Effect of oligosaccharides extract from palm kernel expeller on growth performance, gut microbiota and immune response in broiler chickens

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ABSTRACT This study examined the prebiotic effects of oligosaccharides extract from palm kernel expeller (OligoPKE) on growth performance, cecal microbiota and immune response of broiler chickens. A total of ninety 1-day-old broiler chicks (Cobb-500) were randomly allocated to three treatment groups of six pens (replicates) with five birds per pen. Dietary treatments were: (i) basal diet as control, (ii) basal diet plus 0.5% OligoPKE, and (iii) basal diet plus 1% OligoPKE. Birds growth traits (ADG, ADFI and G:F) were measured during the starter (1–21 day), finisher (22–35 day) and the entire experimental periods. Blood and cecal digesta samples were collected from chickens at 21 and 35 days of age (DOA). Microbial quantification of the digesta samples, white blood cells including heterophil, lymphocyte, monocyte, eosinophil, basophil counts and immunoglobulin (IgA and IgM) were also determined. OligoPKE had no effect on ADG and ADFI throughout the study period, but chickens fed OligoPKE supplemented diet had better ($P < 0.05$) G:F during finisher and overall rearing periods. Supplementing OligoPKE did not significantly alter the birds' microbiota of the cecal digesta. At 21 DOA, blood IgA concentration increased significantly when birds fed 1% OligoPKE in diet recorded compared to the control treatment. Similar observations were also recorded in birds at 35 DOA. Hematological data showed that heterophil and basophil counts of chickens fed OligoPKE supplement were lower than those in control group at 21 DOA. Our findings suggested that OligoPKE improved immune responses in broiler chickens, especially at younger age when the immune system is not still fully developed.

Key words: palm kernel expeller, oligosaccharides, prebiotics, immune system, broiler

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INTRODUCTION

Antibiotics are widely used in animal feed to promote growth and prevent diseases. However, there are increasing concerns in the development of microbial resistance genes due to the use of antibiotics in animal feeds (Hushmand et al., 2011; FDA, 2013). Probiotics are live micro-organisms which when administered in adequate amounts confer a health benefit on the host, while prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of beneficial bacterial species residing in the colon (Saad et al., 2013; Dhama et al., 2015). Prebiotics are mixtures of indigestible oligosaccharides, consisting of three to ten carbohydrate monomers which possibly promote the growth, colonization, or activity of the probiotic microorganisms in the gut (Erdogan et al., 2010). A variety of molecules can be prebiotics, but the great majority is dietary fibers, such as oligosaccharides (Gourbeyre et al., 2011). Studies have shown that oligosaccharides can enhance feed efficiency as they improved villi height and number of goblet cells in the jejunum of broiler chickens (Baurhoo et al., 2007) as well as exerting an influence on the gut immune system via the stimulation of the specific bacteria metabolism (Gourbeyre et al., 2011). Thus, probiotics and prebiotics have been suggested as alternatives to antibiotics in animal production (Alkhalf et al., 2010; Saad et al., 2013).

Palm kernel expeller (PKE) is an important byproduct from the oil palm industry in many tropical and sub-tropical countries, including Malaysia. Being high in fiber, PKE is commonly used as feed ingredient in ruminant diets (Wan Zahari et al., 2012); however, many attempts have been made to use PKE as energy and protein sources in broiler diets, but with inconsistent results (Sundu et al., 2006; Saenphoom et al., 2013). In a study, Sundu et al. (2006) reported that palm kernel meal, a similar by-product as PKE, when supplemented with methionine and lysine could be incorporated up to

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40% in broilers diet with little negative effect on growth rate. However, Saenphoom et al. (2013) demonstrated that the maximum PKE inclusion rates for starter and finisher broiler chicken diets were 5 and 20%, respectively. Since between 58 to 78% of the PKE fiber are made up of insoluble hemicelluloses-mannan (Daud and Jarvis, 1992; Dusterhoft et al., 1991) pre-treating the PKE with cellulolytic enzymes could significantly reduce the fiber components, but no beneficial effects on animal performance were observed (Saenphoom et al., 2013). The above authors postulated that because of the compositions of the PKE fiber, large proportion of the sugars released by enzymatic treatment comprised of mannose which is known to be poorly absorbed (Wilson and Vincent, 1955), particularly when glucose is present in the system. Thus, they suggested that the mannose and mannan-oligosaccharides present in PKE could serve as prebiotics in broiler chicken production.

A recent study in our laboratory demonstrated that extract of PKE, using similar extraction protocol (water as solvent) as in this study, contained a mixture of monosaccharides (including mannose) and oligosaccharides (mainly mannobiose), which could modulate gut microflora, particularly reducing pathogenic bacteria, such as *E. coli*, in rats (Chen et al., 2015). Theoretically, these oligosaccharides can withstand the digestive enzymes and flow to the lower intestines intact to be used by beneficial bacteria. This prebiotic effect has also been demonstrated for mannoligosaccharides (MOS) of other sources (Baurhoo et al., 2007; Corrigan et al., 2012). Many enteric pathogens must attach to the mucosal surface of the gut wall to establish themselves in the gastrointestinal tract. One mode of action to exclude pathogens attachment to the gut wall is by competition for attachment site (Schneitz et al. 1993). Because of attachment is often mediated through binding of bacterial lectins to receptors containing D-mannose (Eshdat et al., 1978), it may be possible to block the lectins with mannose and to inhibit bacterial attachment (Spring et al., 2000). Therefore, the aim of this study was to examine the prebiotic effects of oligosaccharides extract from PKE on growth rate, cecal microbiota population and immune responses in broiler chickens.

MATERIALS AND METHODS

Preparation of Oligosaccharides Extract from PKE

Palm kernel expeller (PKE) was obtained from a local palm kernel oil mill for this study. Oligosaccharides were extracted from raw PKE using water (without enzyme) as solvent, following the procedure of Chen et al. (2015). Briefly, one liter of distilled water was added to 200 g of ground PKE in a one liter Scott bottle, shaken for 1 h at room temperature and later autoclaved. The insoluble materials were removed by centrifugation at 3,000 rpm × 5 min. The supernatant was filtered (Whatman filter paper no. 1) and the excess water was evaporated using a rotary evaporator (Hei-Vap Plug and Play Value 1, Heidolph, Germany), and freeze dried (Freezone 6 Plus, Labconco, USA). The solid extract (OligoPKE) was stored in capped containers at −80°C. The above process was repeated until sufficient OligoPKE for the study was obtained.

Table 1. Chemical composition of the starter (days 1–21) and finisher (days 22–35) basal diets.

Nutrient (g/kg unless otherwise stated)	Starter	Finisher
CP	220.0	199.9
Crude fat	63.1	52.2
Crude fibre	38.0	36.5
Calcium	10.2	9.0
Phosphorus	4.5	3.5
ME (kcal/kg)	3,000	3,200

Animals and Diets

Animals were cared in accordance to the guidelines of the Code of Practice for the Care and Use of Animal for Scientific Purposes, University of Putra, Malaysia. A total of ninety one-d-old male broiler chicks (Cobb-500) were used in a 35-d feeding trial. Chicks were randomly allocated to three treatments in six cages (replicates) of five birds per cage in three-tiered battery cages with wire mesh floor. The three dietary treatments were: (i) basal corn-soybean based diet (control), (ii) basal diet supplemented with 0.5% OligoPKE and (iii) basal diet supplemented with 1% OligoPKE. The birds were fed a starter diet from day 1–21, and the finisher diet from day 22–35 (Table 1). Diets were formulated to meet or exceed the nutrient requirements of broilers as suggested by NRC (1994). Clean drinking water was available at all time to the animals throughout the entire feeding trial. Broilers were weighed weekly on cage basis through the experimental period to determine average BW and ADG. The ADFI per cage was recorded weekly and then G:F was calculated.

Samples Collection and Preparation

On d 21 and 35, two birds from each cage (replicate) were randomly selected, individually weighed and sacrificed. Complete cecum was separated from the carcass, frozen in liquid nitrogen and stored at −80°C for later bacterial quantification analysis. Blood samples were immediately collected from each chicken and transferred to the laboratory in the ice box for hematological analysis and immunoglobulin assay. Heterophils, lymphocyte, monocyte, eosinophil, and basophil were counted manually under the microscope. Plasma was separated by centrifugation (4,000 rpm × 10 min), and immunoglobulin (IgA and IgM) were measured by Abnova Chicken Elisa Kit (version 3) in plasma.

Table 2. Primers used for the determination of the microbial population of chickens cecal digesta.

Total Bacteria	F-5′CAT CCA GTG CAA ACC TAA GAG-3′
	R- 5′GAT CCG CTT GCC TTC GCA-3′
Lactobacillus	F-5′CATCCAGTGCAAACCTAAGAG-3′
	R-5′GATCCGCTTGCCTTCGCA-3′
Bifidobacterium	F-5′ GGGTGGTAATGCCGGATG-3′
	R-5′ TAAGCCATGGACTTTCACACC-3′
Enterococcus	F-5′ CCC TTA TTG TTA GTT GCC ATC ATT-3′
	R-5′ACT CGT TGT ACT TCC CAT TGT-3′
Salmonella	F-5′TCGTCATTCCATTACCTACC-3′
	R-5′AAACGTTGAAAAACTGAGGA-3′
E-coli	F-5′GTG TGA TAT CTA CCC GCT TCG C-3′
	R-5′AGA ACG CTT TGT GGT TAA TCA GGA-3′
Enterobacter	F- 5′CAT TGA CGT TAC CCG CAG AAG AAG C-3′
	R-5′CTC TAC GAG ACT CAA GCT TGC-3′
Clostridium	F-5′GAG TTT GAT CMT GGC TCA G-3′
	R-5′ CCC TTT ACA CCC AGT AA-3′
Campylobacter	F-5′ CTG AAT TTG ATA CCT TAA GTG CAG C-3′
	R-5′ AGG CAC GCC TAA ACC TAT AGC T-3′

Quantitative Real Time PCR

Bacterial quantification was conducted according to the method described by Bahman et al. (2012). The DNA was isolated from the digesta of cecal sample by QIAamp® DNA Stool Mini kit. The PCR products were purified using the MEGA quick-spin™ (Intron Biotechnology, Inc) and the purity and concentration of DNA in each sample were measured using a Nanodrop ND-1000 spectrophotometer and number of copies of a template DNA per ml of elution buffer was calculated accordingly. Standard-curves were prepared using serial dilution of PCR products from pure cultures for the different bacterial groups (Chen et al., 2015).

The populations of total bacteria, *Lactobacillus*, *Bifidobacterium*, *Enterococcus genus*, *Salmonella typhimurium*, *E-coli*, *Enterobacter*, *Clostridium*, and *Campylobacter* were determined by q-PCR. The designed primers used are presented in Table 2. Real-time PCR were performed with the BioRad CFX96 Touch (BioRad, USA) using optical grade plates. The PCR reaction was performed on a total volume of 25 μl using the iQTMSYBR Green Supermix (BioRad, USA). Each reaction included 12.5 μl SYBR Green Supermix, 1 μl of each Primer, 1 μl of DNA samples and 9.5 μl molecular H_2O. The reaction conditions for amplification of DNA were initial denaturation at 94°C for 5 min, followed by 40 cycles of 94°C × 20 s, primer annealing at 55, 58 and 50°C × 30 s for total microbes, *Lactobacillus* and other bacteria respectively, and extension 72°C × 20 s. To confirm the specificity of amplification, melting curve analysis was carried out after the last cycle of each amplification.

Statistical Analysis

Data analyses were performed by comparing birds fed OligoPKE with the control for each parameter using a complete randomized design with unequal group variance using SAS Statistical Software (2008). The significance level was set at $P < 0.05$.

Table 3. Effect of oligosaccharides extract from palm kernel expeller (OligoPKE) supplementation on cumulative ADG, ADFI and G:F of broiler chickens ($n = 30$ per treatment).

	Dietary Treatments			
Parameters	Control	0.5% OligoPKE	1% OligoPKE	SEM
ADG, g				
Day 1 – 21	885	921	890	28.8
Day 21 – 35	929	906	966	73.2
Day 1 – 35	1814	1828	1856	83.8
ADFI, g				
Day 1 – 21	1262	1319	1286	39.2
Day 21 – 35	1749	1635	1664	141.6
Day1 – 35	3011	2954	2918	147.8
G:F, g/g				
Day 1 – 21	1.43	1.43	1.44	0.03
Day 21 – 35	1.88^{a}	$1.81^{a,b}$	1.72^{b}	0.11
Day 1 – 35	1.66^{a}	$1.62^{a,b}$	1.57^{b}	0.06

Data are means ± SEM.

Means within rows with different superscript letters are significantly different ($P < 0.05$).

RESULTS AND DISCUSSION

Broilers Growth Performance

Several previous studies suggested that because of its high content of hemicelluloses-mannan, PKE could be a source of prebiotics for poultry and perhaps other livestock species as well (Sundu et al., 2006; Saenphoom et al., 2013). However, except for the study using experimental rats (Chen et al., 2015), we have not found any study on the effects of oligosaccharides extract from PKE in chickens or other livestock species.

Results of the present study showed that supplementing OligoPKE had no effect on birds ADG and ADFI throughout the experimental period (Table 3). The lack of significant effect of OligoPKE as source of prebiotic on ADG and ADFI of broilers was not as expected, but it is not surprising because supplementation of the OligoPKE at 0.5 and 1% levels did not significantly alter the nutritive contents, including those of energy and protein of the OligoPKE supplemented diets. In a

recent study, Ghasemi et al. (2014) reported that ADG of broilers receiving diets supplemented with commercial prebiotic was higher compared to the unsupplemented-control group only during the grower period (11–28 days). These authors also demonstrated that supplementation of synbiotic (1:1 prebiotic and probiotic) at similar level significantly improved ADG for the entire growth period. Although it was reported that the PKE extract using similar protocol as the present study contained approximately 20 g oligosaccharides/kg PKE primarily in the form of mannobiose (Chen et al., 2015), the extract used in the present, as well as recently by Chen et al. (2015), was in a crude form containing only 60–65% carbohydrates with the remaining fraction made up of 10–15% protein and 15–20% lipids (data not shown). Based on the above information, the lack of significant effect of OligoPKE supplementation on ADG of broilers recorded could be due to the low rate of supplementation, thus could not sufficiently promote growth and activity of probiotic bacteria.

Broilers fed 1% OligoPKE improved their FCR ($P < 0.05$) as compared to the control diet during the finisher (21–35 days) and the entire trial (1 – 35 days). However, the G:F of chickens fed 0.5% and 1% OligoPKE supplements were not significantly different throughout the experimental period. The enhanced FCR of broilers supplemented with 1% OligoPKE compared to those in the control group suggests higher feed efficiency for birds fed 1% OligoPKE diet during the finisher and entire experimental periods. Moreover, the improved G:F for the 1% OligoPKE supplemented group compared to that of control birds was due to the ∼4% higher ADG (but not significantly different) of the former during the finisher period (Table 3).

Microbial Population

The main objective of supplementing OligoPKE in this study was to examine the prebiotic efficacy of OligoPKE to promote the growth or activity of the probiotic microorganisms, and thus to enhance the overall gut health of broilers (Tuohy et al. 2003; Iji et al., 2001). Therefore, the effect of OligoPKE supplementation on microbiota changes in cecal digesta of the experimental chickens are presented.

The influence of dietary OligoPKE on cecal microbiota of broilers at 21 and 35 days old are presented in Figure 1. Results of the present study showed that OligoPKE has no effect on cecal microbiota, however OligoPKE tends to increase *Bifidobacterium* (a common probiotic bacteria) and decrease *Salmonella* at 21 days old samples ($P > 0.05$). Chen et al. (2015) reported that extract from PKE using similar extraction procedure as in this study did not influence population of beneficial bacteria (*Lactobacillus* and *Bifidobacterium*), but significantly decreased ($P < 0.05$) those of pathogens such as *Enterobacter* and *E coli* in rats. On the other hand, by incorporating enzyme and heat treatments in the extraction protocol, the above authors showed many folds increase in proportions of oligosaccharides of lower degree of polymerization (mannobiose and mannotriose) in the extract, which not only further suppressed pathogens population but also significantly increased the population of *Lactobacillus* and *Bifidobacterium*. It was reported by Ibuki et al. (2011) that β-1–4-mannobiose extracted from coconut flour could enhance killing activity of *Salmonella* and activate innate immune responses in chickens macrophages. Therefore, in the present study the inability of OligoPKE to decrease significantly pathogens, especially *Salmonella*, is rather unexpected.

Immune Response

Immunoglobulin is produced by lymphocyte in response to intrusion of foreign substances into the living body. Immunoglobulin binds to the antigen (pathogen) which is then engulfed and digested by macrophages and protects the host (Ohashi et al., 2014). Lymphocytes initially make IgM immunoglobulin with a short lag phase before other immunoglobulin (e.g. IgA) is produced. IgA released from the mucosal surface of gastrointestinal tract plays a major role in the mucosal immune system in inhibition of pathogenic bacteria and neutralizing biologically active antigens (Ohashi et al., 2014). Agunos et al. (2007) demonstrated that β-1–4-mannobiose extracted from coconut flour could act as an immune-modulating agent *in vivo*, preventing *Salmonella* infection in broilers by increasing IgA production.

One of the main objectives of our study was to examine whether supplementation of OligoPKE may enhance immune responses of broiler chickens. Two immunoglobulin, IgM and IgA, were used as indicators and their responses to OligoPKE supplementation at two stages of growth periods (Table 4). Supplementing OligoPKE at 05 and 1% level had no effect on plasma IgM of chickens at 21 and 35 DOA. However, chickens fed OligoPKE supplemented diets had higher IgA concentrations than the control at both 21 and 35 DOA, with birds fed 1% OligoPKE diet recorded approximately one fold increased in IgA compared to control group. Our findings are in agreement with those of Agunos et al. (2007) which showed that β-1–4-mannobiose extracted from coconut flour could act as an immunemodulating agent by increasing IgA production which preventing *Salmonella* infection in broilers. Similarly, Nakamura et al. (2004) demonstrated that dietary fructooligosaccharides upregulate IgA response and polymeric immunoglobulin receptor expression in intestines of infant mice. Further, Scholtens et al. (2008) reported that healthy human infants receiving a formula with short-chain and long-chain fructooligosaccharides resulted in higher

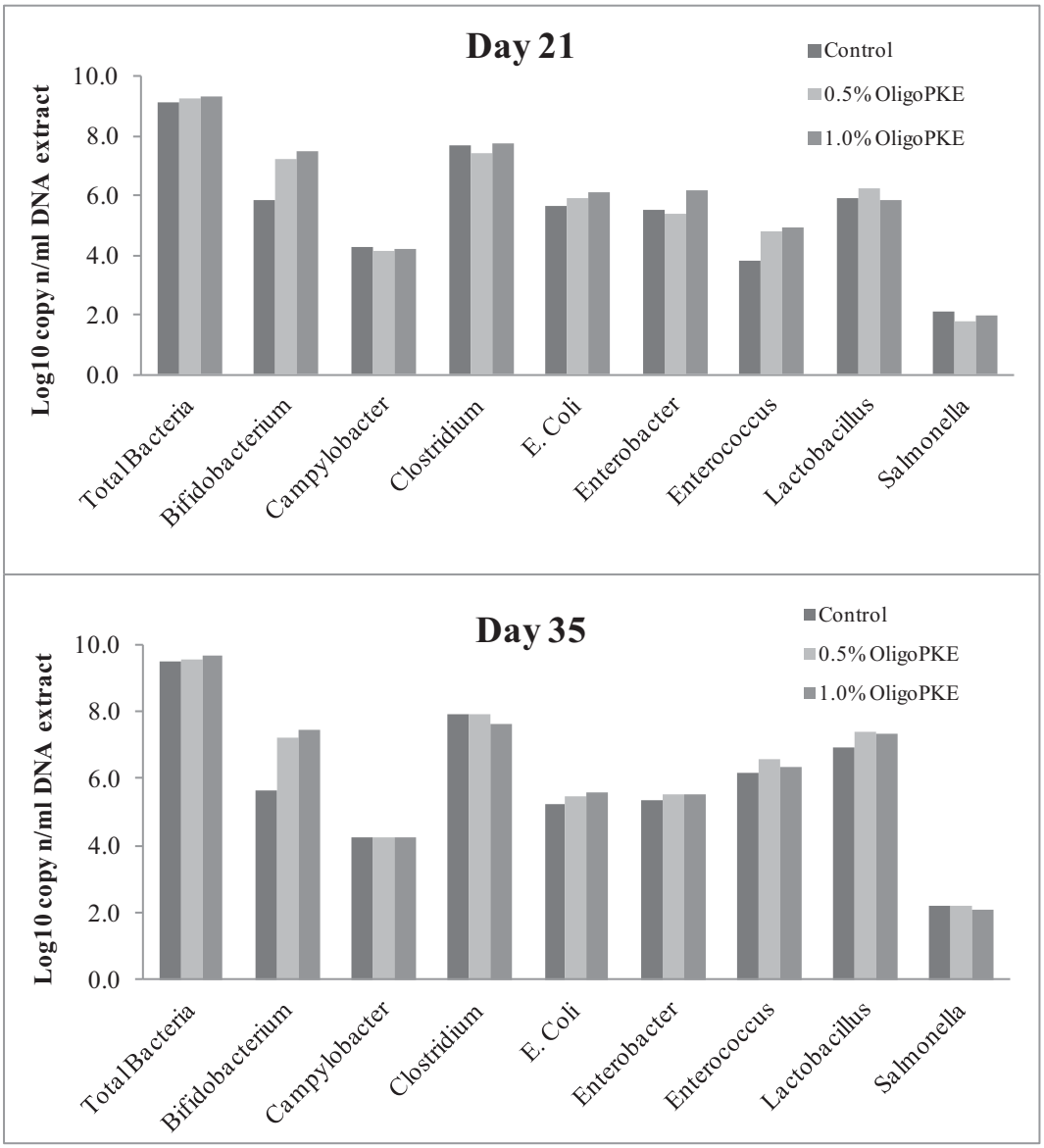

Figure 1. Effect of oligosaccharides extract from palm kernel expeller (OligoPKE) on cecal microbial population of broiler chickens at d 21 and 35, respectively.

fecal IgA. Since the main target area of IgA is the intestine and intestinal pathogen population thus providing protection against mucosal pathogens (Fagarasan, 2008; Ohashi et al., 2014), the higher IgA for OligoPKE supplemented birds suggested that OligoPKE at 1% level enhanced gut health of broiler chickens. However, the non-significant effect of OligoPKE supplementation on population of cecal pathogens (Figure 1), as previously mentioned was somewhat unexpected. Therefore, further investigation is needed to confirm whether the lack of effect of OligoPKE supplementation on gut pathogens is due to the insufficient amount of OligoPKE or other factors such as the presence of oligosaccharides of lower degree of polymerization (Chen, et al. 2015).

Hematological Analysis

An increased production of white blood cells indicates that the body is responding to an infection, a reaction to a drug or immune system disorder (Chechik et al., 1986). In our study, the heterophil count was higher ($P < 0.05$) for 21 DOA chickens fed control diet as compared to those in the OligoPKE supplemented diets (Table 5). Since higher heterophils is an indication of stress response, the lower count of chickens fed OligoPKE demonstrated that OligoPKE supplementation reduced stressors possibly including those from bacterial and fungal infections. In agreement with this study, Brune et al. (1972) and Vleck et al. (2000) reported that oligosaccharides supplementation reduced

Table 4. Effects of oligosaccharides extracted from palm kernel expeller (OligoPKE) on immunoglobulin responses in broiler chickens (ng/mg) at 21 and 35 days of age (DOA) ($n = 12$ per treatment).

	IgM		IgA	
	21 DOA	35 DOA	21 DOA	35 DOA
Control	48	40	204[b]	263[b]
0.5% OligoPKE	42	46	378[a]	421[a]
1% OligoPKE	42	51	492[a]	539[a]
SEM	5.82	5.84	97.96	52.85

Data are means ± SEM.

Means within column with different superscript letters differed significantly ($P < 0.05$).

Table 5. Effect of oligosaccharides extracted from palm kernel expeller (OligoPKE) supplementation on white blood cells counts (10^9/L; $n = 12$ per treatment).

	Control	0.5% OligoPKE	1% OligoPKE
Day 21			
Heterophils	54.8 ± 3.1[a]	50.6 ± 2.88[b]	51.2 ± 6.55[b]
Lymphocyte	28.7 ± 4.32	28.3 ± 3.61	29.5 ± 3.08
Monocyte	5.8 ± 0.84	8.8 ± 2.79	8.8 ± 2.64
Eosinophil	2.8 ± 0.98	3.3 ± 0.82	2.8 ± 0.84
Basophil	9.8 ± 1.89[a]	7.5 ± 1.52[a,b]	6.2 ± 1.64[b]
Day 35			
Heterophils	56.6 ± 5.08	57.3 ± 4.84	62.0 ± 6.24
Lymphocyte	27.2 ± 5.26	26.5 ± 1.52	23.3 ± 3.50
Monocyte	6.4 ± 1.67	5.8 ± 1.47	5.0 ± 0.00
Eosinophil	3.0 ± 0.71	2.8 ± 1.17	4.0 ± 0.82
Basophil	6.8 ± 2.06	7.5 ± 3.02	4.5 ± 2.38

Data are means ± SEM.

Means within rows with different superscript letters differed significantly ($P < 0.05$).

blood heterophils in stressed chickens and penguins, respectively.

Basophils by releasing histamine can control and are responsible for allergy and antigens in the body (Vleck et al., 2000). In chickens, high basophil level in blood indicates the birds are under abnormal environmental condition like heat stress or facing pathogenic infection (Tamzil et al., 2014), while lower level indicated that the birds are in healthier conditions (Maxwell et al., 1992). In our trial, basophil count was lower in birds fed OligoPKE, especially those fed 1% OligoPKE at 21 DOA, suggesting that OligoPKE supplementation enhances immune system in the broilers. Dietary OligoPKE did not affect lymphocyte, monocyte and eosinophil concentrations in birds at 21 DOA. Conversely, OligoPKE supplementation did not affect all the hematological parameters measured in birds at 35 DOA. The above result seems to suggest that supplementation of OligoPKE was more effective in broiler chickens during their younger age when their immune system was not fully developed (Beal et al., 2004).

CONCLUSIONS

Dietary supplementation of OligoPKE at 1% inclusion level enhanced FCR, increased plasma IgA level and decreased heterophil and basophil counts in broiler chickens. In overall, our findings are indicative of a better health status of broilers fed OligoPKE. These results justify further investigations on the use of OligoPKE as prebiotics source to enhance the value of PKE which is currently a low priced agro-industrial byproduct mainly used as feed for ruminant species.

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