *Ruminococcaceae* follwed by *Lachnospiraceae*. These 2 families are known to digest highly recalcitrant polysaccharides, and less recalcitrant nonstarch polysaccharides and starch, respectively (Biddle et al., 2013). Another abundant family was *Bacteroidaceae* that is involved in

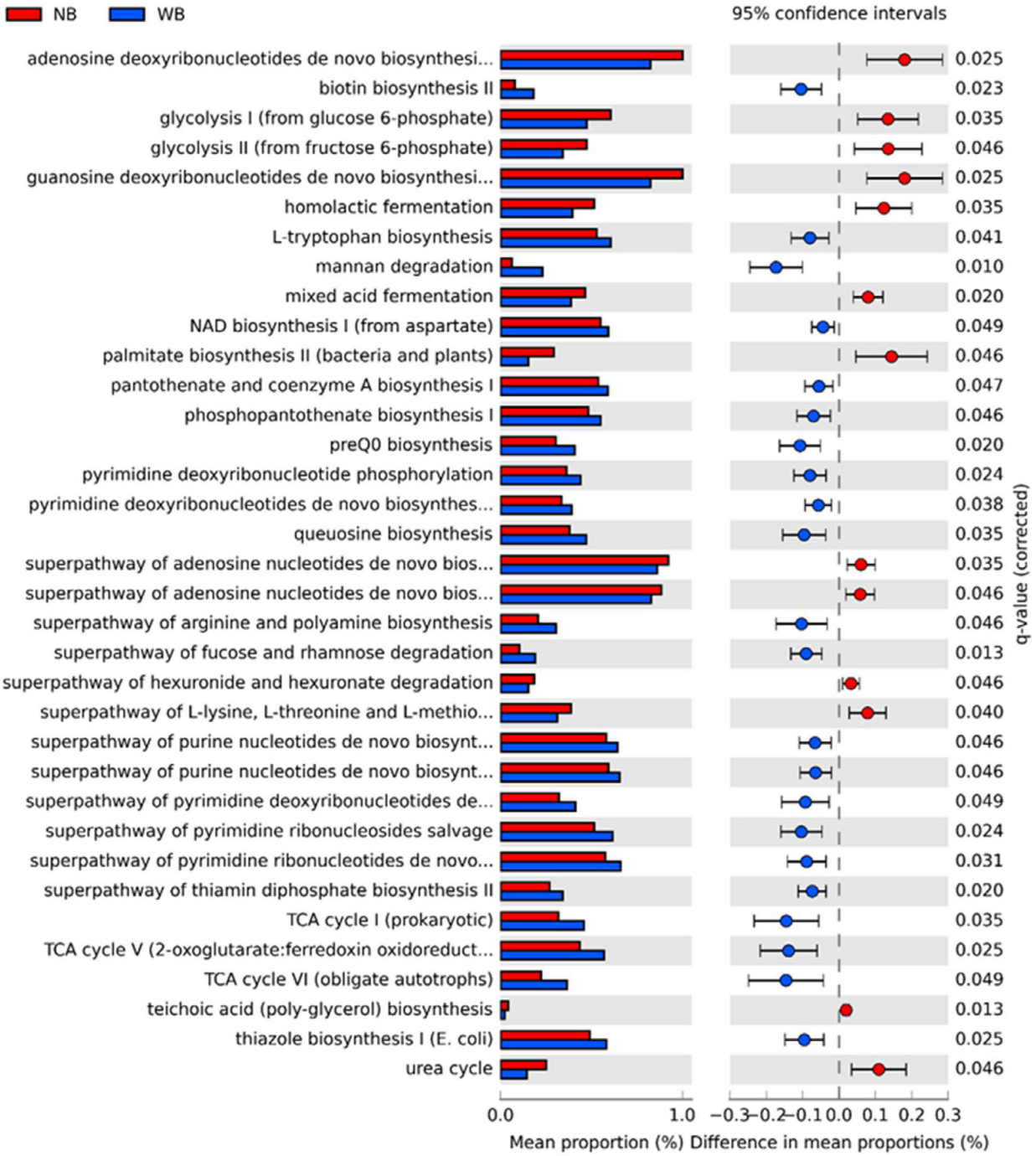

Figure 6. Predicted metabolic pathways in cecal microbiota from broilers that yield normal breast (NB) and woody breast (WB) based on Welch's t-test followed by a Storey FDR correction.

most bacterial functions, such as energy production, carbohydrate metabolism, and synthesis of cellular components. The composition and funciton of these bacteria are dependent on the genetics of birds, the age of birds, the location of the gastrointestinal tract, dietary supplement and environment (Shakouri et al., 2009; Ding et al., 2017).

Broiler strain was not a significant factor with respect to alpha and beta diversities (Figures 1 and 2). This indicates that there were a similar number of bacteria and a similar relative abundace of each bacteria that was identified for the 2 strains of broilers. There were also no differences in the relative abundance of bacteria at any taxonomic levels with the only exception of an uncharacterized genus of the family Ruminococcaceae with strain 1 of 2.1% verse strain 2 of 1.4% (FDR = 0.414). It has been found that chicken gut microbiota was influenced by maternal vertical transmission (Ding et al., 2017), contact with the hens' microbiota as well as external factors (Kubasova et al., 2019; Ocejo et al., 2019). Kubasova et al. (2019) found that hens were not an important source of *Firmicutes*, the most predominant pylum in our study. Although the newly hatched chicks might have different gut microbiota since they were from maternal hens of 2 different genetic strains, the external factors such as diet, litter,

and farm environment became the predominant factor that shaped the cecal microbiota despite of strain type. The environment that contributes to indigenous species could contribute to microbiota colonization. Torok et al. (2009) found that litter type influenced cecal microbiota composition when poultry was raised on new litter, reused litter and other litter materials. Therefore, it is logical that there minimal differences between strains and that diet could have more of an impact on gut microbial variabiltiy than genetic strain.

Reduction of essential amino acids by 20% in broiler diet was sufficient to affect the microbiome beta diversity and the relative abundance of some bacteria (Figures 2 and 5). Dietary interventions have been used in poultry production to enhance broiler's attributes, such as growth performance and processing yields (Pan and Yu, 2014). Modifications of dietary fiber or antimicrobial feed additives can impact the overall intestinal microbial ecology in chickens (Danzeisen et al., 2011; Torok et al., 2011; Walugembe et al., 2015). In general, nutritional requirements differ among genus and in some cases among species. As a result, the availability of substrates in the environment can enhance the growth of some bacteria and at the same time suppress the growth of others (Apajalahti and Vienola, 2016). Changes in amino acid supplementation affect the composition, diversity and activity of gut flora, and therefore intestinal homeostasis (Ma and Ma, 2019). It was expected for the diet to have a greater influence on the microbial diversity in the ceca. In contrast, differences in differentially abundant microbes in the 2 diet groups accounted for less than 2% of the bacterial community (Figure 5).

A large variation in cecal microbiota was found in cecal samples from NB and WB groups. Comparative analysis of NB to WB indicated higher relative abundance (FDR <0.05) for *A. putredinis* (0.039%), *B. pullicaecorum* (1.91%), *D. saccharophila* (0.59%), *L. hamsteri* (0.02%), and *V. vadensis* (0.096%) in NB, accounting for 2.66% of the community compared to 1.76% in WB (Figure 4). The higher relative abundance (FDR < 0.05) of *B. plebeius* and *S. bovis* was found in WB. The total abundance of these 2 bacteria was 24.9% in WB and 6.3% in NB. The higher abundance of *B. plebeius* might contribute to the heavier breast with WB myopathy (Zhang et al., 2021). In addition, the large abundance of *B. plebius* and *S. bovis* as well as the resulting reduced diversity of the bacterial community may contribute to the development of WB myopathy. The reduced diversity of cecal microbiota was observed in outdoor raised compared to indoor raised Dagu chicken (Xu et al., 2016). The authors reported that the chickens that were raised indoor may be exposed to more stresses from the feeding density and limited space. In a mouse study, it was found that long-term stress decreased the diversity in the ceca of stressed mice ` (Bailey et al., 2010). Physiologically, gut epithelial cells produce reactive oxygen species (**ROS**) and reactive nitrogen species (**RNS**) from oxygen metabolism or through gut microorganisms. Under certain conditions, such as heat stress, gut bacteria produce more ROS and RNS, which results in oxidative stress. As for the broilers, the selective breeding of fast-growing birds has increased their susceptibility to heat stress, that may disrupt the immune system and cardiac functions, thus increasing oxidative reactions that compromise meat quality (Hosseindoust et al., 2020). Interindividual variation in response to stress or other environmental stimuli might be different and therefore contribute to the different gut microbioal compostion. Oxidative stress, one of the main causes of WB myopathy (Abasht et al., 2016), might be attributed to the reduced bacterial diversity in birds with WB.

The alteration in bacterial composition and diversity affects birds' digestion and nutrition uptake, the biochemical functions, the intestinal physiological functions and the host immune system (Xu et al., 2016). In this study, we observed a different profile of metabolic pathways in cecal microbiome of woody birds, which may interact with host to affect the host phenotype. In poultry, gut flora activity accounts for 20−36% of the whole-body metabolism (Cant et al., 1996). Carbohydrates are the major source of broiler feed formula, which provides the energy for broilers to grow. Most carbohydrates and fibers that are present in the diet can only be digested by the bacteria in the gut, especially cecal microflora, into short-chain fatty acids (SCFAs, e.g. butyric acid, propionic acid) and lactate to produce ATP. Broilers affected by WB myopathy exhibited decreased glycolysis activities in the cecal microflora (Figure 6), which is similar to the metabolic features of WB tissue (Abasht et al., 2016). The decreased glycolytic activities along with reduced acid-producing activities, homolactic fermentation and mixed acid fermentation (Figure 6) indicated decreased energy metabolism and reduced acid-production capacity in the cecal microflora, which is consistent with the decreased energy metabolism pathways and higher ultimate pH that has been seen in WB tissue in numerous studies. The chemical pathways in the TCA cycle were up-regulated in the cecal microflora of WB affected birds. Abasht et al. (2016) observed elevated fumarate and malate levels in WB affected breast muscle that was attributed to an imbalance in the TCA cycle. In contrast to the findings from our current research, Kuttappan et al. (2017) predicted decreased TCA activity in WB. The down-regulated adenosine deoxyribonucleotides de novo biosynthesis II and guanosine deoxyribonucleotides de novo biosynthesis II pathways in WB affected birds (Figure 6) were also indicators of reduced ATP and GTP production. The increased pathways of purine nucleotides de novo biosynthesis and pyrimide nucleotides and/or their derivatives were indicative of increased energy production, which agrees with the increased TCA cycle activity in our study and the findings from Greene et al. (2020) who observed increased metabolites, for example fumarate, from purine nucleotide degradation and de novo biosynthesis. Although the pathway changes were observed in WB affected cecal microflora, these changes were predicted based on the database of the microbial

taxonomy (16S rRNA) genes not the metabolites of microorganisms, and therefore there might be inconsistent pathway changes in microbiota and tissue due to the unpredicted gut-host interactions. In order to discover the direct cause-effect relationship between gut microbiota and chicken breast myopathy, further studies need to be conducted, such as feeding the bacteria that is overabundant in the ceca of woody birds, to investigate the impact of the microbial compostion on the development of WB myopathy.

Two sugar metabolism pathways were upregulated in the WB group (Figure 6), indicating a potentially enhance ability to metabolize sugar. Most bacteria that were differentially abundant in NB and WB groups can utilize certain types of sugar as the substrate to produce energy. *Bacteroides plebeius* can digest polysaccharides from marine edible algae due to the presence of genes coding for β-porphyranase and β-agarase (Hehemann et al., 2010). *Selenomonas bovis* can utilize glucose, mannose, cellobiose, arabinose, lactose, sucrose, trehalose, melibiose, raffinose, salicin and aesculin but not lactate, xylose, starch, dulcitol, mannitol or sorbitol (Zhang and Dong, 2009). *Lactobacillus hamsteri* was reported to metabolize oligosaccharides that were present in β-glucan hydrolysates in a rat study (Snart et al., 2006). Due to the prefered sugar sources for these differentially abundant bacteria, the related metabolism and accumulated metabolites would be expected to be different in ceca of WB affected birds from not affected birds.

The gut microbiota not only plays a role in energy metablism, but also affects the biosynthesis of vitamins, amino acids, and nucleotides. Four biosynthesis pathways related to vitamin (biotin, pantothenate and thiamin) and/or their phosphorylated products were enriched in WB affected birds (Figure 6). These 3 vitamins are the main organic micronutrients that are needed for bacterial metabolism, as cofactors of enzymes that are involved in biological activities. The altered vitamin metabolism in the ceca of WB affected broilers indicates a different requirement of vitamins for gut bacteria, and therefore an altered biological metabolism in activites such as fatty acid biosynthesis, amino acid metabolism, and the TCA cycle. Amino acids are mainly utilized by the host and small intestinal bacteria. Cecal bacteria mainly digest carbohydrates and only utilize a small concentration of what the host does not digest (Apajalahti and Vienola, 2016). The enriched superpathway of arginine and polyamine biosynthesis suggests a potential increase in polyamine in the WB group. Putrescine and spermidine are the most common polyamines that are produced by bacteria, such as *Bacteroides* spp. and *Fusobacterium* spp. (Noack et al., 2000). Dysregulation of polyamine metabolism impacts the regulation of the glucose, lipid, and energy homeostasis (Ramos-Molina et al., 2019). Polyamines are usually induced in response to stress and therefore function as modulators of oxidative stress and inflammation. Rhee et al. (2007) summarized that physiological levels of polyamines protect DNA from reactive oxygen species (ROS) damage, but excess polyamines that are produced under host defense induce oxidative stress by ROS generation during the catabolic oxidation process of polyamines.

Alistipes putredinis can hydrolyze tryptophan to indole, which may partially explain the lower L-tryptophan biosynthesis due to its lower abundance in the WB group (Figures 4 and 5). Decreased abundance of *A. putredinis* has been seen in human patients with liver fibrosis and other fibrotic diseases (Campion et al., 2019). The decrease in *A. putredinis* decreases the production of short chain fatty acids (SCFA) that have anti-inflammatory abilities, and therefore contributes to advanced fibrosis (Parker et al., 2020), which is a typical feature of WB. Another SCFA producer, *B. pullicaecorum*, is a butyrate-producing probiotic. By activating the SCFA transporter and/or receptor, *B. pullicaecorum* could promote the absorption of neutral amino acids and bile salts, stimulate secretion of immune effectors (Tang et al., 2020), reduce the abundance of *Clostridium perfringens* and *Escherichia Shigella* in the broiler ceca and ileum (Eeckhaut et al., 2016), and improve the clinical outcome of colorectal cancer in mice (Chang et al., 2020). The lower abundance of *B. pullicaecorum* may indicate a microenvironment that is more susceptible to pathogens and weaker in immune responses in the ceca of WB-affected birds. The probiotic, *B. pullicaecorum*, could be investigated in the future to explore its potentials in improving broilers' gut health and reducing WB development.

CONCLUSIONS

This study characterized the microbial communities in broiler ceca using a high-throughput sequencing technique. Phenotype of chicken breast muscle and amino acid supply in the diet impacts colonization of the microbial community in the ceca of broilers. Regardless of diet nutrition, unaffected broilers and WB affected broilers respond differently to the nutritional and environmental stimuli, which can be seen in the differences in their gut microbiota. In WB affected birds, the ceca were characterized with decreased glycolysis and urea cycling, increased TCA cycle and vitamin biosynthesis, and altered amino acid and nucleotide metabolism. The ceca of WB affected birds also contained an increased abundance of *S. bovis* and *B. plebeius*, and decreased abundance of beneficial bacteria including *B. pullicaecorum* and *L. hamsteri*, and a decline in microbial diversity. These differences may be associated with the development of WB. Future studies are needed to characterize the gut microbiota in an early age of birds when the WB myopathy becomes detectable, to supplement broilers' feed with probiotics such as *B. pullicaecorum* to ameliorate the WB development, and to explore the potential sources of *S. bovis* and *B. plebeius* to inhibit their growth in the chicken gut and determine if it contributes to reduced WB incidence.

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DISCLOSURES

The authors declare no conflicts of interest.

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