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RESEARCH ARTICLE

Adjustment Activity of Chinese Herbal Product on Lipid Metabolism and Cholesterol Content in Eggs of Dongxiang Blue-Shelled Layers

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ABSTRACT

Our goal was to study the effects of Chinese herbal products on the lipid metabolism levels of Dongxiang blue-shelled layers (DBS). One hundred healthy 22-week-old layers were randomly divided into 3 groups: control, traditional Chinese herbal product 1 (TCM1) and traditional Chinese herbal product 2 (TCM2) groups. The two TCM groups were treated with the Chinese herbal prescription products 1 and 2. Samples were collected on the 1st, 15th, 30th, 45th, and 60th day after TCM treatments. The blood samples were tested using an automatic biochemical analyzer and the liver samples were analyzed using real-time quantitative polymerase chain reaction. The results showed that the levels of total cholesterol (TC), triglycerides (TG) and low-density lipoprotein-cholesterol (LDL-C) were lower, whereas the levels of high-density lipoprotein-cholesterol (HDL-C) were higher ($P<0.05$) in the TCM treatment groups. Compared to the control group, the liver fat rate in the TCM1 and TCM2 groups was significantly lower. Meanwhile, fat rate of crureus and pectorals, and liver index were also lower than in the control group. There was a significant decrease in cholesterol contents of the eggs. The abdominal fat rate in the two treatment groups was lower than that of the control group. The expression level of apolipoproteinA I and apolipoproteinB 100 in the livers of the TCM1 and TCM2 groups increased; however, this increase was not significant for the in apolipoproteinB 100 levels. In conclusion, the TCM prescriptions have positive effects on the regulation of lipid metabolism through a reduction in the cholesterol levels of eggs and the upregulation of apolipoproteinA I and apolipoproteinB 100 mRNA expression levels of in the liver.

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INTRODUCTION

Lipid metabolism is closely linked to the health of humans and animals. Lipid metabolism disorder leads to liver disease and cardiovascular disease (CVD), which threatens the animal welfare and causes finacial losses in layers (Basiricò *et al.*, 2010; McLaren, *et al.*, 2011). Lipid metabolism disorder is usually observed along with changes in the common blood lipid parameters. Serum triglyceride (TG) content plays an important role in physiological and metabolic functions. TG content is an indicator of some metabolic diseases such as nonalcoholic fatty liver disease (Qasem *et al.*, 2015). Total cholesterol (TC), low density lipoprotein-cholesterol (LDL-C) and high density lipoprotein-cholesterol (HDL-C) contents are correlated with the risk of developing some diseases such as Coronary Artery Disease (CAD) (Abudoukelimu *et al.*, 2015). Additionally, apolipoproteinA I (apoA I) and apolipoproteinB 100 (apoB 100) are also associated with lipid metabolism. Apolipoproteins are proteins that are bound to a lipid core and specialized for the transportation of lipids through the plasma or extracellular fluids (Bertocchini and Stern, 2008). Previous studies have proven that apoA I is the major apolipoprotein in HDL

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and plays an important role in regulating the cholesterol content of peripheral tissues through the reverse cholesterol transport pathway in ducks (Gu *et al.,* 1993; Sontag *et al.,* 2012). Apolipoprotein B has two naturally occurring forms naturally (apoB 100 and apoB 48) (Bostrom *et al.,* 1990). In humans and animals, apolipoproteinB 100 is the main protein component of LDL, which also contains TG, cholesterol and cholesteryl ester (Srivastava and Srivastava, 2000; Wang *et al.,* 2010). Furthermore, the mRNA expressions of apoA and apoB gene were associated with these blood lipid parameters (Lee *et al.,* 2014). It was reported that a change in the apoA content leads to changes in HDL and cholesterol concentrations in plasma and tissues (Gu *et al.,* 1993; Sontag *et al.,* 2012). Meanwhile, apoB content has been reported to influence the LDL-CHOL level (Sontag *et al.,* 2012).

Eggs are an important source of proteins, vitamins, and minerals. However, they have a high cholesterol content, which has negative effects on human and animal health by increasing blood cholesterol levels (Laudadio *et al.,* 2015). As people pay more attentions to their health and diet, the cholesterol contents of chicken and eggs have been more closely considered in recent years, especially the amount of cholesterol contained in eggs (Attia *et al.,* 2015). In order to decrease the cholesterol in chickens and eggs, humans have introduced additives into poultry feeds. However, this action leads to the accumulation of drug residues and threatens human health (Liang, *et al.,* 2013). Herbal products have been used for thousands of years in China, Korea, Japan and other Asian countries to treat diseases and enhance growth, either alone or in combinations (Hayashi *et al.,* 2007*;* Abbas *et al*., 2015; Idris *et al*., 2017). *Astragalus membranaceus* and *Codonopsis pilosula* have been widely used for several thousand years as a part of a variety of herbal mixtures to treat liver disease, CVD, and to lower blood pressure which are related to lipid metabolism for their important pharmacological functions (Wang *et al.,* 2011; Chen *et al.,* 2014). Meanwhile, *Carthamus tinctorius* has been used together with *Crataegus pinnatifida* as a for cardiovascular protective and lipid metabolism regulation regulatory agent (Asgarpanah and Kazemivash, 2013).

Currently, the effects of TCM on the expression levels of apoA I and apoB 100 genes and how the genes influence the lipid metabolism is unclear. In this study, the TCMs were mixed in two prescriptions according to traditional Chinese medicine theory and then administered to Dongxiang blue-shelled layers (DBS), an indigenous, Chinese chicken that is recognized for its blue eggshell color, meat quality, and health care function (Wang *et al.,* 2009). We then observed the effects of the treatments on their growth performance, blood physiological parameters, and lipid metabolism. Meanwhile, the effects of these two TCMs on the mRNA expression levels of apoA I and apoB 100 were also investigated. These results would be useful in finding feed additives for lowering cholesterol and lipid content in DBS and their eggs, which will lead to more profitable DBS meat and eggs and further provide healthier food for humans.

MATERIALS AND METHODS

Preparation of TCMs: All raw materials were purchased from the Chinese Traditional Medicine Pharmacy, Tong Ren Tang (Beijing, China). TCM1 and TCM2 were composed of six and four dry Chinese herbs, respectively. The dried Chinese herbs were filtered through a 10-μm sieve and mixed evenly to constitute TCM1 and TCM2 (Table 1). Both the treatment groups were added to the basal diet at a dose of 10g/kg.

Animals and diets: All experimental protocols were approved by the Committee for the Care and Use of Experimental Animals, Jiangxi Agricultural University, Jiangxi, China.

One hundred healthy, 22-week-old Dongxiang Blue-shelled layers, an indigenous breed from the Jiangxi province, China, were randomly divided into three groups: control group (basal diet) (NRC, 1998) (Table 2), TCM1 group (basal diet with TCM1) and TCM2 group (basal diet with TCM2). All layers were acclimatized for one week before beginning the experiments. They had free access to water and were housed in the cages. The experiment lasted 60 days, and during this period, the feed intake was recorded every day and ten eggs (per group) were collected on days 15, 30, 45, and 60, then labeled and kept at 4°C prior to CHOL content determination using sulfur phosphorus iron reagent spectrophotometry.

Sampling procedures: On the 1st, 15th, 30th, 45th and 60th days, the layers were weighed. Blood samples (5 ml) were collected before treatment with anesthesia from the brachial veins. Serum was obtained by immediate centrifugation (3min, 4°C, 1500g) and stored at -80°C until analysis. Concentrations of aspartate transaminase (AST), total protein (TP), albumin (ALB), globin (GLO), TC, TG, LDL-CHOL, and HDL-CHOL were determined using an automatic biochemical analyzer (BS-380). After being treated for 60 days, the layers were anesthetized and then euthanized. Livers and abdominal adipose tissue were collected and weighed. The livers collected on 1st, 30th, and 60th days were used to measure the mRNA expression levels of apoA I and apoB 100 genes. The crureus and pectoral were also collected and stored at -80°C for analysis. The stored tissues were used for the isolation of liver fat using the Soxhlet extraction method. Liver index and abdominal fat rate were calculated as liver weight/body weight and abdominal fat weight/body weight, respectively.

RNA extraction and Real-time quantitative polymerase chain reaction (RT-PCR): Total RNA was isolated from liver tissue samples using the TRIzol reagent according to the manufacturer's instructions (TaKaRa, Dalian, China). The concentrations of the total RNA samples were determined using the Thermo NanoDrop 2000 (Thermo Fisher Scientific inc., Waltham, USA). First-strand complementary deoxyribonucleic acid (cDNA) synthesis was conducted using the TaKaRa® RT-PCR Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. Primers for the amplification of genes were designed using Primer Premier Software (PREMIER Biosoft International, CA, USA). The primer

sequences and GenBank accession numbers were obtained from GenBank (Table 3). The expression levels of apoA I and apoB 100 were quantified by real-time qRT-PCR using the PrimeScriptTM reagent Kit with gDNA Eraser and the Premix Ex TaqTM (Probe qPCR) master mix. The expression levels of apoB 100 mRNA were quantified using the SYBR Premix Ex Taq™ II (Tli RNaseH Plus) master mix. The internal reference (GAPDH) gene was used as an internal control for the normalization of results. ΔCt(sample)=Ct(target gene)-Ct(reference gene); ΔCt (calibrator)=Ct(target gene)-Ct(reference gene); ΔΔCt= ΔCt(sample)-ΔCt(calibrator), 2-ΔΔCT was used to calculate the expression levels of genes.

Statistical analysis: Data were analyzed using the one-way analysis of variance followed by the Duncan multiple range test. When the *P* value was significant, the capital letters (A, B, C) indicate highly significant differences and lowercase letters (a, b, c) indicate significant differences. All analyses were performed using the SPSS 19.0 software package. Values were shown as the mean±SD. Differences with P<0.05 were considered statistically significant and P<0.01 were considered highly significant.

Table 1: Composition and contents of TCM1 and TCM2 (air dry basis)

Latin name	Used part	Content (g)
TCM1		
Astragalus membranaceus	Dried rhizome	30
Codonopsis pilosula	Dried root	20
Angelica sinensis	Dried root	25
Crataegus pinnatifida	Dried fruit	50
Rhizome Atractylodis macrocephalae	Dried rhizome	20
Atractylodes lancea	Dried rhizome	30
TCM2		
Flos carthami	Dried flower	10
Crataegus pinnatifida	Dried fruit	50
Cyperus rotundus	Dried rhizome	10
Lotus leaf	Dried leaf	20

TCM, traditional Chinese herbal product; TCM I, traditional Chinese herbal product I; TCM II, traditional Chinese herbal product II. [1]Used parts of TCM I and TCM II come from Chinese pharmacopoeia (2005).

Table 2: Ingredients and nutrition content of the feed (air dry basis)

Items		Content (%)
Ingredients	Corn	4.20
	Soybean meal	21.00
	Limestone	8.10
	$CaHPO_4$	1.20
	Soybean oil	4.00
	Salt	0.31
	Premix[1)]	1.00
	Lys	0.12
	DL-Met	0.07
Total		100
Nutrient levels	ME/(MJ/kg) [2)]	11.30
	CP (%)	17.20
	Ca (%)	3.43
	TP (%)	0.63
	AP (%)	0.45
	Lys (%)	0.92
	Met (%)	0.38

[1)] The premix provides following per kg of feed: VA 8 100 IU; VD_3 1 620IU; VE 0.30IU; VK_3 0.90mg; VB_1 0.45mg; VB_2 2.70mg; VB_{12} 0.06g; Nicotinic acid 5.70mg; Folic acid 0.15mg; Biotin 0.045mg; Fe (as ferrous sulfate) 31.30mg; Cu (as copper sulfate) 4.59mg; Mn 41.21mg. Zn (as zinc sulfate) 42.04mg; I (as KI) 0.61mg; Se (as Na_2SeO_3) 0.15mg; choline 448.65mg; rice polishing 3489.53mg; [2)] ME is the calculated value, and the others nutrient levels are measured values.

RESULTS

Effects of TCMs on the growth performance, fat rate in tissues and CHOL content in eggs: The results showed that there was no significant difference (P>0.05) between TCM treated groups and the control group in feed intake (Table 4). The liver fat rate decreased in both treatment groups compared to the control group. The liver fat rate in TCM1 and TCM2 groups decreased by 22.68% and 36.02% (Fig. 1. A), respectively. Compared to the control group, the CHOL contents in eggs on the 60th day (Fig. 1. B) of the two treatment groups decreased significantly by 35.66% and 28.08%, respectively (P<0.05). The pectoral fat rate (Fig. 1. C) decreased by 35.74% in the TCM1 group on the 60th day (P<0.05), whereas the decrease in the TCM2 group was not significant (P>0.05). The crureus fat rate (Fig. 1. D) decreased by 23.58% in the TCM1 group on the 60th day (P<0.05); however, the decrease in TCM2 group was not significant. Additionally, the percentage of abdominal fat (Fig. 2. A) and the liver index (Fig. 2. B) were measured. The liver index decreased in both TCM treatment groups. The abdominal fat rate in theTCM1 treatment group was lower than the control group (except on the 30th day) (P<0.01), and it was also lower in the TCM2 group (P<0.01).

Effects of TCMs on blood biochemical parameters: The effects of TCM1 and TCM2 treatment groups on blood biochemical parameters were measured. Compared to the control group, blood TG levels (Fig. 3. A) of TCM1 group decreased by 7.33% on the 30th day (P<0.01) and by 1.67% on the 60th day (P>0.05), blood TG levels of TCM2 decreased by 10.02% on the 15th day (P<0.01) and by 5.74% on the 60th day (P>0.05). The TC levels of the TCM1 group decreased by 7.98% (P<0.05) on the 30th day and by 8.91% on the 60th day (P<0.01). TC levels (Fig. 3. B) of the TCM2 group decreased by 9.66% (P<0.05) on the 30th day and by 14.98% on the 60th day (P<0.01). The HDL-C content of the TCM treatment groups increased by 44.44% and 19.05% on the 30th day (P<0.01), respectively, and by 13.27% (P<0.01) and 4.08% (P>0.05) on the 60th day (Fig. 3. C). Compared to the control group, the LDL-C content of the TCM treatment groups decreased by 21.38% and 31.45% on the 30th day (P<0.01), respectively, and by 11.83% (P<0.05) and 4.30% (P>0.05) on the 60th day, respectively (Fig. 3. D). The values of AST (Fig. 4. A) in the serum samples showed no significant difference between the treatment and control groups, whereas the GLO values (P>0.05) (Fig. 4. B) decreased and the ALB values (Fig. 4. C) increased. The TP content (Fig. 4. D) declined on the 30th day, then increased on the 60th day (P<0.01).

Effects of TCMs on the expression levels of apoA I and apoB 100 in livers: To elucidate the role of apoA I and apoB 100 in lipid metabolism, the effects of TCMs on the gene expression levels of apoA I and apoB 100 were investigated. There was a significant increase in the mRNA expression levels of apoA I in the livers (Fig. 5. A), respectively, compared to that of the control group. The mRNA expression levels of apoB 100 (Fig. 5. B) in the livers increased, but the differences were not significant.

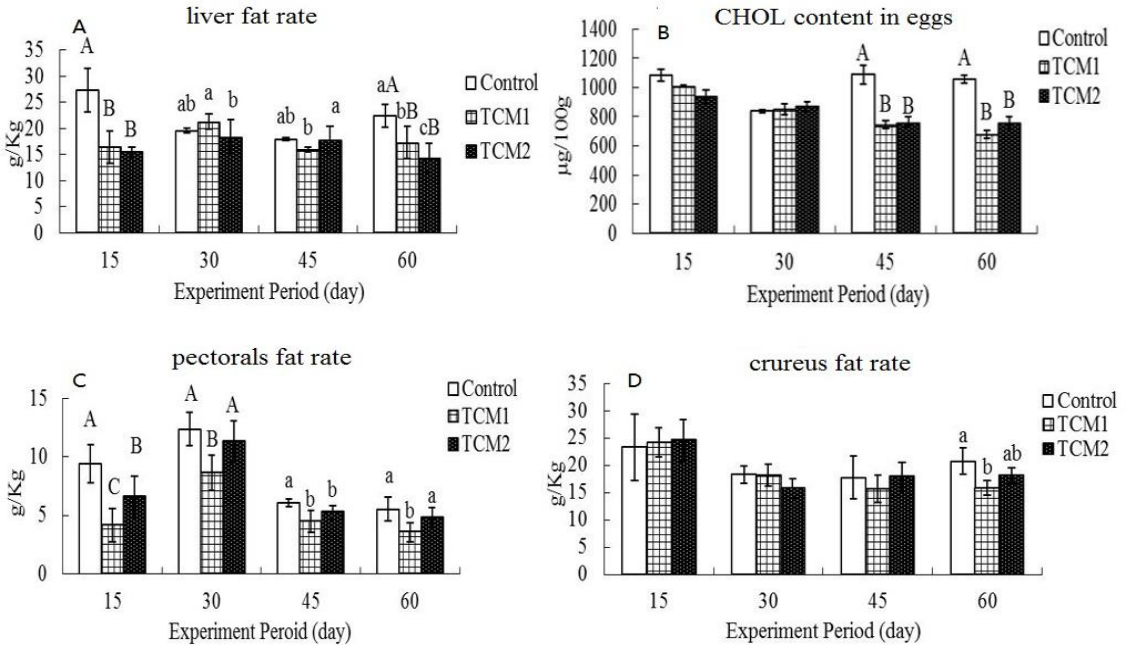

Fig. 1: liver fat rate (A), CHOL contents in eggs (B), pectorals fat rate (C), crureus fat rate (D). [a,b,c] P<0.05. [A, B, C] P<0.01.

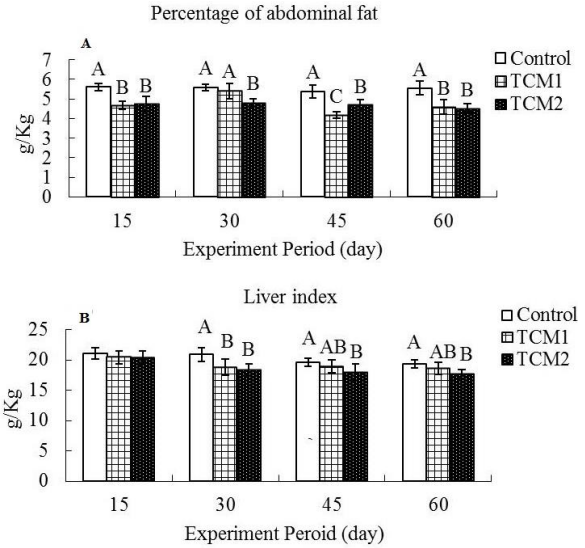

Fig. 2: Percentage of abdominal fat (A). Liver index (B). [a,b,c] P<0.05. [A, B, C] P<0.01.

Table 3: Primer and Probe sequence

Interest Genes	Reference sequence No.	Primer sequences (5'-3')	products Length/ bp
apoA- I	NC-006111.3	F : GGCCAGCGGCAAGGAT R : ACTCAGCGTGTCCAGGTTGTC P: CATCGCCCAGTTCGAGTCCTCTGC	94bp
apoB	NC-006090.3	F: CCTGCCATGGGAAACATTAC R: TGCAGTGCATCAATGACAGA	150bp
GAPDH	NC-006088.3	F : GGTGCTAAGCGTGTTATCATCTCA R: CATGGTTGACACCCATCACAA P: CTCCCTCAGCTGATGCCCCCATG	70bp

Table 4: The values of feed intake

Group	Experimental period(Day)			
	15	30	45	60
Control group	111.76±4.55^{aA}	113.05±4.26^{aA}	113.17±3.88^{aA}	111.74±3.86^{aA}
TCM 1 group	111.73±3.11^{aA}	113.57±4.33^{aA}	113.28±4.43^{aA}	111.47±3.14^{aA}
TCM 2 group	112.29±3.72^{aA}	112.74±3.64^{aA}	112.18±5.88^{aA}	113.04±3.93^{aA}

Data are represented as the mean ± standard error. (n=6). [a-c] within a row with unlike lowercase superscripts, indicates are statistically different with P<0.05. [A-B]within a row with unlike uppercase superscripts, indicates that the samples are statistically different with P<0.01.

DISCUSSION

In this study, liver index and abdominal fat rate declined in both TCM treatment groups when compared to the control group. These results indicate that TCM treatments could inhibit fat accumulation in the liver and abdominal tissue. The results also show that the serum TG, TC, and HDL-C concentrations decreased, but the LDL-C concentration increased in the treatment groups. These outcomes suggest that TCM treatments regulated blood lipid levels. Previous studies have confirmed that TCM treatments can reduce fat accumulation in tissues and regulate lipid metabolism, and studies have confirmed that TCMs affact apoA I and apoB 100 gene mRNA expression levels (Liang, *et al.*, 2013; Guo, *et al.*, 2014). ApoA I is an important apolipoprotein in HDL, which forms HDL and adjusts cholesterol content in tissues via the reverse transport cholesterol pathway. When the mRNA expression levels of apoA I gene were upregulated, both the levels of apoA I increased and HDL content (Srivastava and Srivastava, 2000). The increase in HDL resulted in the reduction of total serum cholesterol because of the reverse cholesterol transport process. Our results showed that the mRNA expression levels of apoA I increased along with the HDL-C levels. However, the TC content and fat accumulation decreased. These results were supported by a previous study (Simo *et al.*, 2009). ApoB 100 is a constituent of LDL, which transports cholesterol through the blood (Srivastava and Srivastava, 2000). Furthermore, a previous study showed that a reduction in apolipoprotein B (apoB 100 in poultry only) secretion led to the accumulation of TG and cholesterol in hepatic cell, therefore, resulting in lower TG and TC contents in the serum (Lee *et al.*, 2014). In a previous study, it was confirmed that apoB was involved in the clearance of LDL in the serum (Gu *et al.*, 2015). In our study, the mRNA expression levels of apoB 100 gene increased significantly in TCM1 and TCM2 treatment groups, whereas the contents of TG, TC, and LDL-C in

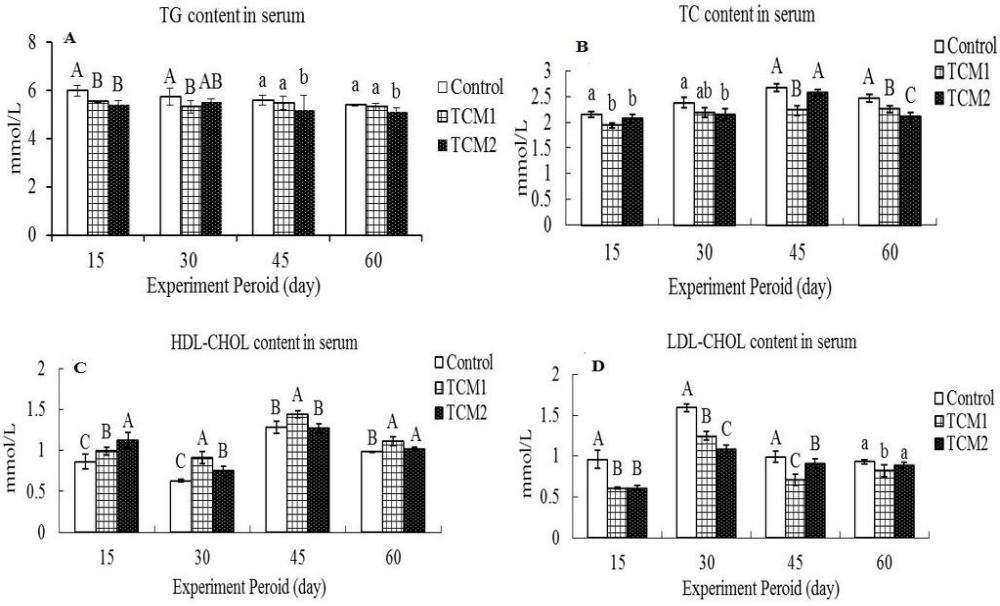

Fig. 3: TG content in serum (A). TC content in serum (B). HDL-CHOL content in serum (C). LDL-CHOL content in serum (D). [a,b,c] P<0.05. [A, B, C] P<0.01.

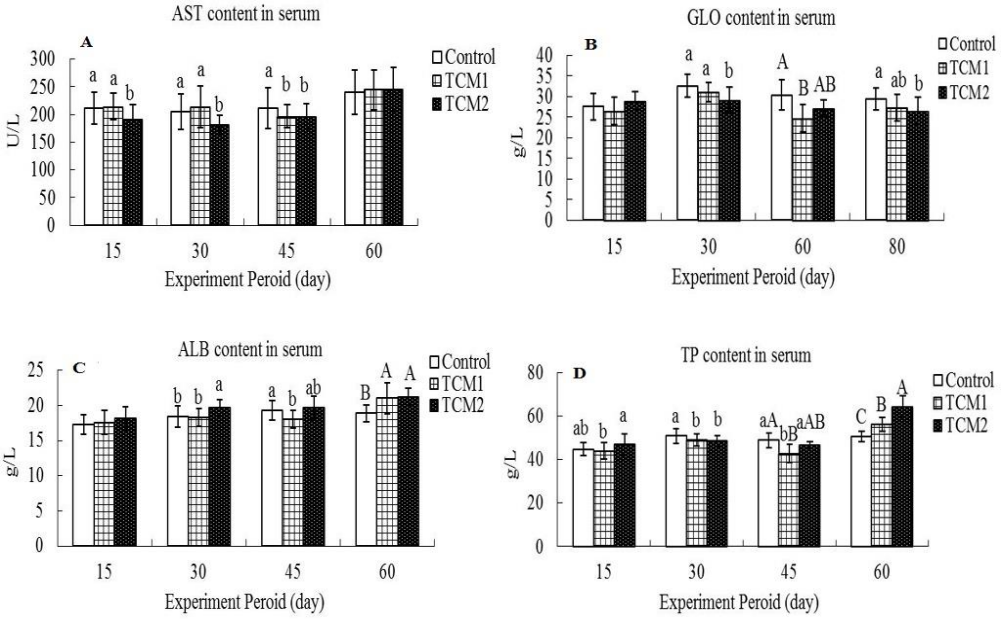

Fig. 4: AST content in serum (A). GLO content in serum (B). ALB content in serum (C). TP content in serum (D). [a,b,c] P<0.05. [A, B, C] P<0.01.

the serum decreased, which are in agreement with the results from a previous study (Khoo, 2015). Additionally, our results showed that the cholesterol contents in the eggs declined in both the TCM treatment groups compared to the control group, indicating that TCM could lower the cholesterol content in eggs.

The major components of TCM1 were *Astragalus membranaceus*, *Codonopsis pilosula*, *Rhizoma atractylodes* and *Crataegus pinnatifida*. *Astragalus membranaceus* and *Codonopsis pilosula* have hepatoprotective activity and are used to regulate metabolism and treat liver and heart diseases (Liu *et al.*, 2015; Zhang *et al.*, 2015). *Crataegus pinnatifida* has several functions, including weight reduction of high-fat animals with high fat content, decreasing the levels of TC, TG, LDL-C, increasing the HDL-C levels, and preventing and treating hyperlipidemia and fatty liver disease (Li *et al.*, 2010; Guo *et al.*, 2015). TCM2 consisted of six dry Chinese herbs, including *Lotus leaf* and *Flos carthami*. *Flos carthami* is used for the treatment of hyperlipidemia (Zhao *et al.*, 2009) and *Lotus leaf* plays an important role in modulating of lipid metabolism (An *et al.*, 2013). Our results demonstrated that these Chinese herbal products have positive effects on lipid metabolism. Additionally, the results showed that there were no significant differences between the control group and TCM treatments groups in feed intake and AST levels. Meanwhile, the levels of TP, GLO, and ALB increased in two the TCM treatment groups. These outcomes indicated that the two TCM prescriptions have no negative effects on growth performance, even though they reduced lipid metabolism levels and fat accumulation in tissues. Furthermore, our study discovered differences between the two TCM formulations. The TCM1 formulation presented more beneficial effects than TCM2 on lipid metabolism by regulating the mRNA expression levels of apoA I and apoB 100 genes, reducing the blood lipid levels, decreasing the cholesterol content in eggs, and lowering fat accumulation in tissues.

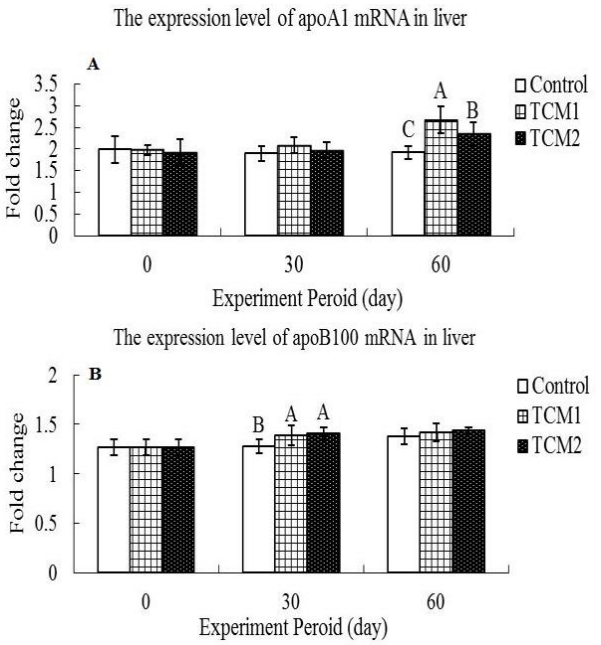

Fig. 5: The expression levels of apoA I mRNA in the liver (A). The expression levels of apoB 100 mRNA in the liver (B). [a,b,c] P<0.05. [A, B, C] P<0.01.

Conclusions: The tested Chinese herbal products have positive effects on lipid metabolism in the Dongxiang blue-shelled layers. These effects are potentially useful for the prevention of lipid metabolism diseases and in providing high-quality and profitable DBS. Furthermore, TCM1 formulation is more effective prescription based on the results of our study.

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Authors contribution: Guoliang Hu and Huabin Cao conceived and designed the study. Yalu Song, Junrong Luo, Tiancheng Wang, Caiying Zhang and Fei Yang executed the experiments. Ping Liu and Xiaoquan Guo analyzed the data. Yalu Song wrote the manuscript. All authors interpreted the data, critically revised the manuscript for technical content and approved the final version.

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