Metagenomics analysis reveals significant modulation of cecal microbiota of broilers fed palm kernel expeller diets

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ABSTRACT The potential use of palm kernel expeller (**PKE**) as an alternative energy source in broiler diets is limited by the high fiber content. Although enzymatic treatment could alleviate the fiber component and increase the nutritive value of PKE, this apparent improvement is not reflected in the growth response of birds fed with the treated-PKE. As chicken's ceca are the most heavily populated with microflora within their gastrointestinal tract, it was hypothesized that any modulation of the intestinal environment by dietary treatments should be reflected by the composition and activities of the cecal microflora. There is a correlation between cecal microbiota composition and the efficiency of the host to extract energy from the diet and to deposit that energy into improved feed conversion ratio. At present, little is known about the changes on cecal microflora of broilers fed with PKE diets. Hence, this study was designed to assess the effects of feeding different forms of PKE; namely untreated PKE (UPKE), enzyme-treated PKE (EPKE), and oligosaccharides extracted from PKE (OligoPKE), on the cecal microbiota of broiler chickens at 14 d old (day 14) and 28 d old (day 28) using 16S rRNA gene high-throughput next-generation sequencing method. The results showed that temporal changes in cecal microbiota of broiler chickens were evident on day 14 and day 28. The relative abundance of phylum Firmicutes, known to be involved in nutrient uptake and absorption in both age groups was higher in the UPKE as compared to EPKE group. In addition, supplementation of OligoPKE increased ($P < 0.05$) the relative abundance of *Lactobacillus* on both D14 and D28, signifying its effect as prebiotics in enhancing growth of indigenous *Lactobacillus*. Our results showed that cecal microbiota was significantly modulated by dietary treatments and that the lower relative abundance of phylum Firmicutes in chickens fed with EPKE could be a reason why broiler chickens fed with EPKE of higher metabolizable energy (ME) content did not show improvement in their growth performance.

Key words: palm kernel expeller, broiler chicken, cecal microbiome

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Palm kernel expeller (**PKE**) is a byproduct from the palm kernel oil extraction. This agro-industrial waste is mainly used as a ruminant feed and its use in poultry feed is constrained by the high fiber content, ranging from 13 to 20% (Sundu et al., 2006). The use of enzyme treatment to enhance the nutritive value of PKE achieved significant reduction in the fiber component which accompanied by an increase in the release of reducing sugars and an enhancement in the metabolizable energy (ME) content (Hanafiah et al. 2017; Chen et al., 2018). However, the above apparent nutritional improvement was not reflected in the growth performance of broiler chickens fed with 20–25% enzyme-treated PKE (EPKE) (Saenphoom et al., 2013; Chen et al., 2018). The reason for this lack of improvement in growth performance is unclear.

It was reported that gut microbiota involved in nutrient digestion and absorption, and energy metabolism (Rinttilä and Apajalahti, 2013). Chicken's ceca are the most heavily populated region of the gastrointestinal tract, harboring diverse microorganisms involved in preventing pathogen colonization, detoxifying harmful substances, recycling nitrogen, digestion of resistant carbohydrates, and absorption of additional nutrients (Clench and Mathias, 1995). In a cecal metagenome study, numerous oligosaccharides and polysaccharides fermentation pathways leading to production of short-chain fatty acids (**SCFAs**) were discovered (Sergeant et al., 2014). The SCFAs were mainly produced through

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microbial fermentation, and could be absorbed and catabolized to produce energy for the host animal (McWhorter et al., 2009). It was estimated that conversion of oligosaccharides into SCFAs by cecal microbiota contributed between 3.5 to 10% of the ME in poultry (Józefiak et al., 2004) and thus serves as a significant energy source for the chickens.

The high-throughput next-generation sequencing (**HT-NGS**) is capable of providing a more comprehensive information of the gut microbiota for better evaluation of dietary alterations on microbial diversity (Zhou et al., 2010). Studies on the age-related changes in gut microbiota (Shaufi et al., 2015), and the replacement of cecal bacterial component in response to different diets on different strain of broiler chickens (Pourabedin et al., 2014; Corrigan et al., 2015; Park et al., 2017) using HT-NGS targeted on 16S rRNA genes have been reported. It is known that cecal microbiota of broiler chickens can be manipulated with different dietary treatments (Apajalahti et al., 2004), and to date, the information on the effects of PKE-containing diets on the gut microbial composition of broiler chickens using HT-NGS method is still lacking. Hence, the present study was conducted to investigate the effects of feeding different forms of PKE; namely untreated PKE (UPKE), EPKE, and OligoPKE (oligosaccharides, mainly mannose and mannanoligosaccharides extract from PKE) on cecal microbial community of broiler chickens at 14 (day 14) and 28 (day 28) d of age using HT-NGS of 16S rRNA gene amplicon. It was anticipated that the outcome of this study would provide an explanation to why there was no improvement in the growth performance of chickens fed with enzyme treated PKE.

MATERIALS AND METHODS

Preparation of PKE-Containing Diets

Enzyme-treated PKE was prepared through solid state fermentation with a crude enzyme extract from *Apergillus terreus* (Chen et al., 2013). Briefly, 1 kg of PKE was weighed into a tray (40 × 30 × 10 cm) and added with distilled water to achieve an initial moisture content of 60%. After that, crude enzyme (9 U mannanase/g PKE) was added into the mixture and incubated at 51°C for 18 h. After incubation, the treated PKE was oven dried at 60°C. Both, the EPKE and UPKE were grounded to 2.5 mm prior to their use in the experimental diet.

OligoPKE was prepared by shaking 500 g of EPKE in 500 mL of distilled water for 1 h in room temperature. Solid particles were removed by centrifuging the mixture at 2,500 × *g* for 10 min and then filtered through a Whatman No. 1 filter. Excess water was removed through evaporation using a rotary evaporator, and lipids and proteins were removed through precipitation by chloroform and acetonitrile as described by Jahromi et al. (2016), followed by lyophilization to obtain solid OligoPKE.

Animal Management

The complete experimental procedure was performed according to the regulations and guidelines established by the Animal Care and Ethics Committee of the Universiti Putra Malaysia. Two hundred and forty day-old male Cobb500 broiler chicks were randomly allocated to 4 dietary treatments: (i) basal diet (control), (ii) UPKE, (iii) EPKE, and (iv) OligoPKE. Each treatment consisted of 6 replicates with 10 birds per replicate (10 birds/cages), making up a total of 60 birds per treatment. All chicks were raised in battery cages and fed with starter diets (day 1 to 21) and grower diets (day 22 to 28) formulated to meet the nutrient requirements of poultry according to the Cobb500 (2015). Feed compositions for experimental diets are presented in Table 1. Feed intake and body weights were recorded weekly for estimations of feed conversion ratio.

Samples Collection, DNA Extraction and Sequencing of the 16S rRNA Genes

Cecal contents were collected at 2 separate periods (i.e., day 14 and day 28) from euthanized birds. At each sampling period, 2 birds were randomly selected from each cage and their cecal digesta samples were pooled and frozen at −20°C for DNA extraction. The total bacterial genomic DNA was extracted from each cecal digesta sample using the QIAampFast DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Extracted DNA was sequenced (2 × 300 bp run configuration) using the Illumina Miseq desktop sequencer (Illumina, California, USA) by Macrogene Inc. (Seoul, Korea), targeting the V3 to V4 hypervariable region of the 16S rRNA gene with forward primer (5′ CCTACGGGAGGCAGCAG 3′) and reverse primer (5′ GGACTACTAGGGTTTCTAAT 3′).

Data Analysis

The raw sequence data obtained were processed and analyzed using QIIME software (Kuczynski et al., 2012). Briefly, paired-end reads were merged using PANDASeq v2.8 (Masella et al., 2012). Overlapping regions were aligned into contigs in which ambiguous base calls (N), shorter than 440 bp and longer than 480 bp were discarded. Quality filtering was conducted using the QIIME default parameters (1.5 allowed barcode error, Phred Q20, and 3 allowed bad quality bases before truncation). All assembled sequences were assigned to the operational taxonomic units (OTUs), picked using UCLUST (Edgar, 2010) with a closed reference protocol and cluster defined at 97% sequence similarity. The OTU sequences were aligned against the Greengenes core database (version 13.8) set using PyNAST alignment algorithm (Caporaso et al., 2010). All OTU lesser than 5 sequences were removed and

Table 1. Compositions of the dietary treatments

	Starter				Grower			
Ingredients (%)	Control	UPKE	EPKE	OligoPKE	Control	UPKE	EPKE	OligoPKE
Corn	55.6	50.9	50.9	54.6	60.4	45.2	45.2	59.4
Soybean meal	36.7	35.6	35.6	36.7	32.4	25.5	25.5	32.4
UPKE	0	5.00	0	0	0	20.0	0	0
EPKE	0	0	5.00	0	0	0	20.0	0
OligoPKE	0	0	0	1.00	0	0	0	1.00
Dicalcium phosphate	1.70	1.60	1.60	1.70	1.40	1.30	1.30	1.40
Common salt	0.30	0.30	0.30	0.30	0.35	0.35	0.35	0.35
Vitamin premix[1]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Mineral premix[2]	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Palm oil	3.60	4.50	4.50	3.60	3.50	6.00	6.00	3.50
Limestone	1.10	1.10	1.10	1.10	1.00	0.80	0.80	1.00
Choline Cl	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
L-lysine HCl	0.32	0.32	0.32	0.32	0.20	0.25	0.25	0.20
DL-methionine	0.16	0.16	0.16	0.16	0.17	0.15	0.15	0.17
Titanium dioxide	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
ME (MJ/kg)	12.6	12.6	12.6	12.6	12.8	12.7	12.7	12.8
Crude protein (%)	20.8	20.9	20.9	20.8	19.5	18.9	19.1	19.5
Ether extract (%)	6.13	7.14	7.10	6.13	6.21	9.27	8.95	6.21
Crude fiber (%)	4.02	4.61	4.48	4.02	3.85	6.12	5.58	3.85
Calcium (%)	0.91	0.89	0.89	0.91	0.80	0.72	0.72	0.80
Lysine (%)	1.33	1.31	1.31	1.33	1.14	1.04	1.05	1.14
Methionine (%)	0.50	0.49	0.49	0.50	0.49	0.43	0.44	0.49

[1]Vitamin premix supplied per ton of diet: vitamin A, 50.00 MIU; vitamin D3, 10.00 MIU; vitamin E, 75.00 MIU; vitamin K3, 20.00 g; vitamin B1, 10.00 g; vitamin B2, 30.00 g; vitamin B6, 20.00 g; vitamin B12, 0.10 g; calcium D-pantothenate, 60.00 g; nicotinic acid, 200.00 g; folic acid, 5.00 g; biotin, 235.00 mg.

[2]Mineral premix supplied per ton of diet: Se, 0.20 g; Fe, 80 g; Mg, 100 g; Zn, 80 g; Cu, 15 g; KCl, 4 g; MgO, 0.60 g; $NaHCO_3$.

the remaining OTUs were then rarefied to the lowest sequence count (n = 21,400) in order to provide an equal depth of sequence analysis for all experimental groups.

Alpha and Beta Diversity Analysis Alpha diversity and rarefaction curve analysis was performed to obtain the community diversity (Shannon and Simpson diversity indices), richness (ACE and Chao1), and sampling effort (Good's coverage) with a cutoff of 0.03 for each sample. Beta diversity analysis was used to compare the diversity among different treatments at different ages. Weighed Unifrac distance (Vázquez-Baeza et al., 2013) was calculated and plotted with Principal Coordinate Analysis using QIIME software. All assigned taxonomy was analyzed using Statistical Analysis of Metagenomic Profiles software (Parks et al., 2014) by applying the Welch's two-sided t-test for comparison among different sample groupings and produced *P*-values for statistical analysis.

Predictive Functional Metagenome of Broiler Cecal Microbiota Predictive functional analysis was performed using PICRUSt software package (Langille et al., 2013). This software estimates the community metagenome using 16S rRNA sequencing data. The OTU table generated from QIIME software was uploaded and processed using the online Galaxy terminal (http://huttenhower.sph.harvard.edu/galaxy/). Differences between dietary treatments were compared based on KEGG orthologs functions predictions, and analyzed using Statistical Analysis of Metagenomic Profiles software package (Parks et al., 2014).

Nucleotide Sequences and Accession Numbers

Illumina MiSeq sequence data from this study have been deposited in the NCBI Sequence Read Archive under the accession number SRP106847.

RESULTS

Growth Performance

Effects of the different dietary treatments on growth performance of broiler chickens are presented in Table 2. The results of the current study showed that there were differences in the daily feed intake (DFI) of broiler chickens on day 14 and day 28. On day 14, OligoPKE fed broiler chickens had higher ($P < 0.05$) DFI as compared to control, whereas broiler chickens supplemented with OligoPKE had lower ($P < 0.05$) DFI on day 28 when compared with other dietary treatment groups (Control, UPKE, and EPKE). However, there were no differences observed in the DFI of broiler chickens in the overall experimental period. Additionally, there were no differences found in the average daily gain and feed conversion ratio among all dietary treatment groups within individual or overall experimental period as shown in Table 2.

Table 2. Growth performance of chicken fed with different dietary combination treatments.

Parameters	Control	UPKE	EPKE	OligoPKE	SEM
Average daily gain, ADG (g/birds)					
Day 7	12.55	13.45	13.81	12.57	0.27
Day 14	28.44	30.73	28.29	31.41	1.05
Day 21	59.14	59.82	58.45	61.16	1.65
Day 28	69.84	66.67	68.15	65.48	1.36
Daily feed intake, DFI (g/birds)					
Day 7	16.28	17.57	18.98	18.22	0.39
Day 14	45.85^{b}	50.38^{a,b}	46.04^{b}	53.96^{a}	0.96
Day 21	76.26	75.23	77.15	78.35	1.12
Day 28	121.19^{a}	122.28^{a}	120.17^{a}	110.44^{b}	1.57
Feed conversion ratio, FCR					
Day 7	1.31	1.31	1.40	1.45	0.03
Day 14	1.63	1.72	1.66	1.59	0.04
Day 21	1.28	1.27	1.27	1.28	0.02
Day 28	1.78	1.84	1.77	1.70	0.03
Overall (g/birds)					
ADG	42.49	42.67	42.18	42.66	0.49
DFI	64.89	66.37	65.58	65.24	0.62
FCR	1.53	1.56	1.53	1.53	0.01
Initial BW	42.90	42.82	44.00	43.11	0.24
Final BW	1,232	1,237	1,225	1,237	13.55
Mortality (%)	4.71	0	0	0	0.19

^{a,b}Means within a row lacking a common superscript differ significantly ($P < 0.05$).

Day 7 = 7-d-old bird, Day 14 = 14-d-old bird, Day 21 = 21-d-old bird, Day 28 = 28-d-old bird, BW = body weight, SEM = standard error of the mean.

Sequencing Data Analysis

Illumina Miseq sequencing of the V3 and V4 regions of the bacterial 16S rRNA gene yielded a total of 4552,873 sequence reads with a median length of 497 bp. After culling the low quality reads, 4370,991 sequence reads were classified into 431 phylotypes at 97% sequence similarity threshold. Sequences were subsampled to the lowest number of sequences per sample (n = 21,400) to ensure an equal sequence depth was used for calculations and comparisons.

Dietary Effect on Cecal Microbial Diversity

The sequence coverage, observed OTUs, species diversity and richness of the cecal microbiome are shown in Table 3. Rarefaction curve plotted from the OTUs (97% similarity) reached asymptote suggesting that high sequence coverage was achieved (Figure 1). Coverage is an estimator of sampling completeness, and in this study, OTU samples at 3% distance were high, indicating that majority of the cecal bacteria were identified at 21,400 sequences per sample. In general, day 28 birds had higher OTUs and species richness (ACE and Chao1) as compared to the day 14 birds. In both age groups, the results showed that there was no significant difference in species richness among treatment groups. However, broiler chickens fed with UPKE, EPKE, and OligoPKE diets had higher diversity (Shannon and Inverse Simpson) indices than control, with only UPKE fed broiler chickens had significantly higher ($P < 0.05$) diversity compared to those in control.

Dietary Alteration on Cecal Bacterial Communities

A Principal Coordinate Analysis plot of the overall diversity based on weighed UniFrac metric is shown in Figure 2. The Principal Coordinate Analysis plot showed a clear temporal shift in bacterial communities as the chicks grew signifying that the clustering distances between samples were highly dependent on age, and to a lesser extent, on the diet. The effect of diets on the overall diversity was more noticeable in the day 14 birds as compared to the day 28 birds. Based on the plot, birds fed with the different PKE diets formed a widespread cluster, distinct from control group.

Comparison of Bacterial Communities among Treatments

In the present study, taxonomic characterization of cecal bacterial communities was reported based on the relative abundance of OTUs. With an OTU definition at similarity cut-off of 97%, a total of 3 most abundant OTUs annotated to the phylum level (Figure 3), 10 most abundant OTUs annotated to the family level (**Supplementary, Figure S1**), and 15 most abundant OTUs annotated to the genus level (**Supplementary, Figure S1**) were evaluated among the 4 dietary treatment groups at day 14 and day 28. Each of the OTU was analyzed for significant enrichments or depletions in dietary treatments based on relative abundance.

The control treatment was used to illustrate the cecal bacterial communities of broiler chickens fed the commonly used corn–soybean meal diet. In the day 14 broiler chicks, majority of the cecal bacterial

Table 3. Analysis of bacterial diversity in broiler's cecal exposed to different dietary treatments at 14- and 28-d-old broiler chicken.

Sample ID	Good's coverage	OTUs	ACE	Chao1	Shannon	Simpson (1/D)
Day 14C	0.998	158 ± 12	194 ± 26	188 ± 25	3.77 ± 0.23^{B}	6.3 ± 1.74^{B}
Day 14U	0.998	171 ± 19	206 ± 17	209 ± 14	4.87 ± 0.25^{A}	15.92 ± 1.14^{A}
Day 14E	0.998	159 ± 12	197 ± 18	190 ± 15	4.41 ± 0.33^{A,B}	11.28 ± 3.91^{A,B}
Day 14O	0.998	180 ± 35	212 ± 42	215 ± 47	4.73 ± 0.6^{A}	13.06 ± 5.47^{A,B}
Day 28C	0.997	264 ± 7	319 ± 4	333 ± 12	4.62 ± 0.15	8.85 ± 1.67^{b}
Day 28U	0.997	282 ± 14	340 ± 14	333 ± 32	5.09 ± 0.3	14 ± 3.3^{a}
Day 28E	0.997	285 ± 18	346 ± 31	350 ± 37	5.03 ± 0.4	12.46 ± 3.45^{a,b}
Day 28O	0.997	271 ± 7	329 ± 9	334 ± 9	4.73 ± 0.15	9.63 ± 0.73^{a,b}

Values are means ± standard deviation (n = 3).
Day 14 = 14-d-old bird, D28 = 28-d-old bird, C = control, U = UPKE, E = EPKE, O = OligoPKE.
^{A,B}Means with different superscripts within a column are significantly different ($P < 0.05$) between treatments at day 14.
^{a,b}Means with different superscripts within a column are significantly different ($P < 0.05$) between treatments at day 28.

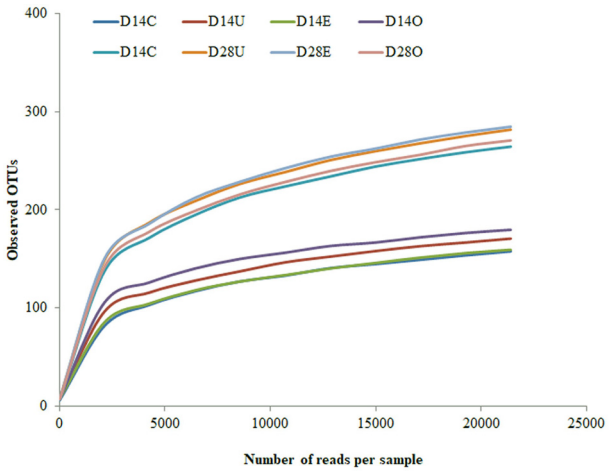

Figure 1. Rarefaction curves of cecal bacteria communities. Rarefaction curves calculated based on the V3-V4 16S rRNA gene sequences of different dietary treatment groups (day 14 = 14-d-old bird, day 28 = 28-d-old bird, C = control, U = UPKE, E = EPKE, and O = OligoPKE) examined at 3% divergence. OTUs were defined by the average neighbor algorithm with 97% similarity using QIIME.

belonged to the phylum Bacteroidetes (60.37%), followed by phylum Firmicutes (37.88%) with the remainder belonging to the phylum Proteobacteria (Figure 3). Temporal shift of bacterial diversity was observed in the day 28 broiler chickens, whereby the phylum Firmicutes (24.34%) was replaced by phylum Bacteroidetes (69.11%) and Proteobacteria (5.68%). These 3 phyla represented approximately 99% of the cecal communities in day 28 broiler chickens (Figure 3).

In the day 14 broiler chicks, replacement of phylum Bacteroidetes by phylum Firmicutes was observed in the 3 PKE-containing diets ($P < 0.05$) (Figure 3A and C). At the genus level, UPKE, EPKE, and OligoPKE diets had higher relative abundance of the genus *Lactobacillus* (6.21, 8.21, and 12.07%, respectively) (Table 4) as compared to the control (1.68%). Similarly, all PKE-containing diets had higher relative abundances ($P < 0.05$) of unclassified Ruminococcaceae, *Oscillospira*, *Blautia*, and unclassified Clostridiales (total of 41.76, 33.78 and 41.30% for UPKE, EPKE, and OligoPKE, respectively) as compared to control (20.24%) at day 14 (Table 4).

There was an increase ($P < 0.05$) in phylum Bacteroidetes and decrease ($P < 0.05$) in phylum Firmicutes in PKE-containing diets as the birds aged (Figure 3). Within the phylum Bacteroidetes, succession of the family Bacteroideceae (34.56%) by the family Rikenellaceae (29.11%) was noticeable in the control group at day 28 (Table 5). In the family level, the effect of dietary treatments on the cecal microbiota of day 28 broiler chickens was prominent in the UPKE and EPKE diets, but not in the OligoPKE diet. The effect of the UPKE and EPKE was directed towards the decrease in relative abundance of the family Rikenellaceae (10.07 and 13.95%, respectively) as compared to control (29.16%). In the case of the genus *Lactobacillus*, although the population of *Lactobacillus* was low in the day 28 broiler chickens (0.77% in control), only OligoPKE supplementation increased ($P < 0.05$) the relative abundance of *Lactobacillus* (2.76%).

Noticeable increases in the phylum Proteobacteria as the broilers aged in all treatment groups, with those fed with UPKE and EPKE diets showing significant increase ($P < 0.05$) from less than 1% in the day 14 broiler chicks to an average of 16.32% in the day 28 broiler chickens (Figure 3). In addition, there was an increase in the family Enterobacteriaceae (13.58 and 12.60%, respectively) and unclassified Clostridiales (11.34 and 9.77%, respectively) as compared to control (3.30 and 5.32%, for Enterobacteriaceae and unclassified Clostridiales, respectively) at day 28.

Predictive Functional Metagenome of Broiler Cecal Microbiota

Functional potential of the cecal microbiota of broiler chickens classified into 3 levels of KEGG orthologs was conducted using PICRUSt (Langille et al., 2013). A total of 328 KEGG pathways were classified into 6 main categories. In the broiler chickens, majority of the predicted pathways belonged to 4 main categories; (1) metabolism, (2) genetic information and processing,

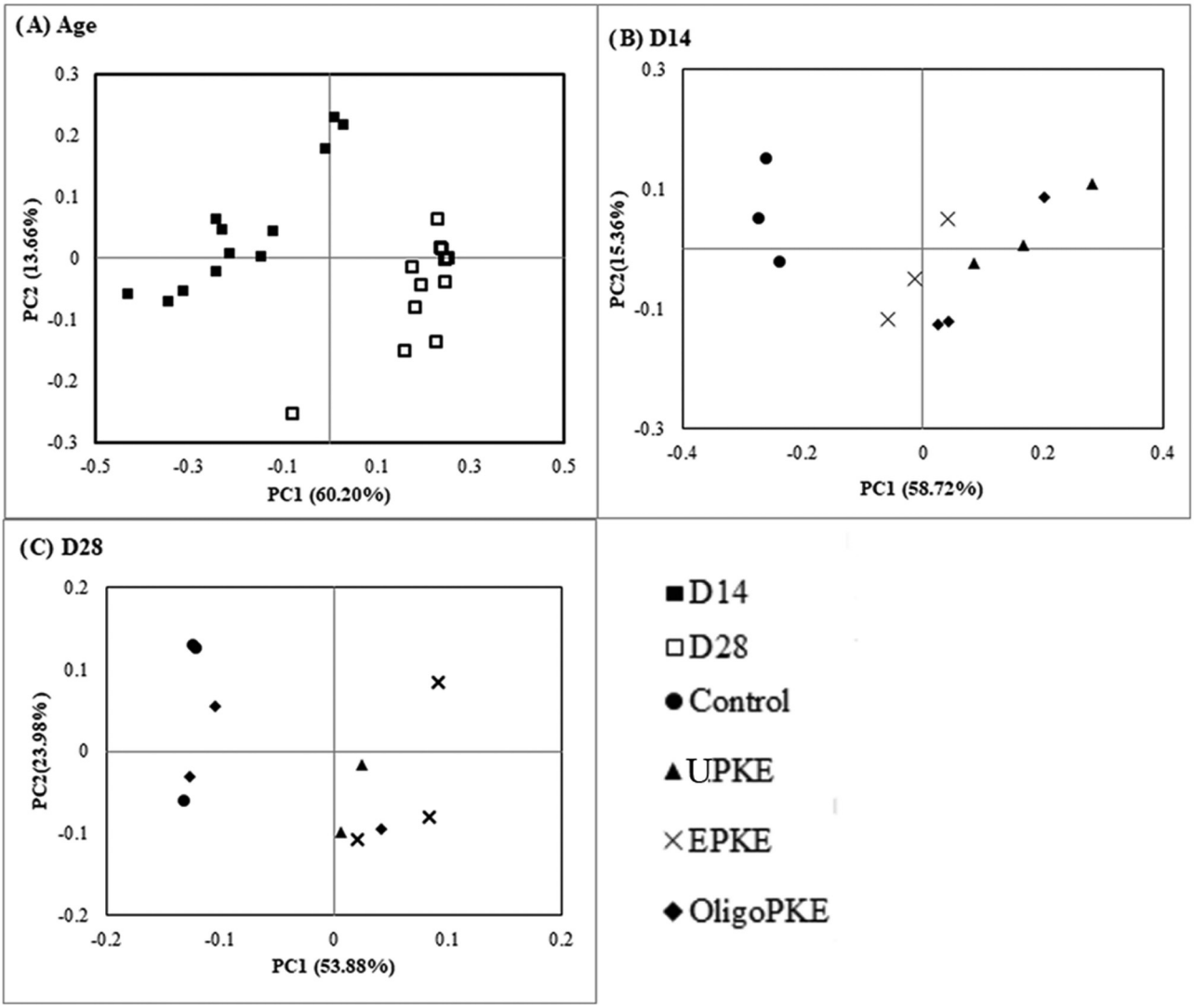

Figure 2. Principal Coordinate Analysis (PCoA) plots of beta diversity. PCoA of weighed unifrac distance in response to broilers at different ages (A), and dietary treatments (B and C).

(3) environmental information processing, and (4) cellular processes and signaling (Table 6). All the 3 PKE fed groups altered an average of 80 affiliated KEGG pathways in the day 14 broiler chicks, mainly by increasing the environmental information processing system accompanied by the decrease in metabolic system (Table 6). However, the effect of dietary treatments on functional potential of cecal microbiota was less prominent in the day 28 broiler chickens, with only those fed with UPKE and EPKE diets showing altered ($P < 0.05$) microbiota functions as compared to control (Table 7).

In the current study, PKE-based treatment groups reduced genes related to metabolism ($P < 0.05$) in the day 14 broiler chicks, but only UPKE and EPKE had significant reduction in metabolism related genes in the day 28 broiler chickens. Because PKE is rich in mannose-based oligosaccharides, the ability of cecal microbiota to utilize mannose and production of SCFAs was evaluated. PKE fed broilers showed an increase in the cecal bacterial population involved in propionate, butyrate, fructose and mannose metabolism in both the day 14 and day 28 groups. However, only day 14 birds fed with UPKE diet increased ($P < 0.05$) the fructose and mannose utilizations. In addition, butyrate metabolism was also higher ($P < 0.05$) for birds fed with UPKE and OligoPKE diets in the day 14 broiler chicks, whereas propionate metabolism was higher ($P < 0.05$) in the day 28 broiler chickens fed with UPKE or EPKE diet (Table 7).

In the environmental information processing category, genes associated with membrane transport systems (ABC transporters and phosphotransferase system) were significantly increased ($P < 0.05$), whilst genes related to signaling molecules and interaction (bacterial toxins secretion and cellular antigens) were reduced in the 3 PKE fed groups at D14. In contrast, UPKE and EPKE increased genes related to bacterial colonization (bacterial motility proteins, bacterial secretion system, and 2-component system) in the day 28 broilers chickens (Table 7).

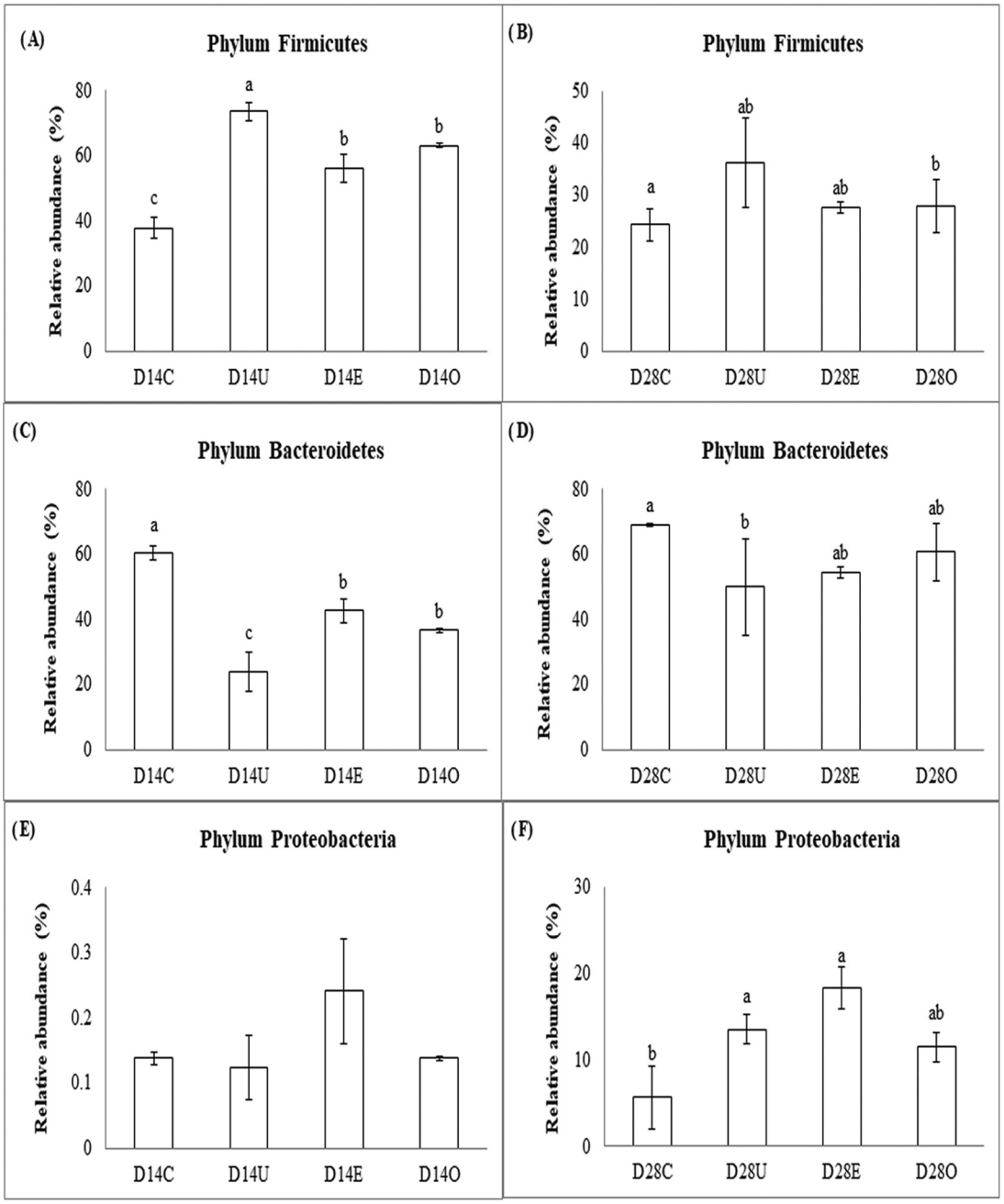

Figure 3. Dietary treatments effect on cecal microbial composition at phylum level. Relative abundances of cecal bacteria at phylum level for each dietary treatment group at 14-d-old (day 14) and 28-d-old (day 28) broilers chickens. Dietary treatment groups are control (day 14C, day 28C), UPKE (day 14U, day 28U), EPKE (day 14E, day 28E), and OligoPKE (day 14O, day 28O). Dissimilar letters (a, b, c) in the figures denote significant difference ($P < 0.05$).

DISCUSSION

Palm kernel expeller contains high level of insoluble fiber mainly in the form of mannan with a very low degree of galactose substitution, resembling very much of cellulose by being hard and highly crystalline (Dusterhoft et al., 1992). Despite the profound reduction in the fiber content coupled with improvement in the production of soluble sugars (Chen et al., 2018), and increase in metabolize energy (Hanafiah et al., 2017) through enzymatic treatment, there was no improvement in the growth performance of broiler chickens in the present study. The above observation, although surprising, is in agreement with those reported previously (Saenphoom et al., 2013; Navidshad et al., 2016). Saenphoom et al. (2013) suggested that the above observation could be due to the poor assimilation of mannose (the main sugar released by enzymatic treatment) by the broiler chickens. It is postulated that this may create a loop of event whereby the poor assimilation of mannose could increase the amount of mannose-based compound in the ceca which may subsequently alter the cecal microbiota. The changes in cecal microbiota will in turn affect the overall functionality of the gut of broiler chickens.

Table 4. Relative abundances of cecal bacterial family (bold type) and selected genera in day 14 broiler chicks fed with different dietary treatments.

	Control	UPKE	EPKE	-OligoPKE
Bacteroidaceae	59.99[a]	27.05[c]	37.68[b]	32.44[a,b]
Rikenellaceae	0.38	0.24	4.95	0.52
Unclassified Clostridiales	4.25[b]	7.14[a]	9.54[a]	7.43[a]
Lactobacillaceae	1.68[b]	6.21[b]	8.21[b]	12.07[a]
Lactobacillus	1.68[b]	6.21[b]	8.21[b]	12.07[a]
Lachnospiraceae	3.74[c]	16.14[a]	10.90[a,b]	7.77[b,c]
Ruminococcus	1.24[b]	7.68[c]	5.76[a,b]	1.61[b]
Blautia	0.69[b]	3.63[a]	2.09[a,b]	2.5[a,b]
Unclassified	0.38	0.24	4.95	0.51
Ruminococcaceae	24.31	35.10	26.33	38.13
Ruminococcus	6.51	3.77	4.15	6.61
Oscillospira	2.41[b]	8.97[a]	2.94[b]	5.56[a,b]
Faecalibacterium	2.39	0	0	0
Unclassified	12.92[b]	22.02[a]	19.21[a]	25.81[a]
Enterobacteriaceae	0.06	0.25	0.87	0.16

Values with different superscripts (a, b, c) in a row are significantly different ($P < 0.05$).

Table 5. Relative abundances of cecal bacterial family (bold type) and selected genera in day 28 broiler chickens fed with different dietary treatments.

	Control	UPKE	EPKE	OligoPKE
Bacteroidaceae	34.56	39.07	35.09	38.00
Rikenellaceae	29.16[a]	10.10[b]	13.95[b]	21.95[a,b]
Unclassified Clostridiales	5.32[b]	11.34[a]	9.77[a]	6.09[b]
Lactobacillaceae	0.77[b]	0.58[b]	0.74[b]	2.76[a]
Lactobacillus	0.77[b]	0.58[b]	0.75[b]	2.76[a]
Lachnospiraceae	3.94	4.77	4.33	4.63
Ruminococcus	1.31	1.58	0.76	1.94
unclassified	29.11[a]	10.07[b]	13.93[b]	21.93[a,b]
Blautia	0.05	0.07	0.04	0.07
Ruminococcaceae	11.86	13.46	11.89	11.19
Unclassified	2.90	4.12	2.98	3.49
Ruminococcus	1.69[b]	3.85[a]	2.31[b]	1.75[b]
Oscillospira	4.82	4.34	5.58	5.20
Faecalibacterium	2.45	1.14	1.01	0.73
Enterobacteriaceae	3.30[b]	13.58[a]	12.60[a]	5.24[a,b]

Values with different superscripts (a, b) in a row are significantly different ($P < 0.05$).

The development of high-throughput sequencing allowed millions of DNA sequences from a sample to be sequenced, providing a greater insight to the microbial diversity, especially towards the less abundant taxa. The combination of V3 and V4 hypervariable regions provides a higher resolution over the single V3 or V4 region of 16S rRNA gene (Cai et al., 2013). In this study, the V3 to V4 region of the 16S rRNA gene amplicon sequencing was conducted using Illumina Miseq platform, applying the paired-end 300-base read chemistry to evaluate the cecal microbiota of broiler chickens fed with different dietary treatments. The generated rarefaction curves have reached plateau with high Good's coverage index (∼1.0) indicated that the 16S rRNA sequencing libraries were able to represent majority of the OTUs present in all the 30 samples sequenced in this study.

Results of the current study showed distinct differences in cecal microbiota richness and diversity between the young (day 14) and adults (day 28) chickens. Based on the rarefaction curves (Figure 1) and diversities indices (Table 3), day 28 chickens showed to have greater richness (ACE and Chao1), diversity (Shannon and Simpson), and variation in community structure (number of OTUs) than the day 14 chicks. These results were consistent with the published data which reported an increase in microbial complexity as chickens aged (Gong et al., 2008; Danzeisen et al., 2011; Shaufi et al., 2015).

At the phylum level, Bacteroidetes was the most predominant phylum found in the cecal content, accounting on an average of 65% of all the bacterial sequences at both ages with the remaining 35% mainly consisting of phylum Firmicutes and Proteobacteria as reported previously (Park et al., 2016). However, these results differed from other studies which showed that broiler cecal was dominated by Firmicutes (Shaufi et al., 2015; Sohail et al., 2015). Although chickens in both age groups (day 14 and day 28) were dominated by the class Bacteroidetes, Clostridia, Gammaproteobacteria, and Bacilli as reported previously (Danzeisen et al., 2011; Shaufi et al., 2015; Park et al., 2016) the proportion of each bacteria may differ. The inconsistency in the taxonomic compositions among studies may be attributed

Table 6. Predicted functional metagenomes classified into first level of KEGG orthologs in cecal microbiota.

	Proportion of sequences (%)			
	Control	UPKE	EPKE	OligoPKE
Day 14				
Metabolism	55.13	53.05*	53.86*	52.52*
Genetic information processing	23.68	23.77	24.01	23.86
Environmental information processing	12.17	14.64*	13.57*	14.66*
Cellular processes and signaling	7.23	7.03	6.94	7.37
Organismal system	1.00	0.79*	0.86*	0.85*
Human diseases	0.80	0.72*	0.76	0.74
Day 28				
Metabolism	56.61	54.80*	54.95*	56.12
Genetic information processing	23.68	23.39	23.34	23.61
Environmental information processing	10.95	12.61*	12.50*	11.54
Cellular processes and signaling	7.07	7.51*	7.53*	7.08
Organismal system	0.89	0.87	0.87	0.86
Human diseases	0.81	0.82	0.82	0.79

Values followed by asterisk "*" in a row are significantly different ($P < 0.05$) from the control value

Table 7. Selected predicted functional metagenomes classified into third level of KEGG orthologs.

Proportion of sequences (%)	Control	UPKE	EPKE	OligoPKE
Day 14				
ABC transporters	2.48	3.34*	2.98*	3.40*
Bacterial motility proteins	0.53	0.59	0.50	0.81
Bacterial secretion system	0.57	0.55	0.56	0.56
Bacterial toxins	0.16	0.14	0.14	0.14
Butyrate metabolism	0.54	0.61*	0.57	0.62*
Cellular antigens	0.06	0.02*	0.04*	0.03*
Fructose and mannose metabolism	1.04	1.16*	1.13	1.07
Phosphotransferase system (PTS)	0.22	0.49*	0.42*	0.35*
Propanoate metabolism	0.54	0.55	0.55	0.55
2-component system	1.24	1.25	1.22	1.35
Day 28				
ABC transporters	2.23	2.62	2.64*	2.40
Bacterial motility proteins	0.39	0.65*	0.66*	0.42
Bacterial secretion system	0.58	0.62	0.61	0.58
Bacterial toxins	0.14	0.13	0.13	0.14
Butanoate metabolism	0.61	0.65	0.65	0.64
Cellular antigens	0.07	0.06	0.07	0.07
Fructose and mannose metabolism	0.95	0.98	0.97	1.00
Phosphotransferase system (PTS)	0.23	0.32	0.29	0.29
Propanoate metabolism	0.48	0.52*	0.51*	0.49
2-component system	1.26	1.42*	1.44*	1.30

Values followed by asterisk "*" in a row are significantly different ($P < 0.05$) from the control value.

to the different analysis methods, as well as other factors such as growth environment, type of broiler chickens, environmental temperature, and feeds (Danzeisen et al., 2011; Wei et al., 2013). For example, using classical cultured based methods, researchers reported that the main cecal genera belong to *Bacteroides*, *Clostridium*, and *Lactobacillus* (Engberg et al., 2004; Bjerrum et al., 2006), which is similar to a culture independent methods by Stanley et al. (2012) who reported high abundances of *Lactobacillus* (24.38%) and C*lostridium* (20.13%). On the contrary, our study showed that cecal microflora of broiler chickens consisted of comparatively low abundance of *Lactobacillus* (0 to 4%) and *Clostridium* (<1%).

The Shannon and Simpson indices which indicate bacterial diversity revealed that broilers fed with UPKE and EPKE diets had higher diversity in both day 14 and day 28 age groups, whereas birds supplemented with OligoPKE had higher diversity only at day 14 of age as compared to the control. This clearly demonstrated that PKE diets could modulate bacterial communities, with the greatest impact on those fed UPKE and that age of bird has higher impact on gut microbiota than dietary prebiotic supplementation, as evident from the present and previous studies (Gong et al., 2008; Danzeisen et al., 2011).

In young chicks (day 14), the relative abundance of phylum Firmicutes was significantly higher in the 3 PKE-based treatment groups when compared to the control. This is in accordance with the results reported by Pourabedin et al. (2014) who observed an increase in the abundance of Firmicutes in the gut of chickens when supplemented with mannanoligosaccharides. In the day 28 broiler chickens, all treatment groups were dominated by the phylum Bacteroidetes. While the increase in phylum Firmicutes was linked to an increase

in nutrient absorption, the increase in phylum Bacteroidetes indicates otherwise (Jumpertz et al., 2011; Tremaroli and Bäckhed, 2012). In human, Firmicutes to Bacteroidetes (F/B) ratio is known to be associated with obesity and higher F/B ratio indicates higher abundance of energy harvesting bacteria (Bervoets et al., 2013). Similarly, higher fecal F/B ratio has also been linked to better bird performance (Singh et al., 2012). The EPKE used in this study had been reported to have higher ME as compared to the UPKE (Hanafiah et al., 2017), and it was expected that the higher ME value of EPKE would promote better growth performance in the broiler chickens. However, the expected improvement in growth performance of birds fed with EPKE diet was not observed in the studies of Saenphoom et al. (2013) and Chen et al. (2018). Results of this study showed that broilers fed with EPKE diet had lower F/B ratio as compared to those fed with UPKE diet at both 14 d and 28 d of age (Figure 3). This result seems to suggest that the effect of additional energy supplied through EPKE is offset by the lower quantity of nutrient harvesting bacteria (Firmicutes) in the EPKE-fed broiler chickens, resulting in a similar growth performance in broilers fed with UPKE and EPKE diets. Nevertheless, the lower F/B ratio may be beneficial in broiler chickens production if leaner birds with higher protein and lower fat contents are desired.

The results of PICRUSt showed significant differences in carbohydrate (butyrate metabolism, propionate metabolism, and fructose and mannose) metabolisms in the ceca of broilers fed the 3 PKE-containing diets as compared to the control. Based on the 16S rRNA microbial abundance, PKE-containing diets tended to increase Firmicutes abundance resulting in higher butyrate metabolism in the day 14 chicks, and later shifted to enhancing proliferation of Bacteroidetes favoring propionate metabolism in the day 28 broiler chickens. This is in accordance to the positive correlation between Firmicutes and butyrate production, and between Bacteroidetes and propionate production reported by Yang et al. (2013). Based on the ontogenic study of broilers, most nutrients were directed to accommodate the rapid turnover of the intestinal cells in young chicks and as the chicks matured, a progressively larger fraction was directed towards body growth (Obst and Diamond, 1992). This provides a plausible explanation for such changes in bacterial population observed, as butyrate is well known in enhancing intestinal cells proliferation and controlling cell apoptosis (Guilloteau et al., 2010), whereas propionate is absorbed and functions in gluconeogenesis and cholesterol metabolism (Ximenes et al., 2005).

At the lower hierarchy, notable increase in the abundance of gram positive bacteria belonging to the genus *Lactobacillus*, *Oscillospira*, and *Blautia* was observed in day 14 broiler chicks fed with the 3 types of PKE-containing diets. The impact of these diets on bacterial genera differed. For example, UPKE mainly increased the relative abundances of *Oscillospira* and *Blautia*, whilst both EPKE and OligoPKE increased primarily the relative abundance of *Lactobacillus*. However, the 3 classified genera (*Lactobacillus*, *Oscillospira*, and *Blautia*) have been shown to be involved in the fermentation of various mono- and oligo-saccharides, producing SCFAs which could contribute around 10% of energy (Józefiak et al., 2004), in addition to their health promoting effects (Mookiah et al., 2014).

Lactobacillus is the better-known probiotic species, important in promoting healthy gut through affecting either intestinal microbial populations, serum lipids, and intestinal morphology (Kalavathy et al., 2003). On the other hand, the function of *Oscillospira* in broilers is less known. However, *Oscillospira* was found to be present in higher abundance in subjects consuming complex carbohydrate diets (Mackie et al., 2003), and an increase in its abundance has shown to reduce inflammatory diseases in human (Konikoff and Gophna, 2016). Nevertheless, *Oscillospira* isolated from human or ruminant shows sequence similarity to *Clostridium* cluster IV obtained from the ceca of broilers (Yanagita et al., 2003). This cluster of *Clostridium* is known to harbor important butyrate producers (Lopetuso et al., 2013). Based on the results of predictive functional profiling in the present study, broiler chicks fed with PKE-containing feeds had higher fructose, mannose, and butyrate metabolisms as compared to the control. This is an indication that the 3 bacterial genera mentioned above (*Lactobacillus*, *Oscillospira*, and *Blautia*) were potentially capable of utilizing mannose to produce butyrate.

As the chicks grew, the family Rikenellaceae in day 28 broiler chickens fed with UPKE and EPKE decreased, whereas the family Enterobacteriaceae and unclassified Clostridiales increased. Interestingly, many unclassified bacteria such as Enterobacteriaceae, Ruminococcaceae, Costridiales, and Rikenellaceae were identified in this study and some of these bacteria were present in high abundance comparable to those of the known bacterial genera and are considered important for cecal ecosystem function of chickens (Wei et al., 2013). The family Ruminococcaceae, specialized in degrading the cellulosic and hemicellulosic components of feeds which cannot be digested by the host (Biddle et al., 2013) was one of the common taxa found in the cecum of chickens (Torok et al., 2011). Clostridiales are a highly polyphyletic order with broad genus placed under this order. Clostridia, depending on *Clostridium* species (Dowd et al., 2008) can positively or negatively influence the host animal. The ability of UPKE and EPKE to reduce Rikenellaceae abundance could be considered as beneficial to the day 28 birds. This is because Rikenellaceae utilize mucin as carbon and energy source (Bomar et al., 2011) and mucin plays an important role in preventing adhesion of various pathogens and toxins present in the intestinal lumen (Macfarlane et al., 2005) and thus, mucin degradation by Rikenellaceae would decrease the intestinal mucosal barrier integrity (Ruas-Madiedo et al., 2008).

The relative abundance of the family Enterobacteriaceae was also increased in broiler chickens fed with UPKE and EPKE diets. It is noted that the family Enterobacteriaceae harbors many known pathogens such as those in genus *Escherichia*, *Klebsilla*, *Proteus*, *Salmonella*, *Serratia*, *Shigella*, and *Yersinia* (Paterson, 2006). However, in this study, these genera of pathogens were detected at considerably low abundance (less than 0.01%), with the remaining 12 to 13% unclassified at the genus level in cecal of broiler chickens fed with UPKE or EPKE diet. This is of concern as these unclassified genera of Enterobacteriaceae may represent an unknown group of potential opportunistic pathogens, which may attack immunodepressed hosts (Healy et al., 2010). Enterobacteriaceae are considered as foodborne pathogens, thus the increase in Enterobacteriaceae in the UPKE and EPKE groups may be caused by the higher inclusion rate of PKE in day 28 diets (20%) as compared to that in day 14 (5%) diet. These results are in agreement with the predictive functional profiling results which showed significant increase ($P < 0.05$) in genes related to bacterial colonization (bacterial motility proteins, bacterial secretion system, bacterial invasion on epithelial cells, and 2-component system) in the day 28 broiler chickens fed with UPKE and EPKE diets, an indication of bacterial colonization through the stimulation of genes involve in fimbriae, flagella, outer membrane, or lipopolysaccharides, leading to adhesion, invasion, and colonization of bacteria (Morgan et al., 2004). Thus, the use of UPKE or EPKE at higher inclusion rate may require further investigation with regards to its safety in terms of microbial manipulation which favors the growth of opportunistic pathogens.

It is apparent that the inclusion of the 3 forms of PKE diets (UPKE, EPKE, and OligoPKE) modulated the cecal microbiota of broiler chickens differently. In the young broilers, all the 3 dietary treatments had positive impact in relation to *Lactobacillus* abundance. However, different effects were observed especially in broilers fed UPKE and EPKE diets at day 28, as both treatments showed a more diverse cecal microbiota with many unidentified species and unknown function. OligoPKE, an oligosaccharides which we extracted from PKE (Jahromi et al., 2016) was included as 1 of the treatments in this study to test for its potential as prebiotics. The supplementation of prebiotics is known to manipulate the microbiome of gastrointestinal tract, typically by shifting the colonic colonization of pathogenic bacteria with beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* (Teo and Tan 2007; Mountzouris et al., 2010). As expected, supplementation of OligoPKE increased the population of *Lactobacillus*, a result in accordance to those reported previously using the same material (Chen et al., 2015; Jahromi et al., 2016). However, the enhancement of *Lactobacillus* in the day 14 chicks was greater than that in the day 28 birds (8.3vs. 2.0% increment as compared to the control). The increase in *Lactobacillus* abundance could probably exclude pathogens competitively by interfering with the initial association of pathogens to the host epithelial cells (Servin and Coconnier, 2003).

CONCLUSION

This study, for the first time, elucidated the microbial community inhabiting the cecum of broiler chickens in response to feeding of different PKE containing diets using the 16S rRNA HT-NGS approach. Our results revealed that broiler chickens fed with UPKE and EPKE diets had higher diversity in the day 14 and day 28 age groups, whereas birds supplemented with prebiotic OligoPKE diet had higher diversity only at day 14 of age as compared to the control. PKE containing diets also altered the cecal Firmicutes/Bacteroidetes (F/B) ratio, with birds fed with EPKE diet had lower F/B ratio than those fed UPKE diet, suggesting that EPKE promotes lower population of nutrient utilizing Firmicutes as compared to UPKE. We postulated that the above finding is a reason for the no difference in growth performance of broiler chickens fed with EPKE (which contained higher ME) diet as compared to those fed untreated PKE diet reported in several previous studies. To confirm the above postulation, we suggest further study such as cecal microbiota transplant to be conducted in future, in order to correlate the relationship between cecal bacterial abundances and growth performance of broiler chickens.

SUPPLEMENTARY DATA

Supplementary data are available at *Poultry Science* online.

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