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Dietary Energy Level Affects the Composition of Cecal Microbiota of Starter Pekin Ducklings

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ABSTRACT

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In this study, we evaluated the phylogenetic diversity of the cecal microbiota of 3-week-old ducklings fed three diets differing in metabolizable energy. The contents of the ceca were collected from ducklings of different groups. The ceca bacterial DNA was isolated and the V3 to V4 regions of *16S rRNA* genes were amplified. The amplicons were subjected to high-throughput sequencing to analyze the bacterial diversity of different groups. The predominant bacterial phyla were Bacteroidetes (~65.67%), Firmicutes (~17.46%), and Proteobacteria (~10.73%). The abundance of Bacteroidetes increased and that of Firmicutes decreased with increasing dietary energy level. The diversity decreased (Simpson diversity index and Shannon diversity index) with the increase in dietary energy level, but the richness remained constant. Notably, *Brachyspira* bacteria were detected with a very high relative abundance (4.91%) in ceca of ducks fed a diet with 11.30 MJ/kg metabolizable energy, suggesting that low energy content may affect their colonization in cecum.

Keywords: duck; bacterial diversity; dietary energy

Ducks have a global population exceeding 1 billion individuals ranking second only to chickens according to annual poultry production in 2014 according to FAO's corporate database. Like other birds, ducks have proportionally smaller intestines and shorter transit times than mammals, but do not appear to be less efficient at digestion than their mammalian counterparts (McWhorter et al. 2009), which rely on microbes in their gastrointestinal tract to accomplish this (Flint et al. 2012). In addition to digestion, microflora in the gastrointestinal tract of poultry influences health and wellbeing (Stanley et al. 2012).

In the past few years, the gastrointestinal microbiomes of economically important birds such as chicken (Callaway et al. 2009; Danzeisen et al. 2011; Singh et al. 2013), duck (Swayne and McLaren 1997), turkey (Scupham et al. 2008), goose (Lu et al. 2009), ostrich (Matsui et al. 2010) have been revealed by high-throughput next-generation se-

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quencing technologies. These studies indicate that avian intestinal microbiomes are more complex and diverse than previously thought, and greatly expand the perspective on the avian intestinal microbiome in terms of species composition, diversity, and community structure.

A recent report revealed that diet plays a more important role than host genotype in shaping microbial ecology (Carmody et al. 2015). Diet energy level variation induces rapid changes in the bacterial composition of the human and mouse gut microbiota, and changes the energy gain from the gastrointestinal tract (Daniel et al. 2014). Dietary changes can increase performance of chickens by optimizing the composition of bacterial species within the microbiota of the distal intestine (Bedford and Cowieson 2012). However, the effect of dietary energy level on poultry intestinal microbiota remains unclear.

Compared with chicken, the duck gastrointestinal microbiome is largely undiscovered. Two reports from a French laboratory revealed that Firmicutes and Bacteroidetes are the dominant phyla in the bacterial community of ducks at 12–14 weeks, and the richness and diversity is significantly affected by overfeeding and dietary probiotic (Vasai et al. 2014). Early life events are critical for the dynamics of microbiota development (Dore et al. 2015). As meat is the major product of ducks slaughtered at 4–6 weeks, discovering the diversity of intestinal microbiota in young ducks is important for implementing a strategy for improving gut health and performance.

In this study we characterized the phylogenetic diversity of the cecal microbiota of ducks at 3 weeks, and investigated the effects of dietary metabolizable energy (ME) on cecal microbiota using high-throughput sequencing after amplification of the V3–V4 region of the bacterial 16S rRNA gene.

MATERIAL AND METHODS

Experimental animals and diets. Thirty healthy newly-hatched male Pekin ducklings with similar weight were divided into three groups. In each group, 10 birds were randomly assigned to one cage (3 × 3 m), and raised on wire floors that contained a self-feeder and water source in an air-controlled house. One 100-W light bulb per cage was provided for ducklings up to 10 days. Different groups were given feed with a different ME content (Table 1). Nine ducks were randomly selected from each group and sacrificed on day 21. Ducks were fasted for 16 h before the sacrifice. Ceca were immediately collected and kept on ice. The contents of the ceca were collected by gently squeezing the organ and were snap frozen in liquid

Table 1. Ingredients and chemical composition of the experimental diets[1]

Diet	Treatment		
	group A	group B	group C
Ingredients[2]			
Corn	54.21	59.41	56.41
Corn DDGS	15.00	15.90	14.80
Soybean meal	3.00	3.00	3.00
Peanut meal	18.50	16.9	18
Rice hulls	4.50	0	0
Soybean oil	0	0	3.00
Dicalcium phosphate	1.40	1.40	1.40
Limestone	1.10	1.10	1.10
Salt	0.30	0.30	0.30
Premix[3]	1.00	1.00	1.00
DL-Methionine	0.16	0.16	0.16
L-Threonine	0.18	0.18	0.18
L-Tryptophan	0.046	0.048	0.047
Nutrients[4]			
ME (MJ/kg)	11.30	11.93	12.58
Crude protein (%)	19.13	19.15	19.15
DL-Methionine (%)	0.45	0.45	0.45
L-Lysine (%)	0.60	0.60	0.60
L-Tryptophan (%)	0.22	0.22	0.22
L-Threonine (%)	0.75	0.75	0.75
Calcium (%)	0.84	0.84	0.84
Non-phytic acid phosphorus (%)	0.38	0.38	0.38

DDGS = distillers dried grains with solubles, ME = metabolizable energy

[1]inclusions of feed ingredients are in kg

[2]supplied per 100 kg diet as-fed

[3]provided per kg of diet: vitamin B_2 4.5 mg, niacinamide 40 mg, D-pantothenic acid 9 mg, choline chloride 500 mg, vitamin B_{12} 20 µg, vitamin E (DL-α-tocopheryl acetate) 30 mg, vitamin K 2.3 mg, vitamin A 12 500 IU, vitamin D_3 5000 IU, biotin 0.1 mg, folic acid 0.5 mg, $FeSO_4 \cdot H_2O$ 147 mg, MnO_2 100 mg, $CuSO_4 \cdot 5H_2O$ 40 mg, $ZnSO_4 \cdot H_2O$ 143 mg, Na_2SeO_3 0.5 mg, KI 2 mg, ethoxyquin 125 mg

[4]ajusted to 87.3% dry matter

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nitrogen. All samples were stored at –80°C. All experimental procedures were approved by the Ethics Committee for Experimental Procedures on Animals of the Jiangsu Normal University.

DNA extraction. Genomic DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Germany) by following the manufacturer's instructions, with some modifications. Mucosal contents were first treated with 25 mg/ml of lysozyme (Vivantis Technologies, Malaysia) in lysis buffer (20 mM Tris-Cl, pH 8.0; 2 mM EDTA, pH 8.0; 1% Triton X-100) for 30 min at 37°C. Samples were then treated with DNase-free RNase (Epicentre, USA). DNA concentration and quality were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and gel electrophoresis, respectively. Samples were then stored at –80°C until required.

High-throughput sequencing and analysis of 16S rRNA gene amplicons. PCR amplifications were carried out using 50 µl of reaction mixtures which contained 25 µl NEBNext® High-Fidelity 2X PCR Master Mix (New England Biolabs, USA) (containing 2.0 mM $MgCl_2$), 25 µM primer, and 50 ng DNA template. The PCR reaction included an initial denaturation step at 98°C for 30 s followed by 25 cycles of 98°C for 10 s, 65°C for 30 s, 72°C for 30 s, and final extension step at 72°C for 5 min in S1000 Thermal Cycler (Bio-Rad Laboratories, USA). Amplicons from the V3 to V4 regions of *16S rRNA* genes of 27 samples were amplified using one degenerate primer pair (V3F: CCCTACACGACGCTCTTCCGATCTARACTYCTACGGRAGGCWG, V4R: AGACGTGTGCTCTTCCGATCTGACTACNVGGGTATCTAATCC). Each primer had a barcode sequence of ten nucleotides at the 5' end, which was unique for each sample. The PCR products were purified with a QIAquick PCR Purification kit (Qiagen) followed by DNA yield quantification and quality estimation using a NanoDrop spectrophotometer. *16S rRNA* gene amplicons were quantified by qPCR using a KAPA Library Quantification kit (KAPA Biosystems, South Africa) and an Eco Real-Time PCR System (Illumina, USA). The amplicons were normalized and sequenced (2 × 300 bp paired-end run) on the Illumina MiSeq sequencer (Illumina) at Biomarker Technologies (Beijing, China).

Bioinformatics analysis. The primers, barcode, and low quality sequences were removed. Illumina reads were analyzed using the mothur software package, Version 1.34.4 (Schloss et al. 2009). Chimeric sequences were removed using the USEARCH software based on the UCHIME algorithm (Dore and Blottiere 2015). Sequences were further analyzed using the open source software package QIIME, Version 1.9.1 (Caporaso et al. 2010) and the Ribosomal Database Project (East Lansing, USA) (Cole et al. 2003). Operational Taxonomic Units (OTUs) were picked using *de novo* OTU picking protocol with a 97% similarity threshold. The alpha and beta diversities were analyzed in QIIME Version 1.9.1 software as per QIIME defaults for that version (Caporaso et al. 2010). The detailed protocol was referred to by Sergeant et al. (2014).

Statistical analysis. Data were analyzed using the GLM procedure of SPSS software for MS Windows by one-way ANOVA. Differences between groups were considered significant when *P*-values were < 0.05.

RESULTS

Characterization of the duck cecal microbiota. After stringent quality trimming on raw data, 549 992 sequences (20 370 ± 5363 sequences/sample), with an average read length of 450 bp, remained for subsequent analysis. The data was submitted to NCBI database with an Accession No. PRJNA326555. The average number of ceca OTUs with a cutoff of 97% sequence homology was 383 ± 55 in each sample, with a total of 1441 OTUs in 27 samples representing 13 phyla (Figure 1). The duck cecal microbiota was mainly composed of Bacteroidetes (65.67 ± 7.08%), Firmicutes (17.46 ± 4.03%), and Proteobacteria (10.73 ± 3.80%) (Figure 1). The top 50 OTUs represented 83.96 ± 4.97% of the sequences. At the class level, the Bacteroidetes was dominated by *Bacteroidia* (65.63 ± 7.12%), Firmicutes was dominated by *Clostridia* (14.03 ± 3.43%), and Proteobacteria was dominated by *Gammaproteobacteria* (5.95 ± 4.11%) and *Deltaproteobacteria* (2.39 ± 1.03%). A total of 161 genera were detected, of which the top 15 are shown in Figure 1.

Dietary energy level affected duck cecum microbial community. Dietary energy level had a significant effect on the microbial community of duck cecum. The Weighted UniFrac principal coor-

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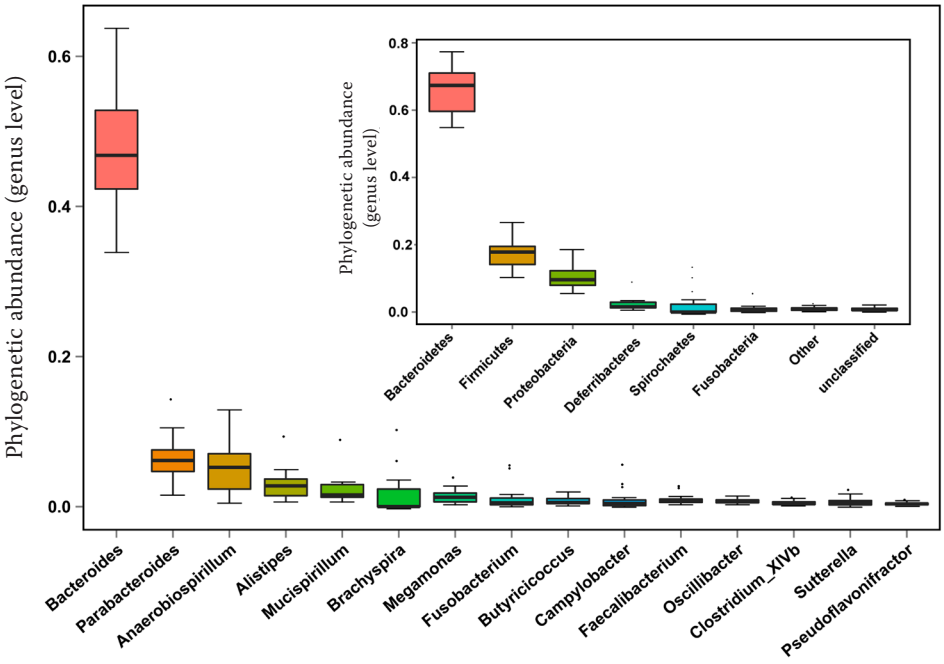

Figure 1. Genus abundance variation box plot for the 15 most abundant genera of Pekin duck cecal microbiota as determined by sequence abundance. Inset shows phylum abundance box plot. Genus and phylum level abundances were measured using reference-genome-based mapping with 85% and 65% sequence similarity cutoffs

dinate analysis showed a diet-responsive structural rearrangement of gut microbiota (Figure 2). The abundance of Bacteroidetes increased and that of Firmicutes decreased with the increasing dietary energy level (Figure 3). *Bacteroides* and *Parabacteroides* were the main contributors to the cecal microbiota variation, and were affected by dietary energy level (Figure 4). The ratio of Firmicutes/Bacteroidetes (0.33 ± 0.07, 0.25 ± 0.08, 0.22 ± 0.04) declined with the increase in energy level ($P < 0.05$). Interestingly, Spirochaetes accounted for 4.91 ± 1.16% of the sequences in group A (ME 11.30 MJ/kg), whereas the proportion was significantly higher than that of group B (0.01 ± 0.01%, ME 11.93 MJ/kg) and group C (0.001 ± 0.001%, ME 12.58 MJ/kg) ($P < 0.001$). The bacteria in *Bacteroides* were largely responsible for the increase of Bacteroidetes, whereas the decrease of Firmicutes was due mainly to bacteria from *Ruminococcaceae* (Table 2, $P < 0.05$). The dietary energy significantly affected taxa diversity ($P < 0.05$), but did not affect their richness (Table 3).

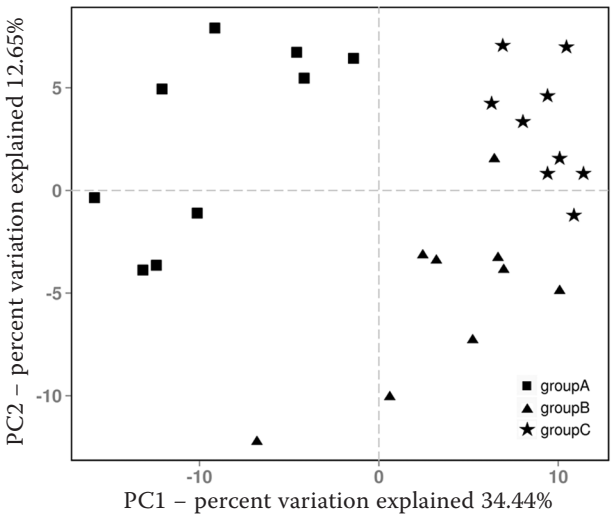

Figure 2. Principal coordinate analysis (PCoA) using the weighted UniFrac metrics based on Operational Taxonomic Units (OUT) compositions of cecal microbiota in each group of Pekin ducks
group A = low metabolizable energy, group B = normal metabolizable energy, group C = high metabolizable energy

DISCUSSION

Previous studies revealed that Firmicutes and Bacteroidetes are the dominant phyla in organisms such as birds, zebrafish, and mammals (Ley et al. 2008; Kohl 2012; Semova et al. 2012), which are known for fermentative metabolism and degra-

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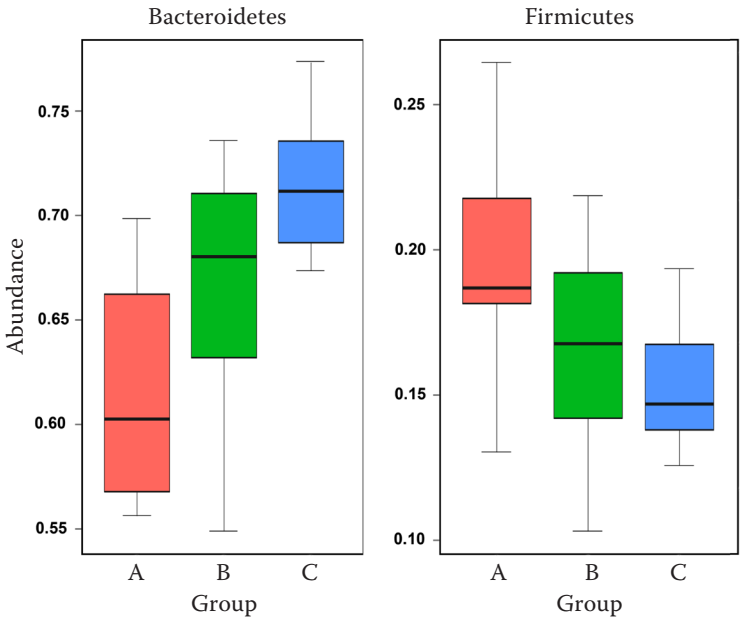

Figure 3. Phyletic differences of Pekin duck cecal microbiota as affected by dietary energy level shown by box plot
group A = low metabolizable energy, group B = normal metabolizable energy, group C = high metabolizable energy

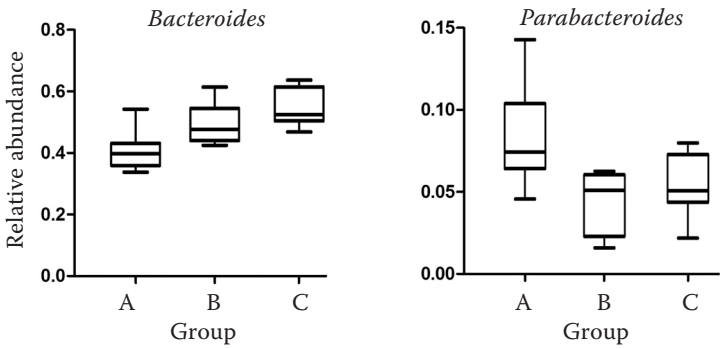

Figure 4. Generic differences of Pekin duck cecal microbiota as affected by dietary energy level shown by box plot
group A = low metabolizable energy, group B = normal metabolizable energy, group C = high metabolizable energy

Table 2. Average abundances of major genera (overall mean abundance > 1%) of the cecal contents of Pekin ducks fed diets with different metabolizable energy levels

Phylum	Genus	Treatment			*P*-value
		A (SEM[1])	B (SEM)	C (SEM)	
Bacteroidetes	*Bacteroides*	40.76 (4.13)[b]	49.33 (4.28)[a]	54.53 (4.39)[a]	0.001
	Parabacteroides	8.30 (1.11)[a]	4.43 (1.26)[b]	5.37 (1.29)[b]	0.011
	Alistipes	3.35 (0.77)[a]	1.25 (0.72)[b]	3.78 (0.65)[a]	0.024
Firmicutes	unclassified *Ruminococcaceae*	6.49 (0.81)[a]	4.64 (0.73)[b]	4.14 (0.77)[b]	< 0.001
	Butyricicoccus	1.00 (0.27)[a]	0.60 (0.31)[ab]	0.52 (0.15)[b]	0.049
Proteobacteria	*Sutterella*	0.33 (0.25)[b]	0.33 (0.24)[b]	1.12 (0.32)[a]	0.001
Deferribacteres	*Mucispirillum*	1.58 (0.55)	3.41 (0.65)	1.88 (0.53)	0.099
Spirochaetes	*Brachyspira*	4.91 (1.72)[a]	0.01 (0.02)[b]	0.00 (0.03)[b]	0.001
Fusobacteria	*Fusobacterium*	0.30 (0.41)[b]	1.63 (0.45)[a]	1.41 (0.45)[a]	0.035

A = low metabolizable energy, B = normal metabolizable energy, C = high metabolizable energy
[1]standard error of the mean for $n = 9$
[a,b]values with different superscripts in the same row differ significantly between treatments ($P < 0.05$)

dation of plant polysaccharides (Danzeisen et al. 2011; Flint et al. 2012). The Pekin duckling cecal microbiota was also dominated by Bacteroidetes and Firmicutes (more than 80%), which is in close agreement with a previous study on 12–14-week-old duck, suggesting an important conserved role

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Table 3. Alpha diversity of microbial profile of the cecal contents of Pekin ducks fed diets with different metabolizable energy levels

Item	Treatment			*P*-value
	A (SEM[1])	B (SEM)	C (SEM)	
OTUs	416.75 (25.22)	353.33 (15.01)	378.25 (15.19)	0.169
Ace	622.50 (27.45)	547.85 (21.44)	569.37 (23.46)	0.660
Chao	588.67 (24.79)	493.48 (22.65)	523.01 (23.52)	0.183
Simpson	0.06 (0.003)[b]	0.07 (0.005)[ab]	0.08 (0.007)[a]	0.045
Shannon	3.77 (0.12)[a]	3.48 (0.11)[ab]	3.37 (0.09)[b]	0.019

A = low metabolizable energy, B = normal metabolizable energy, C = high metabolizable energy, OTUs = number of operational taxonomic units, Ace = ACE richness, Chao = Chao1 richness, Simpson = Simpson diversity index, Shannon = Shannon diversity index

[1]standard error of the mean for $n = 9$

[a,b]values with different superscripts in the same row differ significantly between treatments ($P < 0.05$)

in the intestinal microbiota metabolism (Vasai et al. 2014). However, the ratio of Firmicutes/Bacteroidetes was quite different between host species. In this and previous studies, duck cecal segments were dominated by Bacteroidetes, whereas Firmicutes was the major phylum in broiler chickens and ostrich (Matsui et al. 2010; Vasai et al. 2014). Interestingly, the major phylum in ceca of turkeys and laying hens was also Bacteroidetes regardless of the phylogenetic distance between these bird species, further confirming that environmental conditions and diet could play a more important role than genetics (Callaway et al. 2009; Carmody et al. 2015).

The relative abundance of duck cecal Firmicutes is lower (17.5% vs 27.5%) whereas that of Proteobacteria is higher (10.7% vs 3.9%) than that reported in a previous study on 12–14-week-old ducks (Vasai et al. 2014). Notably, the most diet-responsive *Streptococcaceae* (Vasai et al. 2014) were in a very low abundance in the present study, to the extent of being undetected in two of the 27 cecal samples. Moreover, diversity index, including Chao1 richness, Simpson diversity, and Shannon diversity index were all lower than those of the previous study (Vasai et al. 2014). Proteobacteria is also a dominant phylum present in newborn mice, but it is suppressed in normal adult microbiota (Mirpuri et al. 2014). Similarly, the differences between the present study and the previous report of Vasai et al. (2014) might be attributed to the increased age of ducks, a situation which has been recorded in chickens and humans (Gong et al. 2008; Nicholson et al. 2012). Research shows that early gut colonizers have the potential to exert physiologic, metabolic, and immunologic functions for most, and perhaps all, of host life (Faith et al. 2013).

The relative abundance of major *Ruminococcaceae* (phylum Firmicutes) increased with the decreasing dietary energy level. This is in agreement with the report on monkey (Amato et al. 2015). Most of the known *Ruminococcaceae* originate from rumen ecosystems where they act as major degraders of resistant polysaccharides such as starch and cellulose. Interestingly, *Ruminococcus* sp. strain 16422 has been associated with high efficiency of energy extraction from feed in chicken (Stanley et al. 2012). *Butyricicoccus* includes several taxa of butyrate-producing bacteria, which have been reported to prevent inflammatory bowel disease (Faith et al. 2013). These findings suggest that significant increasing the dietary energy concentration could increase the risk of bowel problems.

The spirochaete *Brachyspira* can cause enteric disease in avian, porcine, and human hosts, amongst others, with a potential for zoonotic transmission (Mappley et al. 2014). In the present study, *Brachyspira* bacteria were detected with a high relative abundance (4.91%) in group A, but were almost undetected in groups B and C. Interestingly, only one OTU that had 6898 read tags was mapped to *Brachyspira*. We compared the representative sequences of this OTU with 16S ribosomal RNA sequences (Bacteria and Archaea) database by BLASTN, and found that it has 99.6% similarity with the 16S ribosomal RNA sequence of *Brachyspira pilosicoli*. This indicated that low

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dietary energy might favour colonization by *Brachyspira pilosicoli*. *Brachyspira pilosicoli* (Swayne and McLaren 1997) has been isolated from asymptomatic wild ducks (*Anas platyrhynchos*), and its presence has been associated with increased typhlocolitis, renal degeneration, and hepatic/splenic amyloidosis in ducks (Glavits et al. 2011).

In summary, dietary energy level significantly affected duck cecal microbiota. Increased dietary energy level reduced the diversity of duck cecal microbiota. Low dietary energy content may reduce the defense of duck cecum to *Brachyspira*. From a practical point of view, the gut microbiota should be considered when formulating duck feed.

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