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Original Article

Improving broiler chickens' health by using lecithin and lysophosphatidylcholine emulsifiers: a comparative analysis of physiological indicators

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Abstract

Background: Lipids play a vital function in a bird's body and to improve the lipids utilization and their absorption in a bird's digestive system, emulsifiers are suggested. **Aims:** This study evaluated and compared the effects of lecithin (LEC) and lysophosphatidylcholine (LPC) emulsifiers on broiler chicken's productivity traits and physiological indicators such as blood plasma parameters, intestine traits, short-chain fatty acids (SCFA) profile in caecum's content, caecum's villus height and crypt depth and their ratio. **Methods:** 900 Ross 308 broiler chickens were assigned to 3 groups with 6 replicate pens and fed with a standard compound diet (SCD) and an SCD supplemented with 0.05% LEC and 0.05% LPC. Body weight (BW), average daily gain (ADG), daily feed intake (DFI), feed conversion ratio (FCR), and dry matter (DM) content of the litter were recorded. At the end of the trial, 10 birds from each group were randomly selected and euthanized. Blood samples were collected, and blood plasma analysis was performed. Intestinal samples were collected post-mortem and intestinal traits, SCFA profiles, and intestinal histomorphometric were measured. **Results:** The inclusion of lysophosphatidylcholine significantly increased the broilers' BW and ADG at their fifth week of age ($P<0.05$). Lecithin increased the low-density lipoprotein cholesterol (LDL/C) concentration in blood plasma ($P<0.05$). Butyric and isovaleric acid concentrations significantly increased by LPC and reduced by LEC ($P<0.05$). Lecithin and LPC caecum's villus heights were significantly increased ($P<0.05$), and caecum's crypt depth was also increased in LEC compared to SCD ($P<0.05$). **Conclusion:** As an emulsifier, lysophosphatidylcholine can improve the broilers' weight, but LEC showed better effects on their physiological indicators by improving intestinal mucosal absorption areas in caecum.

Key words: Intestine, Lecithin, Lysophosphatidylcholine, Physiology, SCFA

Introduction

Poultry consumption is growing rapidly around the world, but so is the price of feed, which affects the costs of poultry production. One approach to minimize production cost is the dietary manipulation of nutrient supplies through improved feed efficiency. Therefore, special attention needs to be given to feed composition. Poultry feed is supplemented with oils and fats to increase energy content and achieve a growth performance parallel to the industry needs (Abbas *et al*., 2016). However, fat utilization, level, and digestibility vary in poultry with age, due to lack of several digestive enzymes (Farman *et al*., 2017). Fats are water-insoluble, so an emulsion is required to improve their absorption. Emulsifiers maintain the distribution of the oil droplets in the emulsion to improve the utilization and absorption of lipids in a bird's digestive system.

Lecithin (LEC) and lysophosphatidylcholine (LPC) emulsifiers can enrich poultry diets. Lecithin supplemented feed not only provides energy to broilers but also serves as an emulsifier to improve the digestibility of dietary fats. In his study, Hertrampf (2001) claimed that feeding diets enriched with LEC affected and improved poultry nutrient digestibility. lysophosphatidylcholine is another feed additive used as an emulsifier. It is an important metabolite produced by many cells, widely distributed in a variety of tissues, and capable of increasing ion permeation in membranes and altering mucosal barrier functions (Nakano *et al*., 2009);

LPC can modify the activity of various membrane associated enzymes (Maingret *et al*., 2000). In terms of animal production, supplementing feed with LPC can improve body weight (BW) during the starter period (Zhang *et al*., 2011), feed conversion ratio (FCR) (Zampiga *et al*., 2016), and growth performance through increasing nutrient utilization (Raju *et al*., 2011; Jansen *et al*., 2015). Many emulsifiers have been evaluated for growth performance, nutrient utilization, and digestibility in various animal species. However, information and comparative analysis on the effects of different emulsifiers, in this case, LEC and LPC as emulsifiers in the diet, on physiological properties of ROSS 308 broiler chickens is limited. Therefore, this experiment was conducted to evaluate and compare the effects of LEC and LPC on broiler chicken's productivity traits and physiological properties (blood plasma parameters, intestine traits, short-chain fatty acids (SCFA) profile in caecum's chymus, caecum's villus height and crypt depth, and their ratio).

Materials and Methods

This experiment was carried out in accordance with the Law of the Republic of Lithuania on the Care, Storage and Use of Animals. Complied with the following directives: directive 2010/63/EU of the European Parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes and directive 2007/43/EC, which describes rules for the protection of chickens, kept for meat production.

Experimental design

The feeding test was performed with 900 Ross 308-line combination male broiler chickens, which were individually weighed and randomly assigned to 1 control group and 2 dietary treatments (300 broilers in each) with 6 replicate pens (50 birds in each pen). Birds in each pen had free access to feed and fresh water from hanging feeders and drinkers. Broiler chickens were fed *ad libitum* with a standard compound diet (SCD) and an SCD supplemented with 0.05% LEC and 0.05% LPC. The diets were formulated to meet the nutrient and energy requirements for Ross 308 broiler chickens (Aviagen Inc., 2014). Table 1 shows the feed ingredient composition and nutrient content.

Productivity traits

Individual BW was recorded on day 1 of age of broiler chickens, and later with a 1-week and 2-week interval until 5 weeks of age. During the feeding trial, the following parameters were determined: average daily gain (ADG), daily feed intake (DFI), FCR for periods 1-7, 8-21, and 22-35 day of age; and the dry matter (DM) content of litter at 1, 3 and 5 weeks of age, which was determined by drying it at 105°C and measuring the constant weight.

Table 1: Feed ingredient composition and nutrient content (1-35 day of age)

Indicator	SCD	Treatment	
		LEC	LPC
Ingredient (%)			
Soybean meal	33.40	33.12	33.12
Maize	20.00	19.23	19.23
Wheat	36.50	37.53	37.53
Vegetable oil	4.84	4.79	4.79
Limestone	1.35	1.35	1.35
Monocalcium phosphate	0.93	0.93	0.93
Lysine sulphate	0.49	0.50	0.50
Methionine	0.49	0.49	0.49
Wheat flour	0.30	0.30	0.30
Threonine	0.20	0.21	0.21
Sodium sulphate	0.19	0.19	0.19
Sodium chloride	0.18	0.18	0.18
Mineral premix[1]	0.10	0.10	0.10
Vitamin premix[2]	0.03	0.03	0.03
Lecithin	-	0.05	-
Lysophosphatidylcholine	-	-	0.05
Calculated analysis[3] (%)			
ME (MJ/kg)	13.15	13.15	13.15
Crude protein	21.50	21.50	21.50
Crude fat	8.11	8.11	8.11
Crude ash	6.09	6.09	6.09
Crude fibre	2.57	2.59	2.59
Ca	0.88	0.88	0.88
P	0.60	0.60	0.60
Na	0.16	0.16	0.16
Mg	0.08	0.08	0.08
K	0.96	0.96	0.96
Cl	0.16	0.16	0.16
Lysine	1.34	1.34	1.34
Methionine	0.69	0.69	0.69
Met + Cysteine	1.03	1.03	1.03
Tryptophan	0.27	0.27	0.27

SCD: Standard compound diet, LEC: Standard compound diet + 0.05% lecithin, LPC: Standard compound diet + 0.05% lysophosphatidylcholine, ME: Metabolisable energy, and Met: [1] Mineral premix (per kg of feed): Fe 20.00 mg; Mn 120.00 mg; Zn 110.00 mg; Cu 16.00 mg; I 1.25 mg; Se 0.30 mg, [2] Vitamin premix (per kg of feed): vitamin A 11995.20 IU; vitamin D_3 4998.00 IU; vitamin E 94.98 mg; vitamin K_3 3.50 mg; vitamin B1 2.50 mg; vitamin B_2 8.00 mg; vitamin B_6 5.00 mg; vitamin B_{12} 29.98 µg, and [3] Calculated values were according to meet the nutrient and energy requirements for Ross 308 broiler chickens (Aviagen Inc., 2014)

Physiological methods

At the end of the five-week experiment, 10 birds from each group (total n=30) were randomly selected from each pen and euthanized using electrical stunning. Slaughter was carried out at a commercial slaughterhouse in accordance with established procedures and following the laws of the Republic of Lithuania. Blood samples were collected directly in test tubes after decapitation and intestinal samples were collected post-mortem. The remaining test broiler chickens were utilized after the feeding test.

Blood plasma parameters

The following parameters were found in broiler

chickens' blood plasma based on Tietzs' methodology (1998): total and high-density lipoprotein cholesterol (HDL/C), low-density lipoprotein cholesterol (LDL/C), albumin, globulin, total protein, triglyceride, and glucose levels. These parameters were determined using blood analyser COBAS-Integra 400/700/800 (Roche Diagnostics, USA).

pH determination

The pH of *duodenum*, *ileum*, and caecum contents was determined using an Inolab 730 pH-meter (WTW, Germany).

Dry matter determination

D*uodenum*, *ileum*, and caecum DM contents were measured by drying the chymus at 105°C for 3 h and calculating the difference between the dried and non-dried contents of the intestinal sections (Naumann and Bassler, 1993).

Length and weight of intestines

Digestive tracts were removed post-mortem and weighed with chymus. Their length was measured on a glass surface using flexible tape Hoechstmass (Hoechstmass, Germany). The intestinal walls were washed with physiological solution, dried up with filter paper, and weighed without chymus (Lentle *et al*., 1998).

Short-chain fatty acids assay

The profile of the SCFA was determined using a gas chromatography system Shimadzu GC - 2010 (Shimadzu Corp., Japan) with a 2.5 mm × 2.6 mm glass tube filled with 10% of stationary phase (SP) -1200/1% 2-oxoheptylphosphonic acid (HPO) on 80/100 Chromosorb W AW column, tube temperature 110°C, flame ionizations detector's (FID) temperature 108°C, injector's temperature 195°C. The value of the SCFA accumulation was calculated as the concentration of separate SCFA in digestive content (Zdunczyk *et al*., 2004).

Intestinal histomorphometric measurements

Caecum's samples from the middle were fixed with 10% neutral formalin solution, using standard procedures for histologic evaluation. The tissues were then embedded into paraffin, cut with a rotary microtome by 4 µm-thick tissue sections, and stained with haematoxylin and eosin. All groups caecum's samples villus heights and crypt depths were morphometrically and microscopically measured. The histological samples thus prepared were examined using an Olympus BX63 microscope (Olympus Corp., Japan), Olympus DP72 video camera (Olympus Corp., Japan) and the computer Image-Pro Plus program system.

Statistical analysis

SPSS for Windows, version 25.0 was used for data analysis (IBM SPSS Inc., Il., USA, 2017). One-way analysis of variance (ANOVA) and post-hoc test (Fisher-LSD) was used to calculate and detect differences between groups; p-values less than 0.05 ($P<0.05$) were considered statistically significant.

Results

Productivity traits

Broiler chickens grew gradually during the complete growing period (Table 2). At the fifth week of age, significantly higher BW and ADG were reached in LPC, compared to LEC ($P<0.05$). During all growing periods,

Table 2: Effect of feed supplemented with lecithin and LPC on productivity traits of broiler chickens and litter DM content

Indicator	Period	SCD	Treatment		SEM	P-value
			LEC	LPC		
BW (g)	1 day	43.90	43.96	43.90	0.31	0.488
	1 week	159.49	152.23	154.42	6.15	0.255
	3 weeks	938.72	894.66	955.96	36.95	0.117
	5 weeks	2594.20^{ab}	2503.35^{a}	2628.85^{b}	50.65	0.025
ADG (g)	1-7 day of age	16.76	15.44	16.11	0.88	0.154
	8-21 day of age	56.25	53.64	58.17	2.51	0.090
	22-35 day of age	117.89^{ab}	116.34^{a}	121.45^{b}	2.48	0.050
DFI (g)	1-7 day of age	19.15	20.72	20.03	1.75	0.385
	8-21 day of age	70.67	73.13	71.37	2.28	0.302
	22-35 day of age	161.64	167.20	163.06	7.29	0.461
FCR	1-7 day of age	0.86	0.85	0.88	0.06	0.539
	8-21 day of age	1.26	1.30	1.26	0.03	0.190
	22-35 day of age	1.47	1.50	1.48	0.06	0.622
DM of litter (%)	1 week	93.01	93.05	94.09	2.53	0.431
	3 weeks	73.32	76.25	78.55	13.09	0.070
	5 weeks	58.81	61.77	64.61	6.60	0.392

SCD: Standard compound diet, LEC: Standard compound diet + 0.05% lecithin, LPC: Standard compound diet + 0.05% lysophosphatidylcholine, SEM: Standard error of means, BW: Body weight, ADG: average daily gain, DFI: daily feed intake, FCR: feed conversion ratio, and DM: Dry matter. $^{a, b}$ The means with different superscripts small letters in a row show significantly difference ($P<0.05$)

Table 3: Effect of feed supplemented with lecithin and LPC on blood plasma indicators of broiler chickens (35 day of age)

Indicator	SCD	Treatment		SEM	P-value
		LEC	LPC		
Total protein (g/L)	36.37	38.00	36.83	2.93	0.592
Albumin (g/L)	1.28	1.35	1.40	0.14	0.203
Globulin (g/L)	1.23	1.19	1.25	0.05	0.301
Cholesterol (mg/dL)	120.45	122.38	122.60	5.20	0.071
HDL/C (mg/dL)	92.17	92.90	94.00	1.68	0.307
LDL/C (mg/dL)	36.63[b]	39.07[a]	37.07[b]	0.88	0.024
Triglyceride (mg/dL)	92.40	95.30	90.40	2.31	0.066
Glucose, mmol/L	10.33	10.56	9.04	0.96	0.110

SCD: Standard compound diet, LEC: Standard compound diet + 0.05% lecithin, LPC: Standard compound diet + 0.05% lysophosphatidylcholine, SEM: Standard error of means, HDL/C: High-density lipoprotein cholesterol, and LDL/C: Low-density lipoprotein cholesterol. [a, b] The means with different superscripts small letters in a row show significantly difference (P<0.05)

Table 4: Effect of feed supplemented with lecithin and LPC on broiler chickens' intestine traits

Indicator	Intestine	SCD	Treatment		SEM	P-value
			LEC	LPC		
Weight (g)	Intestine with chymus	43.90	43.96	43.90	12.92	0.807
	Intestine without chymus	159.49	152.23	154.42	6.62	0.181
Length (cm)	Intestine	213.8	200.3	188.00	17.82	0.167
pH	Duodenum	6.34	6.20	6.29	0.14	0.327
	Ileum	6.75	6.74	6.80	0.26	0.212
	Caecum	0.86	0.85	0.88	0.20	0.543
DM (%)	Duodenum	17.00	18.79	18.13	1.90	0.360
	Ileum	16.33	19.10	16.38	2.14	0.213
	Caecum	19.15	19.21	20.72	3.29	0.471

SCD: Standard compound diet, LEC: Standard compound diet + 0.05% lecithin, LPC: Standard compound diet + 0.05% lysophosphatidylcholine, SEM: Standard error of means, and DM: Dry matter. P-values more than 0.05 (P<0.05) indicate no statistically significant difference

the highest DFI and FCR were observed in LEC; nevertheless, these findings were not statistically significant (P>0.05). None of the emulsifiers had a significant effect on the DM content of litter (P>0.05).

Blood plasma parameters

Table 3 shows the broiler chickens' blood plasma parameters. None of the emulsifier's additives showed significant changes in the broiler chickens' blood plasma profile (P>0.05), except for LEC which significantly increased LDL/C concentrations in the blood plasma. This indicator was lower in SCD and LPC (P<0.05).

Intestine traits

After evaluating several intestine traits, no significant differences were found between the groups (Table 4). The heaviest intestines with and without chymus and the longest ones were found in the SCD group (P>0.05). pH varied evenly across all groups, and the model of chymus DM changes remained unclear as no trends were observed.

Short-chain fatty acids' profile

Table 5 shows the results of different emulsifiers on SCFA profile changes in the caecum's chymus. The results showed that LEC and LPC inclusion did not have significant effects on acetic, propionic, isobutyric, and valeric acid concentrations in the caecum's chymus (P>0.05). However, compared to the SCD group, butyric acid concentrations were significantly higher in LPC samples but lower in LEC (P<0.05). Likewise, LPC increased isovaleric acid concentrations, while slightly lower concentrations of the following SCFA were found in LEC (P<0.05).

Table 5: Effect of feed supplemented with lecithin and LPC on SCFA profile changes in caecum chymus of broiler chickens

SCFA (µmol/g)	SCD	Treatment		SEM	P-value
		LEC	LPC		
Acetic acid	4.74	5.85	4.86	0.72	0.141
Propionic acid	2.75	2.80	2.68	0.08	0.163
Isobutyric acid	1.64	1.55	1.74	0.10	0.083
Butyric acid	1.79[a]	1.70[a]	1.98[b]	0.09	0.006
Isovaleric acid	1.05[ab]	1.01[a]	1.18[b]	0.07	0.032
Valeric acid	1.44	1.38	1.49	0.07	0.148

SCFA: Short-chain fatty acids, SCD: Standard compound diet, LEC: Standard compound diet + 0.05% lecithin, LPC: Standard compound diet + 0.05% lysophosphatidylcholinem, and SEM: Standard error of means. [a, b] The means with different superscripts small letters in a row show significantly difference (P<0.05)

Intestinal histomorphometric measurements

Table 6 shows the caecum's villus height and crypt depth measurements and its estimated ratio. The lecithin treatment positively affected caecum's villus by increasing its height (P<0.05). The lecithin group's

caecum's villus height was the highest, compared to that of the SCD (P<0.05), and a lower caecum's villus height was found in the LPC treatment compared to LEC (P<0.05). The highest caecum's crypt depth was found in LEC samples; likewise, slightly lower measurements were observed in SCD (P<0.05). However, no significant emulsifier effects were found on the villus height and crypt depth (V/C) ratio (P>0.05).

Table 6: Effect of feed supplemented with lecithin and LPC on broiler chickens' caecum's villus height, crypt depth and V/C ratio

Indicator	SCD	Treatment		SEM	P-value
		LEC	LPC		
Villus height (µm)	586.80[a]	682.14[b]	607.38[a]	33.73	0.012
Crypt depth (µm)	207.57[a]	247.30[b]	219.72[ab]	17.25	0.035
V/C ratio	2.83	2.76	2.77	0.22	0.444

V/C: villus height and crypt depth, SCD: Standard compound diet, LEC: Standard compound diet + 0.05% lecithin, LPC: Standard compound diet + 0.05% lysophosphatidylcholine, and SEM: standard error of means. [a, b] The means with different superscripts small letters in a row show significantly difference (P<0.05)

Discussion

Broiler chickens in all treatments grew steadily, remained healthy and consumed their daily feed throughout the experiment. The feed supplemented with LPC caused the biggest increase in the broiler's BW at their fifth week of age. Our results are in line with the data reported by Melegy *et al*. (2010) and Wealleans *et al*. (2020), who claimed that feed supplemented with lysolecithin increased broilers BW and that chicks fed only with a lecithin-oil based diet were much lighter. However, we found no significant results regarding BW during earlier growing periods. Likewise, the highest ADG was reached in the LPC treated group. Nevertheless, no significant differences were found between treatments comparing DFI, FCR, and DM of the litter.

Biochemical blood plasma evaluation can provide valuable information. It indicates the broiler chickens' general health state and is often helpful in revealing health disorders. The concentration of triglycerides, LDL/C, and total cholesterol in blood plasma presents a similar pattern of variation as a function of age. In our study, higher values of these indicators were obtained, which were similar to the results obtained by other researchers (Oguz *et al*., 2002). The higher values of these indicators in LEC-treated broiler chickens' blood plasma correspond to the LEC emulsifier's low mobilization in the tissues and the intense synthesis by the liver (Szabo *et al*., 2005). Moreover, triglycerides, LDL/C, and total cholesterol increase are related to higher dietary energy supplies (Rajman *et al*., 2006). The lower values at 35 days of age can indicate a bird's high-energy requirement caused by high body development (Almeida *et al*., 2006). Nevertheless, in our study total protein, albumin, globulin, cholesterol, HDL/C, triglyceride, and glucose levels in blood plasma did not reveal any significant trends or differences between treatments.

Feeding plays a very important role in the life of every living organism and has a direct relationship with the gastrointestinal tract. The growth and development of the gastrointestinal tract is vital for broiler chickens as it helps utilize nutrients in their diet (Uni *et al*., 1998). Size, morphology, and functionality of the digestive system are very important in helping broilers to adapt to various growing conditions. In our study, better gut development was found in the SCD intestinal samples, which were heavier and longer. However, no intestinal trait was statistically significant. Different intestine chymus pH and DM content measurements did not reveal any changing model for these parameters either and remained unclear as no significant trends were observed during our study.

Short-chain fatty acids are saturated aliphatic organic acids that consist of one to six carbons. The established profile of SCFA can indicate many things: for example, butyrate, which is a by-product of microbial fermentation products, is important for the normal development of epithelial cells (Pryde *et al*., 2002). Butyric acid appears to play a role in the development of the intestinal epithelium and, therefore, seems to be both bactericidal and a stimulant of villus growth (Leeson *et al*., 2005). In our study, butyric acid concentrations significantly increased in LPC-treatment caecum's content samples but decreased in LEC. In fact, butyric acid is the major energy source to enterocytes and is essential to the health of intestinal mucosa (Isolauri *et al*., 2004). Short-chain fatty acids can decrease the enzyme activity of hepatic 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), which can lower the cholesterol synthesis rates and plasma glucose levels by increasing the gut hormone peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) (Rodwell *et al*., 1976). Consequently, gut hormone peptides PYY and GLP-1 play an important role in the communication between tissues, and GLP-1 indirectly regulates blood glucose levels by increasing the secretion of insulin and decreasing the secretion of glucagon by the pancreas (Den Besten *et al*., 2013). In our study, this was reflected when much lower blood plasma glucose levels and higher butyric and isovaleric acid concentrations were found in the caecum's content in the LPC-treated group. Nevertheless, only the mentioned SCFA results were statistically significant.

Understanding macroscopic intestine development is important, as digestive tract growth and development are factors that contribute to the efficient improvement of a bird's digestive processes. Intestinal morphology indicators like villus height, crypt depth, and their ratio are the main indicators of gut health (Mitchaothai *et al*., 2010). The intestinal villus plays an important role as the nutrient absorption site in the gastrointestinal tract, where the absorption area can be influenced by feed quality and digestibility (McDonald *et al*., 2002). Therefore, nutrient absorption can be maximized, only when the intestinal villus is not corrupted. Our research

shows that both emulsifiers can significantly increase the height of caecum's villus, which indicates that this kind of supplement can improve nutrient absorption. When comparing different emulsifiers, the LEC treatment showed a better effect on the histomorphometric indicators of higher caecum's villus height in LEC, compared to experimental LPC treatment. The same trend was reflected in caecum's crypt depth results: LEC emulsifier deepened caecum's crypts, which also indicates that this emulsifier benefits from improved nutrient uptake and absorption. In general, the increased caecum's villus height and crypt depth of birds fed with pellets was in line with adequate growth performance and increased metabolizability of nutrients. Improved villus height may increase the total luminal villus absorptive area and subsequently result in satisfactory digestive enzyme action and a much higher transport rate of nutrients to the villus surface (Tufarelli *et al*., 2010). Similarly, a deeper crypt may indicate faster tissue turnover to permit the renewal of the villus, which suggests that the animal's intestinal response mechanism is trying to compensate for normal sloughing or villus atrophy caused by inflammation from pathogens and their toxins (Gao *et al*., 2008). Moreover, the higher villus height and crypt depth ratio in the broilers fed with experimental treatments resulted in a decreased turnover of the intestinal mucosa. A slower turnover rate of the intestinal epithelium results in a lower maintenance requirement, which can finally lead to a higher animal growth rate or efficiency (Van Nevel *et al*., 2005). Bearing in mind that a higher ratio of villus height and crypt depth refers to a greater capacity of nutrient digestibility and absorption in chickens (Silva *et al*., 2009), our histomorphometric results are highly important as well, even though they did not show any statistically significant V/C differences between the treatments.

Our results show that using emulsifiers reflects higher fat absorption that might decrease fermentation in the small intestine, leading to lower intestinal villus surface damage and therefore better broiler chicken growth performance (measured by increased BW at 35 days of age). To conclude, our results show that to significantly improve broiler chickens' BW and ADG, chicks should be treated with LPC dietary treatments. However, to improve their general health, gut state, and intestinal mucosal absorption, it is recommended to supplement broiler chickens' diet with an LEC emulsifier.

Conflict of interest

Authors declare no conflict of interest.

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