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Effects of Phytosterols on Growth Performance and Fat Metabolism in Broilers

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Abstract.- The purpose of this study was to investigate the effects of diet supplemented with different levels of phytosterols (PS) on growth performance and lipid metabolism in broilers. A total of four hundred 1-day-old chicks (female: male =1:1) were randomly assigned into 4 treatments, each of which replicated 5 times with 20 chicks per replicate. Birds were fed with a basal diet without any additive (control), or supplemented with PS at 30, 40, or 50mg/kg for 42 d. The results showed that PS had no significant (P>0.05) effect on growth performance during the whole experimental period of 1 to 42d of age. The highest levels of serum free fatty acid were found in diets supplemented with PS at 40mg/kg and 50 mg/kg in female and male chickens, respectively (P<0.05). PS had no significant influence (P<0.05) on the serum triglycerides compared with the controls in chickens. Serum fatty acid synthetase was significantly decreased (P<0.05) in all chickens supplemented with PS compared with the control. In addition, hepatic fatty acid synthase and sterol regulatory element-binding transcription factor 1c mRNA expressions in the 40 mg/kg and 50mg/kg PS treatments were found to be lower (P<0.05) than those in other treatments. It is concluded that PS, as feed additives, had a positive effect on lipolysis by inhibiting the enzymes related to lipolysis. Diets supplemented with 40mg/kg and 50mg/kg of PS had a better effect in female than in male chickens, respectively.

Key words: Phytosterols, growth performance, lipid metabolism, broilers.

INTRODUCTION

Economic prosperity has increasingly improved our living standards, as a result, consumers are growingly yearn for health-promoting effects of functional foods enriched with natural ingredients. Thus the high quality of animal production has attracted more attention; the more intensive breeding practices have, however, led to making high energy levels for broilers, which leads to excess fat deposition (Hoffman, 2010). Reduction fat deposition in broilers has eventually become more challenging.

Phytosterols (PS) are structurally similar to cholesterol, which are present in fruits, seeds, vegetables and their oils and reduce intestinal cholesterol absorption. Therefore, the interest in studying PS was initially due to their effectiveness in reducing the solubility of cholesterol that inhibit cholesterol biosynthesis (Fernandez *et al*., 2002; Stelmach-Mardas and Przyslawski, 2013). Previous studies suggested that PS may have a protective effect against inflammation and subsequent cardiovascular cancers and improve the antioxidant capacity (De Jong *et al*., 2008; Rubis *et al*., 2010; Rudkowska, 2010).

The U.S Food and Drug administration was the first to approve a health claim for PS in the year 2000, and the Ministry of Agriculture of China has also approved PS as a new type of additive in 2008. Furthermore, it was reported that PS could be responsible for facilitating the growth performance (Li *et al*., 2011), lowering the level of blood cholesterol and may have effect on fat metabolism (Klingberg *et al*., 2008; Chen *et al*., 2010). However, there is little known about the effects of PS on lipid metabolism defense in male and female chickens. Studies are therefore, needed to determine the effect of PS on the growth performance, and lipid metabolism in chickens. The aims of this study were to evaluate the effects of different dietary levels of PS on overall growth performance, lipid metabolic enzymes and hepatic mRNA gene expressions of sterol regulatory element-binding transcription factor 1c (SREBP-1c), and fatty acid synthase (FAS) in female and male chickens.

MATERIALS AND METHODS

Phytosterols

Phytosterols were purchased from Jiangsu

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Spring Fruit Biological Products Co., composed of PS ≥91%.

Animals, experimental design and management

A total of 400 one-day-old AA broilers were purchased from a commercial hatchery. The chickens were allocated to 4 treatments in a randomized complete block design. Each treatment had replicated 5 times with 20 birds (10 females and 10 males) per replicate. Chickens were fed with 1) a basal diet (maize-soyabean meal type) without PS supplementation (control), 2) a basal diet with 30 mg/kg of PS supplementation, 3) basal diet with 40 mg/kg of PS supplementation, 4) basal diet with 50 mg/kg of PS supplementation. The diets were formulated in accordance with the NRC (1994) guidelines to meet the nutrient requirements of broilers. Diet compositions are shown in Table I. Chickens were fed a starter diet for 21d and a grower diet from 22 to 42 d of age. All chickens were vaccinated against Newcastle and infectious bursal diseases at 7, 14, and 21 days of age, the temperature of the room was maintained at 34-36ºC during 1 to 14d of age and then was gradually reduced to 26ºC, after which it was maintained at room temperature and kept constant throughout the experiment of 42d of age. Chicks received a 24h light program. Feed and water were provided *ad libitum.*

Growth performance

Body weight and feed intake for each replicate were recorded at (1-21d, 22-42d and 1-42d of age to calculate average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCRs body weight gain/feed intake).

Sample collection

At 42 d of age, two chickens (1 male and 1female) per replicate were randomly selected, weighed and then were killed by exsanguinations humanely. Blood samples were collected and serum samples were separated by centrifugation at 3,500x g for 15 min at 4ºC. Serum samples were frozen at -20ºC for the further anslysis. After collection of blood samples, liver tissue samples of all killed chickens were excised and stored in liquid nitrogen for the further analysis of hepatic FAS and SREBP-1c mRNA gene expressions.

Table I. Ingredients and nutrient levels of basal diets.

Ingredients(%)	1-21days	22-42days
Maize	61.78	65.67
Soybean meal	30.5	25.5
Maize protein meal	3.5	4.3
Soybean oil	2.8	3.2
Sodium chloride	0.42	0.33
1 % Premix[a]	1	1
Total	100	100
Nutrition levels*		
ME(MJ/kg)	12.46	12.62
Crude protein	20.54	19.02
Calcium	1.00	0.87
Available Phosphorus	0.45	0.40
L-Lysine	1.10	1.00
DL-Methionine+cystine	1.00	0.82

* the nutrient values used were form which standard.

[a]: Provided per kg of diet: Iron, 69 mg; Copper, 7.5 mg; Zinc, 65 mg; Manganese, 110 mg Iodine, 1.1 mg; Selenium, 0.4 mg; Bacitracin Zinc, 30 mg;Vitamin A, 4500 IU; Vitamin D_3, 1000 IU; Vitamin K, 1.3 mg; Vitamin B_1, 2.2 mg; Vitamin B_2, 10 mg; Vitamin B_3, 10 mg; Choline chloride, 400 mg; Vitamin B_5, 50mg; Vitamin B_6, 4mg; Biotin, 0.04 mg; Vitamin B_{11}, 1 mg; Vitamin B_{12}, 1.013 mg.

Measurements of serum index

To determine the levels of serum free fatty acids (FFA) and triglycerides (TG), commercial kits were purchased from Jiancheng Biochemical Reagent Co., Nanjing, People's Republic of China.

Serum hormone sensitive lipase (HSL) and fatty acid synthase (FAS) enzyme activities of chickens were analyzed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. The kits were purchased from Shanghai Blue Gene Biotech Co., Ltd. People's Republic of China.

Quantitative real time PCR analysis

Total RNA was extracted from liver tissues of chickens by using TRIZOL reagent according to the manufacturer's protocol, and quantified by measurement of optical density at 260 nm. Ratios of absorption (260/280 nm) of all preparations were between 1.8 and 2.0. Aliquots of RNA samples were subjected to electrophoresis in a 1% ethidium

bromide stained 1.4 % agarose formaldehyde gel to verify their integrity. Reverse transcription was performed using 2 µg of total RNA: 5.0 µg 5 × RTbufer, 1.0 µg 106 RT Random Primer (Promega, Belgium), 2 µl 256 d NTP (Promega, Belgium), 0.5 µl Multiscribe Reverse Transcriptase (Promega, Belgium), 0.2 µl RNase inhibitor (Promega, Belgium), and the addition of nuclear free water to final volume of 25 µl. Reaction system was run at 37 ºC for 60 min and 95 ºC for 5 min.

Quantitative real-time RT-PCR was performed in a 25 µl reaction buffer that included 12.5 µl SYBR GREEN, 0.25 µl of forward primer, 0.25 µl of reverse primer, 2 µl of cDNA, and 10 µl ddH_2O were incubated in a Strategene MX 3000 PTM Detection System (Applied Biosystems). The reaction mixture was subject to program to conduct one cycle (95 ºC for 30 s) and 40 cycles (9ºC for 5s and 60 ºC for 31 s). The primer sequences are listed in Table II. Each sample was assayed in duplicate and the *m*RNA expression levels of the target genes were standardized against β-actin. The results(fold changes)were expressed as $2^{-\Delta\Delta C(t)}$ with $\Delta\Delta C(t)$=[C*(t)* ij−C*(t)* β-actinj]−[C*(t)* i1−C*(t)* β-actin1], where Ct ij and C*(t)* β-actinj are the Ct for gene i and for β-actinj in a pool or a sample(named j)and where Ct i1 and C*(t)* β-actin1 are the Ct in pool 1 or sample l, expressed as the standard.

Statistical analysis

Growth performance was analyzed by one-way ANOVA using the SPSS program (version 16.0). The rest of the data were performed by the GLM procedures and differences among treatment means were determined by Duncan's multiple range test, comparing the effects of diets, genders and the interaction between them. Effects were considered significant at $P< 0.05$.

RESULTS

Effects of dietary PS on growth performance in AA broilers

The effects of the dietary PS on growth performance in AA broilers are shown in Table III. Compared with the control, a significant increased ($P<0.05$) in the ADG were found in chickens fed diets with 30 mg/kg PS in the period of 1 to 21 d. ADG, ADFI and FCR of chickens fed on different dietary treatments in the periods of 21- 42d and during the whole experimental period (1-42d) were not significantly different ($P>0.05$).

Effects of dietary PS on serum lipid in female and male AA broilers

As shown in Figure 1, serum TG levels in chickens fed different dietary treatments had no significant difference ($P>0.05$) in the periods of (1-21d), (22-42d) and during the whole experimental period (1-42d). Serum FFA levels were significantly higher in male chickens than the female chickens. The highest levels of serum FFA were found in the female chickens fed diets supplemented with PS at a level of 40mg/kg (23.43%), while, in male chickens at a level of 50mg/kg (10.98%) as compared with the control chickens.

Effects of dietary PS on serum FAS and HSL enzyme activities in female and male AA chickens

The effects of PS supplementation in chickens on serum FAS and HSL enzyme activities are shown in Table IV. When chickens were fed diets with PS, serum FAS enzyme activity of chickens fed PS was significantly increased ($P<0.05$), but were not affected by sex. However, the interaction between PS diets and sex of chickens was significant for serum FAS enzyme activity. The serum FAS activities were down-regulated by PS supplementation in chickens ($P>0.05$). Dietary treatments supplemented with PS at levels of 40 and 50 mg/kg significantly decreased ($P<0.05$) the levels of serum FAS enzyme activities in female (50.93%) and male (75.23%) chickens, respectively at 42d of age. As shown in Table IV, the PS supplementation in diets and sex interaction had involved the serum HSL enzyme activity. The HSL enzyme activities were significantly up-regulated ($P<0.05$) in chickens fed diets supplemented with PS at levels of 40 and 50mg/kg in female (23.91%) and in males (48.42 %), respectively as compared with the control group. The interaction between PS diets and sex of chickens was significant for serum HSL enzyme activity.

Effects of dietary PS on hepatic SREBP-1c and FAS mRNA expressions in female and male AA broilers

Effects of PS addition to the diets on the

Table II.- Gene-specific primer of the lipid metabolism related enzyme.

Gene	Accession No.	Primers sequences(5'→3')	Product size
FAS	NM205155	F:GTGTTCGTGACGTGAGCAGT R:TCCTCTGCTGTCCCAGTCTT	190bp
SREBP-1c	AY029224	F:GCCTCTGTGCCTTGTCTTC R:ACTCAGCCATGATGCTTCTTCC	130bp
β-actin	L08165	F:TGCGTGACATCAAGGAGAAG R:TGCCAGGGTACATTGTGGTA	300bp

FAS: fatty acid synthetase. SREBP-1c: sterol regulatory element-binding proteins 1c

Table III.- Effect of PS on growth performance of AA broilers

	PS added in feed (mg/kg)				Mean	SEM	P value
	0	30	40	50			
1-21d							
ADFI, (g/d/bird)	65.02[a]	68.39[b]	68.37[b]	67.04[ab]	67.20	0.52	0.056
ADG, (g/d/bird)	38.05[a]	40.26[b]	38.80[ab]	39.32[ab]	39.11	0.32	0.079
FCR	1.71[a]	1.70[a]	1.76[a]	1.71[a]	1.72	0.02	0.582
22-42d							
ADFI, (g/d/bird)	150.31[a]	145.03[a]	145.11[a]	146.29[a]	146.68	1.37	0.513
ADG, (g/d/bird)	81.81[a]	82.30[a]	82.67[a]	84.34[a]	82.76	0.77	0.738
FCR	1.84[a]	1.76[a]	1.76[a]	1.74[a]	1.77	0.02	0.271
1-42d							
ADFI, (g/d/bird)	107.66[a]	106.71[a]	106.74[a]	106.67[a]	106.94	0.52	0.902
ADG, (g/d/bird)	59.93[a]	61.28[a]	60.74[a]	61.78[a]	60.93	0.46	0.553
FCR	1.80[a]	1.74[a]	1.76[a]	1.73[a]	1.76	0.01	0.341

[abc] Values in the same row with different superscript differ significantly ($P<0.05$).
PS: phytosterols. ADFI: average daily feed intake. ADG: average daily gain. FCR: feed conversion ratio.

Table IV.- Effects of PS on serum FAS and HSL enzyme activities in AA broilers

	PS added in feed(mg/kg)				**Mean**	**SEM**	**P value**		
	0	**30**	**40**	**50**			**Diet**	**Sex**	**Diet×Sex**
FAS (pg/mg*pro)									
Female	6.06	3.64	2.98	3.19	3.97	1.47			
Male	9.27	2.59	2.51	2.30	4.17	3.09			
Means	7.67[a]	3.12[b]	2.74[b]	2.74[b]	4.07	2.37	<0.01	0.445	<0.01
Pooled SEM	1.84	1.24	0.79	0.45					
HSL (pg/mg*pro)									
Female	25.80	29.28	31.97	29.52	29.14	5.13			
Male	19.04	20.31	20.89	28.26	22.12	4.12			
Means	22.42[a]	24.79[ab]	26.43[b]	28.89[c]	25.63	5.10	<0.01	<0.01	<0.01
Pooled SEM	3.95	5.34	6.45	1.27					

[abc] Values in the same row with no common superscript differ significantly ($P<0.05$).
PS: phytosterols. FAS: fatty acid synthetase. HSL: hormone-sensitive lipase.

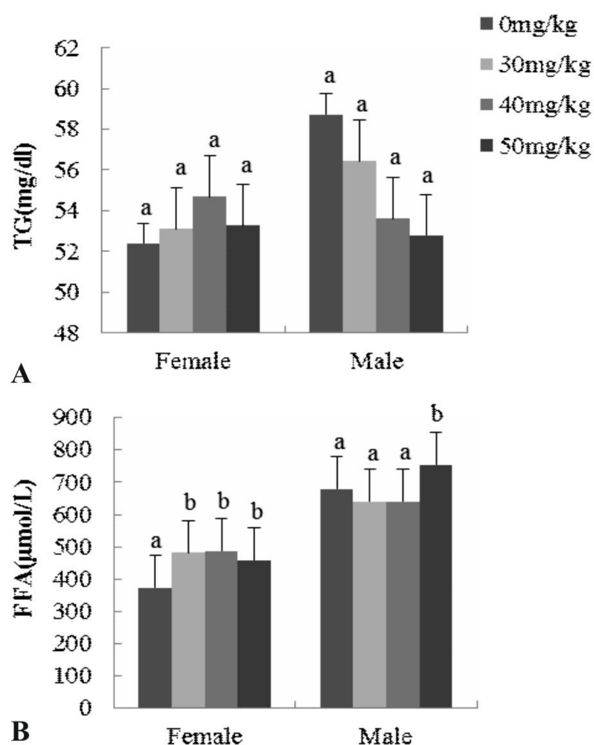

Fig.1. Effect of PS on serum TG (A) and FFA (B) in male and female AA broilers.
[abc]Different letters showing a significant difference between mean values (P<0.05); PS, phytosterols; TG, triglyceride; FFA, free fatty acid.

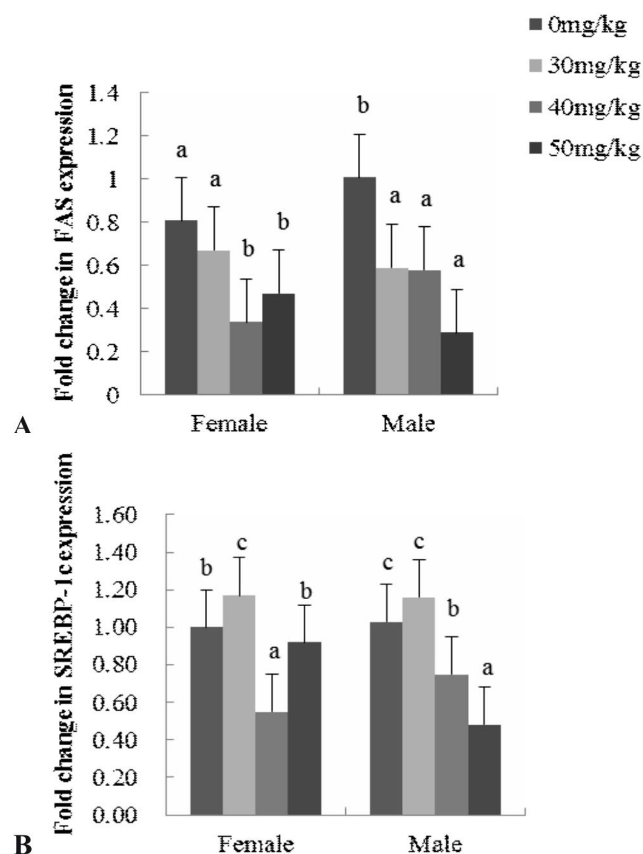

Fig. 2. Effect of PS on hepatic FFA mRNA expression (A) and SREBP-1c mRNA expression (B).
PS, phytosterols; FAS, fatty acid synthetase; SREBP-1c, sterol regulatory element-binding proteins 1c.

mRNA expressions of hepatic SREBP-1c and FAS mRNA expression are shown in Figure 2. The diets supplemented with PS significantly decreased (P<0.05) the mRNA gene expression of hepatic FAS as compared with the control group in chickens at 42d of age and this expression was lower in diets contained PS levels of 40 and 50 mg/kg in female and male chickens, respectively. However, mRNA gene expression of hepatic SREBP-1c was significantly increased (P<0.05) in diets supplemented with a level of 30 mg/kg PS in both female and male chickens. In addition, supplementation with higher level of 40mg/kg PS significantly decreased (P<0.05) hepatic SREBP-1c mRNA expression in female chicks. The diets supplemented with PS at levels of 40 and 50 mg/kg were significantly down-regulated (P<0.05) the hepatic SREBP-1c mRNA gene expressions in male chickens as compared with the other groups and the decrease was 45.0% and 58.02% for hepatic SREBP-1c and FAS enzymes in female chickens, while, for male chickens, the decrease was 53.39 % and 71.28 % for hepatic SREBP-1c and FAS enzyme gene expressions, respectively.

DISCUSSION

In this study, the results show that poultry diets supplemented with PS had no significant effect on the growth performance in chickens from day 1 to day 42. These findings are in agreement with the study in layer hens (Elkin and Lorenz, 2009), which showed that there was no effect of PS (1g per 100 g of diet) on weight gain, feed consumption, and feed efficiency in layer hens at 28d of age. Similarly, Liu *et al*. (2010) also reported

that plant sterol supplementation had no significant effect on feed intake and body weight gain in layers at 8 weeks of age. However, studies in pigs (Li *et al*., 2011; Harvey *et al*., 2014) showed that the dietary supplementation with PS improves the growth performance, whereas in broilers (Naji *et al*., 2013) phytosterol showed non-significant effect on FCR, the body weight though increased in the phytosterol group fed with 25, 50, and 75 g/kg diet in 21 days. The expected effects of PS may vary because of the animal species, and the chemical composition and concentration (Maguire *et al*., 2004).

Most of the circulating FFAs are bound to albumin and are involved in supplying fat to various tissues as well as for oxidation in the fasting state. Our study suggested that the PS facilitated the release of FFAs in the bloodstream and accelerated fat mobilization in females (40 mg/kg of diet) as compared to males (50 mg/kg of diet). These results are in agreement with the findings that PS can decrease the concentration of serum FFA in diabetic fatty rats (Misawa *et al*., 2012; Gunawan *et al*., 2008). However, some studies reported that PS cannot change the concentration of serum FFA in rats (Katamoto *et al*., 1991; Amiot *et al*., 2011).

All diets had similar influence on serum TG in female and male chickens at 42d. These results are in agreement with the finding in rats (Ling and Jones, 1995) as well as in humans (Racette *et al*., 2010). This might be due to that the basic poultry diets in all treatments were composed of the same basic ingredients and providing the same amount of fat from these ingredients to all chickens during the whole period. However, some researchers have reported that supplementation with PS decreases the levels of serum TG in hamsters (Misawa *et al*., 2012), in fish (Gilman *et al*., 2003) and in humans (Sialvera *et al*., 2012). The differences in results might be due to the variations in the basic feed ingredients composition and its total fat content.

A previous study proved that there is a difference in lipid metabolism between female and male in human (Blaak, 2001). However, very little information is available on the lipid metabolism differences in chickens supplemented with the PS. It is well known that reduction in lipogenic activity are typically associated with reductions in enzyme activities and related gene expressions (Katsurada *et al*., 1987; Clarke, 1993; Mourot *et al*., 1995). Fatty acid synthatase plays a crucial role in *de novo* lipogenesis in birds. HSL has opposite effects of mobilizing FAs from adipocytes into the bloodstream for lipolysis in the adipocyte (Zubair and Leeson, 1994; Kokta *et al*., 2004). The results of our present study suggested that poultry diets supplemented with PS (40 mg/kg of diet) were more effective in increasing serum HSL and FAS enzyme activities in female chicks, while, 50mg/kg PS levels in diets increased serum HSL and FAS enzyme activities in male chicks. Many studies have shown the similar results that the plant sterol can regulate fat enzyme activities, such as FAS and HSL (Bennett *et al*., 1995; Schoonjans *et al*., 2000; Marinangeli *et al*., 2006). Thornton *et al*. (2011) also suggested that the dietary supplementation of PS reduced fat accumulation by facilitating the genes related to lipolysis in obese mice. Sterol regulatory element-binding transcription factor 1c, as one of the potential regulators, that directly stimulate the transcription of genes encoding FAS enzyme. The results of the present study showed that the PS 40 and 50 mg/kg of diets decreased the mRNA expressions of both hepatic SREBP-1c and FAS gene expressions in female and male chicks, respectively. The results of the present study showed that the 40 and 50 mg/kg PS of diets decreased the mRNA expressions of both hepatic SREBP-1c and FAS enzymes genes in female and male broilers, respectively. Our present study results are in agreement with the previous study that the oral feeding of anti-diabetic PS (lophenol and cycloartanol) in rats significantly reduced FAS and SREBP-1c in mice (Misawa *et al*., 2012). Similarly, Rideout *et al*. (2010) also found that the diets supplemented with PS increased hepatic lipogenic gene expressions of SREBP-1c (2.4-fold) and FAS (6.5-fold) enzymes as compared with the control group in mice. Therefore, it was concluded that female chickens are more sensitive to diets supplemented with PS as compared to male chickens, and PS may alter the expressions of various downstream enzyme genes that are involved in the synthesis of hepatic TG, and enhanced the lipolysis.

In our study, it is interesting to find that

female broilers are more sensitive to PS diets as compared to male broilers. The results suggested that the diets supplemented with 40 mg/kg of PS in females, and 50 mg/kg of PS had better effects in males. This study showed that the female and male can deposit fat in different ways and result in different consequences. Women generally have a larger proportion of body mass as fat, and are more likely to deposit fat subcutaneously and on their lower extremities, while men are more likely to deposit fat in the abdominal region (Böttner *et al*., 2004; Regitz-Zagrosek *et al*., 2006). A previous study suggested that the difference between female and male in lipid metabolism is associated with oestrogen (Power and Schulkin, 2008), and PS is found to have (phyto) oestrogenic potential and act as an effective oestrogen-like agonist (Waalkens-Berendsen *et al*., 1999). It also appears that females could have higher rates of oxidation by the estrogen, however, exogenous estrogen in males have an effect on decreasing carbohydrate and amino acid metabolism (Hamadeh *et al*., 2005). Thus, it would appear that females are more physiologically sensitive to dietary PS, and may use fat as a metabolic fuel under conditions.

CONCLUSIONS

It is concluded that the diets supplemented with PS had no influence on growth performance in chickens. Free fatty acids and genes related to fat metabolism were affected, which suggests that fat deposition and metabolism were improved by supplementation of PS in chickens. Therefore, the use of PS as a beneficial feed additive in broiler diets is recommended. Further experiments are required to understand and clarify the molecular mechanisms of fat metabolism and regulation by PS supplementation in chickens.

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Conflict of interest declaration

There is no conflict of interest in this study.

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