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Effects of Alfalfa Saponins on Cholesterol Metabolism in Broilers

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Abstract

This experiment was conducted to evaluate the effect of different levels alfalfa saponins on cholesterol metabolism in broilers. A total of 320 Arbor Acres (AA) broilers of 1 day-old were randomly allotted to 4 treatments with five replicates in each treatment, and with 16 broilers in each replicate. The experiment last 49 days. During the stage from 0 to 3 weeks, broilers in the control group were fed basal diet and birds in the test groups were all fed the basal diets added with 0.02% alfalfa saponins. while from 4 to 7 weeks, birds in the test groups were fed the basal diets added with 0.04%, 0.08% and 0.12% alfalfa saponins, respectively. The results showed that: Supplemented 0.08% and 0.12% alfalfa saponins to broilers diets could significantly decrease cholesterol content of serum and liver ($P<0.05$), increased HDL-C content in serum and bile acids content in feces ($P<0.05$), highly decreased LDL-C content and HMG-CoAreductase mRNA abundance ($P<0.01$), implicating alfalfa saponins could block enterohepatic circulation of bile acids, thus supress the synthesis of cholesterol to some extent. 0.08% alfalfa saponins also could significantly reduce insulin content in serum ($P<0.05$). Added moderate alfalfa saponins to broilers feed could reduce TG content and Hydrocortisone content in serum to a certain extent, effectively improve cholesterol metabolism.

Keywords: Alfalfa saponins; Broilers; Cholesterol; Bile acids

Introduction

Hyperlipidemia is common worldwide, and is considered as a highly modifiable risk factor for coronary heart disease and peripheral artery diseases. Cardiovascular disease is a major cause of death worldwide, and is related to the level of cholesterol [1]. Increased serum low-density lipoprotein (LDL) level, can accelerate atherosclerosis. Limiting the intake of dietary cholesterol could be an effective approach to prevent Cardiovascular disease. People are advised to limit their intake of poultry products because of their high levels of cholesterol. Thus, lowering the content of cholesterol in poultry products is important to peoples health. In recent decades, increasing attention has been paid to new natural agents with lipid-reducing activity [2-4], and it may be an important way to lower the cholesterol level. Alfalfa (*Medicago sativa*) is used as a food additive in the USA, Russia, China and North Africa, for its high contents of vitamin and bioactive ingredients [5,6]. Saponins is extracted from *Medicago sativa* L., and its the main active component. Saponins is traditionally a natural surface-active glycosides. It is famous with forming a stable soap-like foam. In the meanwhile, as a natural alfalfa plants extractive, Alfalfa saponins have a variety of biological active fuctions, and played a positive role in lowering the cholesterol content [7-9]. A large number of research found that saponins could improve animal performance, increase antioxidant capacity, anti-tumor, reduce cholesterol, improve immunity and other useful biological functions [10-13]. Yu et al. [9] had fond that Saponins significantly reduced the level of TC and LDL-C in serum [9]. In view of the biological characteristics of alfalfa saponins, this experiment was conducted to determine the effects of different levels of alfalfa saponins on cholesterol metabolism in broilers, as well as provide the scientific basis in the application of broiler feed.

Materials and Methods

Experimental materials

Alfalfa saponin, extract by ethanol extraction from the alfalfa meal and macroporous resin column purified, was obtained from Bo Yan Biological Technology Co., Ltd. in Cangzhou City, Hebei Province. Alfalfa saponins was tested with Thin Layer Chromatography. The purity of alfalfa saponins tested by UV spectrophotometry was 20%.

Experimental birds and grouping

A total of 320 one-day-old Arbor Acres broilers (42 ± 2 g) were randomly divided into 4 treatments, with 5 replicates in each treatment, and with 16 broilers in each replicates, using the complete randomized blocks single factorial design. Feeding experiment was conducted from 2007-4-19 to 2007-6-6 in Erqi District Houzhai town of zhengzhou city.

Experimental diet and design

During the stage from 0 to 3 weeks, broilers in the control group were fed basal diet and birds in the test groups were all fed the basal diets added with 0.02% alfalfa saponins. while from 4 to 7 weeks, birds in the test groups were fed the basal diets added with 0.04%, 0.08% and 0.12% alfalfa saponins, respectively (Table 1).

Feeding and management

All broilers were fed in three-dimensional cages in accordance with routine immunization and disinfecting quarantine procedures. This experiment was carried out in strict accordance with the recommended AA broiler feeding and management manual. The temperature was set at 33 at the initiation of experimentationand lowered by 2-3°C each week thereafter, until a final temperature at

21-22 in the fourth weekend. From the initial daily 23 h, light gradually decreased to natural light. Maintain good ventilation. All broilers were free to feed and water.

Ingredient (%)	0-3 weeks	4-7 weeks
Corn	59.7	63
Soybean meal	32.5	28
Cottonseed meal	0	1.6
Fish meal	2	1
Soybean oil	1.5	2.5
Limestone	1.4	1.5
Phosphorous	1.4	1.2
Salt	0.3	0.3
DL-methionine	0.2	0.1
Premix	1	0.8
Nutrient level		
DE (MJ/kg)	12.2	12.59
CP	20.79	19.01
Lys	1.11	0.97
Met	0.54	0.41
Met + Cys	0.89	0.74
Ca	0.98	0.93

Note: Each kilogram of premix supplied such nutrients for the complete feed: V_A: 8000 IU; VD_3: 1500 IU; V_E: 30 IU; V_K: 2 mg; V_{B1}: 5 mg; V_{B2}: 8 mg; V_{B6}: 6 mg; V_{B12}: 1 mg; Nicotinic acid: 40 mg; Pantothenic acid: 20 mg; Folic Acid: 1 mg; Biotin: 0.4 mg; Choline chloride: 1500 mg; Composite trace elements: 1000 mg; Methionine: 2000 mg; Salinomycin: 60 mg. Calculated values for ME, Lys, Met, Analyzed values for others.

Table 1: Composition and nutrient levels in basal diet for broilers of different week age.

Sample collection

At 49 days of ageone broiler whose weight is similar to average weight was selected from each replicate. Blood samples were collected from heart and serum was separated by centrifugation at 3000 rpm and frozen at -70 for later analysis. 0.5 g liver of each broiler was homogenized in Phosphate Buffered Saline (PBS, pH 7.2) (0.25 g/mL) at 4 uC. The supernatant was then centrifuged at 4000, 4 for 10 min. The preparation was adjusted to indicated concentration and stored at -70 for later analysis of total cholesterol, as well as the abundance of HMG-CoA reductase and mRNA. At 47 days of age, Feces of each broiler were collected during the last 3 days experimental period and dried at 60. Feces were weighed and grinded into 0.5 mm diameter powder for bile acid concentrations analysis.

Parameters measured

Determination of serum indices: HITACHI 7170A automatic biochemical analyzer was used to detect total cholesterol (TC), Triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) Cortisol and insulin concentrations.

Determination of fecal total bile acid: Weigh faeces 0.5 g, added 5 mL distilled water, homogenized and centrifuged at 4,000 rpm for 10 min. Using Total Bile Acid Kit, TBA provided by BIOSINO BIO-TECHNOLOGY AND SCIENCE INC, the supernatant was examined with HITACHI 7170A automatic biochemical analyzer.

The identification of liver cholesterol concentrations and HMG-CoA reductase mRNA express production by RT-PCR HITACHI 7170A automatic biochemical analyzer was used to detect liver cholesterol concentrations. The method of Liver HMG-CoA reductase mRNA express production by RT-PCR, as follows:

Primer design and synthesis: According to the broiler (Gallus gallus) liver HMG-CoA reductase catalytic domain cDNA sequence (sequence number AB109635), β-actin sequence (serial number L08165) recorded by Gen-bank, we use Oligo 6.0 primer design software to design relevant sequence primer, and their primer are as follows:

HMG-CoA reductase primer sequence:

U 5'-CACTGGGTTTGGTTCTTGTT-3'

L 5'-GGATTCGGTCTCTGCTTGTT-3'

β-actin primer sequence:

U 5'-CAGAAAGACAGCTACGTTGG-3'

L 5'-TTCATAGATGGGCACAGTGTGG-3'

Primers above were synthesized by ThreeBoYuanZhi from Beijing Biotech Co., Ltd. PCR products of HMG-CoA reductase, β-actin were predicted at the lengths of 248 bp, 357 bp respectively.

Liver total RNA extraction: Total RNA was extracted Using one-step Trizol extraction reagent obtained from the Gene Technology Co., Ltd. Beijing Parkson, and the concentrations and purity were tested by GeneQuant pro from the Biochrom Ltd. Detecting A260 was used to calculated the concentration. The ratio of A260 and A280 determine the purity of RNA (1.8-2.0), while electrophoresis was used to detect the quality.

RT-PCR

Reverse transcription reaction: Fermentas (MBI) provided the M-MuLV kit for reverse transcription of total RNA, and changing the quantity of RNA to ensure the amount of RNA is at the same level in different group.

PCR: HMG-CoA reductase reaction program: 94°C initial denaturation for 5 min; 94 denaturation 30 s, 53 30 s, and then 72 extension for 1 min, 37 cycles, at last extension at 72°C for 10 min.

β-actin reaction program: 94°C intial denaturation for 5 min; 94°C denaturation 50 s, 54.5°C; 50 s, 72°C extension for 1 min, 37 cycles; at last extension at 72°C for 10 min.

PCR products analysis: We take 7 μL HMG-CoA reductase PCR products and β-actin PCR products from the same sample, and 5 μL DNA marker, respectively. Using the agarose gel electrophoresis concentration of 2.5% and imaging system (AlphaImager) to get some camera shooting pictures. We use the PCR product ratio (IOD) of HMG-CoA reductase to β-actin to show the relative PCR products contents of HMG-CoA reductase.

Sequencing of HMG-CoA reductase PCR products: The selection of HMG-CoA reductase PCR products were purified and then send to Treasure biological engineering Co., Ltd. for sequencing.

Reaction System	Size
10 × PCR Buffer	2.5 μL
10 mM dNTP (2.5 mM)	3 μL
Forward Primer 20 μM	0.5 μL
Reverse Primer 20 μM	0.5 μL
Template DNA	1 μL
ddH$_2$O	17 μL
Total	25 μL

Statistical analysis

The statistical analysis was performed using the SAS 8.12 statistical program. A one-way ANOVA program was used to analyze the experimental data. Significant differences between treatment groups were further analyzed using Duncan's multiple range test (Duncan, 1955) and the results using "M ± SE". Differences were considered significant at $P<0.05$.

Results and Analysis

Effect of Different Levels of Alfalfa Saponin on Lipid Metabolism in Broiler

The serum TG, TC, HDL-C and LDL-C levels of different groups was described in Table 2. The TG content of broiler given 0.04%, 0.08% and 0.12% of alfalfa saponins was lower than the control group$P>0.05$, and the TG content was decreased linearly with alfalfa saponins concentration ($P>0.05$). Likewise, with the addition of alfalfa saponins, serum TC levels decreased by sequence. But there was no significant difference between the 0.04% group and the control group ($P>0.05$), while the 0.08% and 0.12% group were significantly lower than the control group ($P<0.05$); Compared with the control group, serum HDL-C levels in each treatment increased, but no significant difference ($P>0.05$). Increasing with the addition of alfalfa saponins, the substance gradually increased. In contrast with serum HDL-C levels, serum LDL-C levels decreased compared with the control group, and 0.08% and 0.12% groups were significantly lower than the control group ($P<0.01$), while 0.04% group was no significant difference ($P>0.05$). Between three treatments, there were no significant difference $P>0.05$ on serum HDL-C levels except 0.12% group was lower than 0.04% group ($P<0.05$).

Effects of Different Alfalfa Saponins Levels on Hormone Levels and Total Fecal Bile Acid in Broilers

Serum Hydrocortisone and insulin levels were described in Table 3. The concentrations of serum Hydrocortisone in 3 treatments were highter than the control group, but the difference was not significant ($P>0.05$). On the contrary, Serum insulin levels in treatment groups were lower than the control group. Compared with the control group, there were significant difference ($P<0.05$) in 0.08% group, while 0.04%, 0.12% groups had no significant difference ($P>0.05$).

Addition	TG (mmol/L)	TC (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)
0	0.38 ± 0.08	3.99 ± 0.63[a]	2.27 ± 0.30	1.15 ± 0.12[Aa]
0.04	0.34 ± 0.03	3.55 ± 0.48[ab]	2.31 ± 0.14	0.97 ± 0.14[ABab]
0.08	0.30 ± 0.03	3.22 ± 0.24[b]	2.38 ± 0.20	0.78 ± 0.30[Bbc]
0.12	0.29 ± 0.03	3.13 ± 0.30[b]	2.43 ± 0.34	0.68 ± 0.05[Bc]

Note: Within the same row followed by different superscripts, a lower case letter means a significant difference ($P<0.05$), a capital letter means a highly significant difference ($P<0.01$).

Table 2: Contents of TGTCHDL-CLDL-C in serum [A,B,a-c].

Total fecal bile acid was described in Table 3. Total fecal bile acid of 0.04% group was similar with the control group, both had no significant difference, while the other two groups were significantly higher than the control group ($P<0.05$), and 0.12% group was significantly higher than the control group ($P<0.01$). Comparison between each treatment group, 0.12% group was significantly higher than 0.04% group ($P<0.05$).

Addition	Hydrocortisone (μg/dL)	Insulin (μIU/ML)	Total bile acid (μmol/L)
0	0.07 ± 0.01	0.57 ± 0.16[a]	110.40 ± 2.55[Bc]
0.04	0.09 ± 0.02	0.45 ± 0.15[ab]	121.80 ± 11.41[ABbc]
0.08	0.11 ± 0.04	0.22 ± 0.06[b]	132.60 ± 2.45[ABab]
0.12	0.08 ± 0.01	0.51 ± 0.10[ab]	141.90 ± 7.13[Aa]

Table 3: Contents of hormone in serum and total bile acid in soil [A,B,a-c].

Effects of Alfalfa Saponins On Liver Cholesterol and Hmg-CoA Reductase mRNA Abundance in Broilers

Effects of alfalfa saponins on liver HMG-CoA reductase mRNA expression products in broilers

Total RNA extraction results: The electrophoresis results of broiler liver HMG-CoA reductase mRNA RT-PCR products was shown in Figure 1. The ratio of A260 and A280 was used to determine the RNA purity (1.8-2.0).
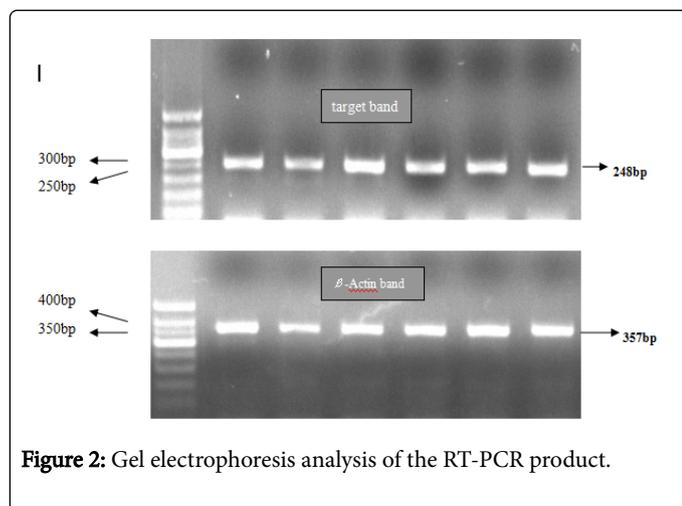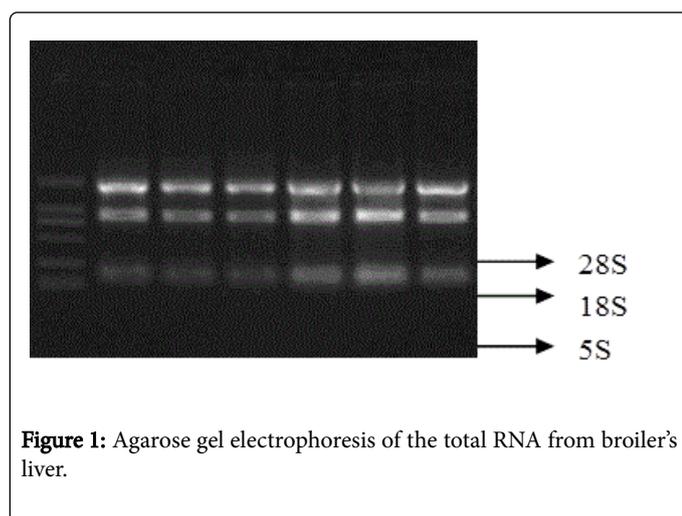
RT-PCR and gel electrophoresis results: 2.5% agarose gel electrophoresis were used to test HMG-CoA reductase and β-actin RT-PCR products, and obtained two fragments close to 248 bp and 357 bp respectively, which were consistent with the expected results (Figure 2).

Sequencing: Compared with the GenBank nucleotide sequence of genes, it's homology was 98.56%.

Effect of adding alfalfa saponins on liver cholesterol contents and HMG-CoA reductase mRNA relative contents in broilers

Liver cholesterol contents and HMG-CoA reductase mRNA relative contents in broilers were described in Table 4. Compared with the control group, liver cholesterol contents and HMG-CoA reductase mRNA relative levels of 0.08% and 0.12% groups were significantly lower than the control group ($P<0.05$). Meanwhile, HMG-CoA reductase mRNA relative levels of 0.08% and 0.12% groups were

significantly lower than the control group (P<0.01), while the 0.04% group had no significant difference, but lower than the control group. Comparison between each treatment, only liver HMG-CoA reductase mRNA relative contents of 0.08% group was significantly lower than the 0.04% group (P<0.01), while others were not significantly different (P>0.05).

Figure 1: Agarose gel electrophoresis of the total RNA from broiler's liver.

Figure 2: Gel electrophoresis analysis of the RT-PCR product.

Addition	Liver (nmol/L)	Relative contents of HMGR
0	377.50 ± 28.72^a	1.04 ± 0.09^{Aa}
0.04	353.33 ± 15.28^{ab}	0.95 ± 0.08^{ABab}
0.08	317.50 ± 10.61^b	0.71 ± 0.14^{Cc}
0.12	300.00 ± 40.00^b	0.80 ± 0.10^{BCbc}

Table 4: Contents of cholesterol in liver and relative contents of HMGR [A,B,a-c].

Discussion

Effect of alfalfa saponins on cholesterol metabolism in broilers

There are two main sources of cholesterol in broilers: One is the dietary supply, the other is its own synthesis. Most broiler feeds made of plant which Cholesterol intake is low. Therefore, cholesterol is almost synthesis by its own. The liver is the most active organ in which about 3/4 or more cholesterol is synthesized [14]. Cholesterol biosynthesis is a complex process. The substrate in cholesterol synthesis, acetyl CoA, can be converted to cholesterol through a series of multi-step enzymatic reaction intermediatesand HMG-CoA reductase is the rate-limiting enzyme of cholesterol synthesis. The activity of HMG-CoA reductase plays an important role on the regulation of cholesterol synthesis, That is phosphorylation by inactivation of the enzyme to reduce cholesterol synthesis. Hunger and satiation, cholesterol feedback regulation, hormones can regulate cholesterol synthesis. As we all know, hunger can inhibit the synthesis of cholesterol, but the cholesterol synthesis will increases when the intake of high sugar, high saturated fat diet. As for hepatic cholesterol synthesis of the cholesterol feedback inhibition is achieved by inhibiting the synthesis of HMG-CoA reductase. However, the regulation of hormones is more complex. Insulin-induced hepatic HMG-CoA reductase synthesis, Insulin can Induce hepatic synthesis of HMG-CoA reductase, However the cortisol and glucagon inhibit and decrease the activity of the enzyme, while thyroid hormone can increase the synthesis of the enzyme and promote the transition of cholesterol into the liver bile acid at the same time, and the effect of the latter is stronger than the former. Hepatic synthesis cholesterol and assemble most of the cholesterol (including exogenous and endogenous) in free into VLDL secreted into the blood, together with other lipid and apolipoprotein (ApoB, etc.) then utilized by other tissues and organs [14,15]. Liver is an important excretory organ of cholesterol, and almost all cholesterol are excreted by liver, except some from the intestinal mucosa. Liver bile acid formation is the most important way to reduce cholesterol, Primary bile acid of Cholesterol as the raw material is the main form of hepatic clearance of cholesterol, In addition, cholesterol can be converted into steroid hormones, vitamin D_3 and cholesterol ester [14].

Serum total cholesterol and low density lipoprotein cholesterol levels of adding 0.08% and 0.12% alfalfa saponins groups decreased significantly, while high density lipoprotein cholesterol contents was not significant effects, and Malinow MR had reported the similar result [16]. Yuan et al. [17] reported that adding with alfalfa saponins significantly decreased serum cholesterol and LDL cholesterol levels in rats [17]. Yu et al. [9] reported that the total alfalfa saponins significantly reduced the serum total cholesterol and LDL cholesterol in broilers [9], which were in agreement with the result of Ma [18]. These results are consistent with the current results. The mechanism of Alfalfa saponins reducing serum total cholesterol may be the formation of insoluble complexes of saponins combined with cholesterol, thus contributing to lower cholesterol levels. Low-density lipoprotein is the principle form of carring the endogenous cholesterol [14]. Alfalfa saponins can be combined with the endogenous cholesterol which is discharged by bile, thus preventing re-absorption of cholesterol [19]. It may be the reason that alfalfa saponins reduced serum low-density lipoprotein cholesterol but didn't effect serum high density lipoprotein cholesterol. The current result was that alfalfa saponins had not noticeable effect on serum triglyceride levels, and

this is not consistent with the result Yu [19] reported. It is still need further study.

The liver HMG-CoA reductase mRNA expression levels of 0.08% and 0.12% alfalfa saponins groups were decreased by 31.73% and 23.08% (P<0.01) respectivelyeffectively reducing cholesterol synthesis in liver. 0.08% and 0.12% alfalfa saponins groups mRNA expression levels of HMG-CoA reductase in the liver decreased by 31.73% and 23.08% (P<0.01), respectively, which effectively reducing cholesterol synthesis in liver. The author speculated that alfalfa saponins may reduce liver HMG-CoA reductase mRNA abundance, thereby reducing cholesterol contents. This experiment showed that breast muscle, leg muscle and liver cholesterol levels significantly decreased in 0.08% and 0.12% alfalfa saponins groups. Wang et al. [20] also reported that fed with alfalfa extract, rat liver cholesterol was significantly decreased [20]. These results are consistent with the above, the mechanism may be that alfalfa saponins convert hepatic cholesterol into bile acids, thus contributing to the emission of cholesterol from the body, reducing the deposition of cholesterol in muscle. Result of serum hormone showed that serum insulin levels significantly reduced in 0.08% group, while no significant effect on cortisol levels. Compared with the other test groups, 0.12% alfalfa saponins group decreased cortisol, increased insulin contents, which may be caused by feedback inhibition of cholesterol synthesis. The mechanism of Alfalfa saponins reducing serum total cholesterol may be the formation of insoluble complexes of saponins combined with cholesterol, thus contributing to lower cholesterol levels. The mechanism may be that due to adding with 0.12% alfalfa saponins significantly reduced the hepatic cholesterol and serum cholesterol levels, thereby enhanced the HMGR (HMG-CoA reductase acid mono) activity, promoted the synthesis of hepatic cholesterol. Because of cortisol and insulin are effected by HMGR activity, high levels of alfalfa saponins have an effect on the levels of cortisol and insulin. Detection of fecal bile acid showed that addition of alfalfa saponins increased fecal bile acid excretion. Liu et al. [21] indicated that alfalfa saponins can affect or block the bile acid enterohepatic circulation, Bile acid in the body can not be converted to cholesterol but only discharged from the body by the intestinal tract [21]. These results are agreement with the current results.

Conclusions

Alfalfa saponin has a significant effect on total cholesterol and LDL cholesterol in broiler serum, and significantly reduced cholesterol levels in broiler breast muscle, leg muscle and liver. The addition of alfalfa saponins decreased the expression level of liver HMG-CoA reductase mRNA, reduced hepatic cholesterol synthesis effectively, and improved the excretion of feces bile acid in broiler. In conclusion, the broiler dietary supplemented with 0.08% alfalfa saponins can obtain the optimal performance.

Acknowledgement

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