Effects of dietary alfalfa saponins on laying performance, egg cholesterol concentration, and ATP-binding cassette transporters G5 and G8 expression in laying hens

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ABSTRACT
This experiment was designed to evaluate the potential for dietary alfalfa saponins (AS) to influence cholesterol (Ch) concentrations and the mRNA expressions of the ATP-binding cassette (ABC) that concludes transporters G5 (ABCG5) and G8 (ABCG8) in the livers of laying hens. Hundred and fifty laying hens (27 weeks old) were divided into five treatment groups randomly. Five replicates were assigned to each treatment group consisting of six birds per replicate. The same basic diet was used for all groups with supplementation of 0 (control), 60, 120, 240, and 480 mg/kg of AS, which was given ad libitum to hens for 60 days. The results showed that AS in the diets of laying hens could effectively reduce egg Ch concentrations, and increase the excretion of bile acid by the liver with the expression levels of ABCG5 and ABCG8 improving. Our study found that dietary AS could effect cholesterol metabolism by up-regulating the expression of G5 and G8 in hens, and that AS is a potential agent for reducing Ch concentrations and 120 mg/kg of AS in the diet was suitable and effective for laying hens.

Introduction
Cardiovascular disease (mainly heart disease and stroke) is a major cause of death worldwide, which occurs more in developed countries. Cholesterol (Ch) is a crucial risk factor of this phenomenon. The accumulation of plant and animal sterols in the blood, which is the normal route of sterols exit in the body, is caused by increasing the sterols fraction absorption in the diet and decreasing the sterols secretion into the bile (Salen et al. 1989; Miettinen et al. 2006). The fraction of dietary Ch which is absorbed from 15% to 75% (50% on average) with the remainder excreted in the faeces (Cohn et al. 2010), and the entry of dietary sterols into the bloodstream is limited by multiple physiological mechanisms. Ch being absorbed excessively or the body being inordinate may lead to cardiovascular disease. In many countries, egg consumption has been decreasing considerably because Ch can increase the risk of heart disease (Hargis 1988; Spence et al. 2012). This has been confirmed in the National Cholesterol Education Program Diet (Dimitrova-Karamfilova et al. 2012) and the American Heart Association Dietary Recommendations (Lichtenstein et al. 2006).

In recent years, people have been looking for alternatives to lower Ch levels. Supplying broiler chickens with diets of 0.06% alfalfa extract produced a fat weight decrease at five weeks and six weeks of age (Dong et al. 2007). Ch and triglycerides (TG) (Aulchenko et al. 2009) of yolks of Japanese laying quails were significantly reduced by adding karaya saponins (Afrose et al. 2010, 2011). Previous studies also suggested that 60 mg/kg of alfalfa saponin (AS) obviously reduced the contents of yolk Ch and egg Ch (Hou et al. 2009). Saponins are steroid triterpenoid glycosides that occur widely in plants that are consumed by animals and humans (Johnson et al. 1986; Mayer 1996). They are produced by plants and some lower marine animals or bacteria (Riguera 1997; Yoshiki et al. 1998). Recently, some pharmaceutical companies have been marking saponin feed additives in poultry production to reduce the content of Ch. The exaction of AS is from alfalfa leaves and stems, and its main active component is AS (50%). It also contains a small amount of flavonoids and polysaccharides. AS has pleiotropic effects of physiology, including the aphid population reduction (Golawska et al. 2008; De Geyter et al. 2012), nutrient material digestion and fermentation alteration in rumen (Lu and Jorgensen 1987; Klita et al. 1996), antimicrobial activity (Oleszek 1993; Avato et al. 2006), and regulation of Ch and lipid metabolism (Malinow et al. 1977, 1981; Yu et al. 2011). Actually, it is well known that saponins form insoluble complexes with cholesterol and bile acids. However, the underlying molecular mechanisms remain undefined.

The absorption of phytosterols is limited more than that of Ch significantly. Less than 5% of dietary phytosterol is absorbed in humans (Schoenheimer 1931) and only a small amount of phytosterol can reach the liver, which is secreted into the bile preferentially (Gould et al. 1969). Two recent studies (Elkin and Lorenz 2009; Liu et al. 2010) both reported that dietary
phytosterols failed to affect egg Ch contents and that very little were actually deposited into the egg, suggesting that they are either poorly absorbed or very efficiently re-secreted into the intestine via the hepatocyte or enterocyte genes. Our main goal, therefore, was to decrease Ch synthesis and absorption, and promote Ch elimination.

A series of studies have provided the molecular basis for two major pathways of the sterols trafficking: dietary sterols absorbed in the intestine and the biliary sterols secreted from the liver. The relevant diseases are caused by mutations in two oppositely way, closely apposed genes by encoding the ATP-binding cassette transporters G5 and G8 (Peet et al. 1998; Lee, Lu, Hazard et al. 2001). Both ABCG5 and ABCG8 are members of the transporters super-family and are involved in the Ch and lipid metabolism regulation (Chawla et al. 2001; Edwards et al. 2002). These genes are coordinately up-regulated by LXRα (the receptor of nuclear hormone) in response to the dietary Ch (Lee, Lu, Hazard et al. 2001; Repa et al. 2002). They are expressed predominantly in the small intestine and the liver (Repa et al. 2002). The expression levels of G5 and G8 are coordinately increased in mice, which have consumed a high Ch diet (Berge et al. 2000). LXRα, a nuclear receptor which plays an important role in regulating genes involved in Ch transportation, is required to increase the expression of ABCG5 and ABCG8 in response to dietary Ch of mice (Peet et al. 1998) or key factors of pathways responsible for dietary Ch trafficking.

It is also not known whether it is sufficient to promote Ch secretion when ABCG5 and ABCG8 expression levels increase, or whether other steps are rate-limiting in the transport process. Furthermore, the crucial transport substrate of ABCG5 and ABCG8 has not been identified, and the effects of these genes increasing expression level on phospholipids and bile acids are still unknown. To solve these issues, we aimed to identify the relationship between biliary Ch secretion and ABCG5 and ABCG8 expression levels in liver.

In view of the biological characteristics of AS on efflux of Ch, ever since the 1970s, research efforts focused on reducing egg Ch content in various ways. These ways included genetic selection, changing the hens’ diets with different nutrient materials and probiotics, and treating the hens using drugs, some phyto-genic extracts, and non-nutritive components (Washburn and Nix 1974; Pesti and Bakalli 1998; Chowdhury et al. 2002; Kim et al. 2004). The role of liver ABCG5 and ABCG8 in Ch excretion have rarely been studied. This study was conducted to determine the effects of different dietary levels of AS on Ch and the liver genes ABCG5 and ABCG8 in laying hens.

Materials and methods

Experimental materials and ethics statement

AS was obtained from Hebei Bao’en Biotechnology Co. (Shijiazhuang, China) that was extracted from alfalfa leaves and stems as a standardized product, and the purity was 61.64%, and also contained flavonoids (10.97%), polysaccharides (8.12%), moisture (7.11%), and unknown components (12.16%). Diets were prepared and stored at room temperature with in validity period (eight weeks).

Animal feeding experiments were carried out according to the guide that has been made by the National Institutes of Health for the Care and Use of Laboratory Animals. The experimental schemes were approved by the Institutional Animal Ethics Committee of Henan Agricultural University (Permit Number: 11-0085). All animal experiments were performed in accordance with the guidelines made by the Ministry of Science and Technology of the People’s Republic of China (Approval number: 2006–398). Efforts were made to avoid the suffering of experimental animals to the most extent.

Experimental birds, grouping, and diets

Hundred and fifty Hy-Line Brown laying hens at age 27 weeks were randomly divided into five dietary treatment groups. The birds were caged in wire cages (two hens per cage, measuring 48 × 38 × 34 cm) with individual feed-troughs with a common water-trough, and the wire cages were placed in a clean and open-sided house. They were fed according to the College of Animal Science and Veterinary Medicine of Henan University guidelines about animal experimentation. Before the experiment, the birds were fed with a balanced basic diet for two weeks to allow them to adapt most and not affect the rate of egg production. The 27-week-old laying hens were divided into five treatment groups of five replicates in each group with six pullets of each replicate. The basic diet was supplemented with 0 (control), 60, 120, 240 and 480 mg/kg of AS. The birds were provided clean drinking water and feed with 18 h of light daily during 60 days experiment, which has not included preliminary trial period two weeks. The diet meets the basic nutrient requirements of laying hens according to the National Research Council (NRC 1994) nutrient requirements. The composition of the experimental diet is shown in Table 1.

Sample collection

Four eggs were collected randomly from each replicate every 15 days and the yolk Ch levels were analysed. Yolk samples were made to saponification and detection using high-performance liquid chromatography (Zhang et al. 1999).

| Ingredient                  | % Nutrient level | %     |
|-----------------------------|-----------------|-------|
| Corn                        | 67.0            | Crude protein | 16.11 |
| Soybean meal                | 17.5            | Calcium | 3.69  |
| Limestone powder            | 8.5             | Phosphorus | 0.64  |
| Peruvian fishmeal           | 4.5             | Available phosphorous | 0.44  |
| Dicalcium phosphate         | 1.0             | Lysine | 0.92  |
| Common salt                 | 0.2             | Methionine | 0.48  |
| Choline chloride            | 0.1             | Methionine + cysteine | 0.75  |
| DL-methionine               | 0.2             | Metabolic energy, MJ/kg | 11.40  |
| Trace mineral and vitamin premix$    | 1.0             |         |

$Obtained by calculation.

$Premix provided the following per kilogram of diet: 60.1 mg of iron, 80 mg of manganese, 6.8 mg of copper, 80 mg of zinc, 1 mg of iodine, 0.011 mg of selenium, 12,000 IU of vitamin A, 6000 IU of vitamin D3, 30 IU of vitamin E, 4.0 mg of vitamin K, 5 mg of vitamin B12, 2.5 mg of biotin, 3 mg of folic acid, 35 mg of niacin, 10 mg of panthenic acid, 8.5 mg of pyridoxine, 6.5 mg of riboflavin, and 2.6 mg of thiamin.
Serum was isolated by centrifugation at 3000×g for 10 min at 20°C and the samples were stored in a −70°C refrigerator until further analysis. Two hens were killed from each replicate when the experiment came to an end. The breast and thigh muscles were collected and then frozen immediately in liquid nitrogen for later analysis. The liver tissues were isolated from the same location and washed with physiological saline, blotted dry by filter paper, chopped and frozen in liquid nitrogen for later analysis. The liver tissues were isolated from the same location and washed with physiological saline, blotted dry by filter paper, chopped and frozen in liquid nitrogen at once, and then stored at −80°C until RNA extraction and analysis.

Faeces samples from each group were collected three days before the experiment was over and then dried at 60°C. Faeces samples were ground into 0.5 μm diameter pieces for analysis.

**Primer design and synthesis**

According to the laying hens (Gallus gallus) liver ABCG5 (sequence number: XM419457.4) and ABCG8 (sequence number: XM419458.4) catalytic domain mRNA sequences, primers of ABCG5 and ABCG8 were designed using Primer Express Software 5.0 based on sequence data available from GenBank. The primer sequences are shown in Table 2.

**RNA extraction and reverse transcription reaction**

Total RNA was extracted using Invitrogen Trizol reagent (Code No.: 15596-018) in accordance with the manufacturer’s instructions after grinding the liver with liquid nitrogen. The degradation and contamination of RNA were checked by 1% agarose gels. According to manufacturer’s instructions, the concentration and purity of RNA was assessed by the NanoDrop ND-1000 spectrophotometer and Agilent 2100 Bioanalyzer. The ratio of A260 and A280 was used to determine the RNA purity (1.9–2.1). Then we used reverse transcriptase M-MLV (RNase H-) (Takara Co., Code No.: D2639A) to reverse transcribe RNA using Oligo (DT) 18 Primer (Takara Co., Code No.: RR420A). β-actin of the hens livers was served as a control gene. RT-QPCR was performed eight copies on a 96-well PCR plate with 20 μl reaction volume that contains 2 μl cDNA, 10 μl SYBR Green Mastermix, and 0.5 μl of each specific forward primer and reverse primer, respectively, and 7 μl enzyme free water. The reaction was carried out on Roche for 2 min at 95°C for pre-degeneration, which was followed to the program: 38 cycles at 95°C for 15 s, 58°C for 30 s, and 72°C for 20 s. In order to confirm the absence of primer dimerization, the reaction mixture was considered as the negative control with no cDNA. Based on the expression level of β-actin, ABCG5, and ABCG8 quantitative variations were calculated using the relative quantitative method (2−ΔΔCt).

**Statistical analysis**

All data were analysed with a one-factor analysis of variance (ANOVA) using SPSS 19.0 software. Significant differences of statistical analysis of laying hens in different groups were assessed by least significant difference (LSD) analysis and a Duncan ANOVA. The LSD was calculated to compare differences among treatments, where an ANOVA showed significant differences. Statistical significance was defined with a two-tailed probability (P < .05).

**Results**

**AS and cholesterol in eggs**

On day 15, day 30, and day 45 of the experiment, the egg Ch concentrations of AS supplemented groups were significantly reduced compared with the control group (Table 3), however, those of the AS supplemented groups at day 60 were increased

| Items | Cholesterol content in eggs. | AS in diets (mg/kg) |
|-------|-------------------------------|---------------------|
|       | Period                        |                     |
|       | 0                             | 60                  | 120                   | 240                  | 480                  |
| 15 d  | 207.49 ± 3.37a                 | 194.83 ± 4.79c      | 189.55 ± 5.34d        | 204.99 ± 4.22b       | 204.09 ± 2.34b       |
| 30 d  | 230.38 ± 3.45a                 | 196.95 ± 3.32c      | 194.22 ± 5.24d        | 205.63 ± 6.23b       | 205.46 ± 4.32b       |
| 45d   | 234.18 ± 3.22a                 | 216.25 ± 3.32e      | 216.25 ± 3.32e        | 222.99 ± 1.22b       | 224.78 ± 5.25a       |
| 60d   | 228.24 ± 9.21b                 | 233.22 ± 3.22a      | 209.09 ± 9.12c        | 232.11 ± 8.23a       | 228.56 ± 7.21b       |

Notes: Data in the table show the cholesterol content of eggs per 100 g (mg/100 g, egg), the different superscript lowercase letters within the same column represent significant differences (P < .05).

**DNA sequences of ABCG5 and ABCG8**

The selections of depuration for ABCG5 and ABCG8 PCR products were conducted and sent to Treasure Biological Engineering Co., LTD for sequencing.

**Real-time PCR and gel electrophoresis**

We used real-time quantitative PCR (RT-QPCR) to detect the expression differences of ABCG5 and ABCG8 in liver tissues among treatments. We adjusted the level of cDNA to the same level and then performed RT-QPCR in accordance with the instructions using SYBR® Taq for RT-PCR (Takara Co., Code No.: RR420A). β-actin of the hens livers was served as a control gene. RT-QPCR was performed eight copies on a 96-well PCR plate with 20 μl reaction volume that contains 2 μl cDNA, 10 μl SYBR Green Mastermix, and 0.5 μl of each specific forward primer and reverse primer, respectively, and 7 μl enzyme free water. The reaction was carried out on Roche for 2 min at 95°C for pre-degeneration, which was followed to the program: 38 cycles at 95°C for 15 s, 58°C for 30 s, and 72°C for 20 s. In order to confirm the absence of primer dimerization, the reaction mixture was considered as the negative control with no cDNA. Based on the expression level of β-actin, ABCG5, and ABCG8 quantitative variations were calculated using the relative quantitative method (2−ΔΔCt).

**Table 2. SYBR green primer sequences used for RT-QPCR.**

| Genes   | Primer sequences (5′–3′) |
|---------|-------------------------|
| β-actin | 5′-GACTGCTGCTGACACTT3′ |
| ABCG5   | 5′-CCACCCGAAATGCTTCTA3′ |
| ABCG8   | 5′-TGACCCGCTGTAGACCTT3′ |
|         | 5′-AGATCCCCCTGATGAGA3′ |

Note: The primer sequences came from GenBank.
except for 120 mg/kg AS group. Feed consumption and feed efficiency were decreased by supplying AS in the diets, but only that of 120 mg/kg was significantly lower than the controls (Table 4). Egg production and egg mass were not significantly different among the five groups.

**AS and liver and feces**

Adding AS in diets had an effect on Ch content of liver, muscle, and the concentration of bile acid (Tables 5 and 6). The bile acid of the 480 mg/kg group was similar to the control group, with both having no significant difference (P > .05). The other experimental groups were significantly higher than control groups (P < .05) regarding the faeces results (Table 6). The 240 mg/kg AS group was the highest and was significantly higher than those of other treatment groups. The bile acid concentrations in the liver of the 60 mg/kg and 240 mg/kg AS groups were significantly lower (P < .05) than the control group; however, those of the 120 and 480 mg/kg AS groups had no significant difference, indicating AS could reduce the Ch levels within a certain range and a suitable level of AS is essential. The results also indicated that 240 mg/kg addition of AS reduced liver Ch concentrations significantly.

At the same time, thigh and breast muscle Ch concentrations were decreased significantly with the addition of 120 and 240 mg/kg of AS. Supplementation of AS (60, 120, and 240 mg/kg), however, resulted in a significant increase in faeces bile concentrations and a decrease in liver bile acid concentrations.

**AS and serum lipid levels**

The serum Ch reduction at the end of eight weeks in this study varied with the content of AS in the feed; feeding 120 or 240 mg/kg AS resulted in an 8.33% or 14.86% reduction of serum total cholesterol (TC) concentration compared with the controls (P < .05). The other experimental groups were significantly higher than control groups (P < .05) regarding the faeces results (Table 6). The 240 mg/kg AS group was the highest and was significantly higher than those of other treatment groups. The bile acid concentrations in the liver of the 60 mg/kg and 240 mg/kg AS groups were significantly lower (P < .05) than the control group; however, those of the 120 and 480 mg/kg AS groups had no significant difference, indicating AS could reduce the Ch levels within a certain range and a suitable level of AS is essential. The results also indicated that 240 mg/kg addition of AS reduced liver Ch concentrations significantly.

AS and genes in liver

When AS addition in the diet was 60 or 120 mg/kg, the RT-PCR data, listed in Table 8, showed that gene expression levels of ABCG5 and ABCG8 in the livers of laying hens were up-regulated compared with the control group (P < .05). On the contrary, for more than 120 mg/kg of AS, the genes expression levels were down-regulated but had no significant difference contrasted to the control group (P > .05).

**Discussion**

**AS, cholesterol, and serum lipid levels**

The results of our study indicated that AS may have a Ch-depressing effect in both the serum and eggs of laying hens. TC, LDLC, and VLDL were all decreased, however, HDLC was enhanced in the serum and showed no significant difference contrasted to the control group. HDLC has been found to be the most pivotal factor among the information of serum lipids due to increased penetration of oxidated LDL into the arterial walls (Gotto Jr and Brinton 2004) and the excess of LDL can deposit into the blood vessel walls easily. Ch and Ch esters from different peripheral tissues are carried to the liver by HDL and translated into the bile acids. Therefore, HDL plays a key role in decreasing Ch levels in the peripheral and blood tissues and inhibiting atherosclerotic formation of plaque in the aorta (Whitney et al. 2005).

Therefore, reducing TC and LDL levels and increasing HDL levels in the serum are very important for reducing the risk of atherosclerosis (Goldstein and Brown 2001). In contrast to this, birds that were supplied with a diet containing yucca saponin showed no effect regarding serum Ch concentrations (McGonigle and McCracken 2002). The discrepancy might be due to either different concentrations or different sources. The present observations indicated that AS could reduce lipoprotein cholesterol (LDLC) was significantly reduced by adding 240 or 480 mg/kg AS. However, very low density lipoprotein cholesterol (VLDLC) concentrations were significantly decreased in the 120 and 240 mg/kg AS groups.
LDLC and increase HDLC, for instance, saponins could reduce LDLC in the serum of rats or humans selectively (Potter et al. 1993; Matsuura 2001). In this study, the addition of 120 mg/kg AS may be the threshold to elicit changes in Ch metabolism for laying hens.

**AS and cholesterol in eggs**

The content of egg Ch with 120 mg/kg AS was the lowest in all groups at all time points, and the Ch content decreased about 8.65% compared to the controls in our study. This suggested that the optimum content of AS in the diets of laying hens is 120 mg per kg (Table 3). However, those values for all treatment groups increased with an increase in the extension of the test period. This may be caused by laying hens own physiological functions, because Ch is an essential structural component and indispensable material for animal tissue and cell membranes. It not only participates in the formation of cell membranes, but also is involved in the synthesis of bile acid, steroid hormones, and vitamin D. Ch plays a key role in embryonic development, membrane fluidity, the conduction of nerve, and cell differentiation. Therefore, in order to meet the requirements of the body or meet the needs of the egg itself, this may lead to increased Ch synthesis in the liver and promote Ch to the eggs, although the source was inhibited from exogenous AS. Although Ch levels in the other groups had a tendency to be decreased except for the 120 mg/kg AS group, the decreased levels were lower. This may be due to the concentration of the dietary AS being too high for Ch-related metabolic disorders in laying hens.

In the present study, the reduced concentrations of Ch in eggs observed were similar to the conclusion of 11.7% reduction in yolk Ch, which was reported with feeding on a 0.15% aqueous diet of alfalfa extract supplemented in hens (Deng et al. 2012). The concentration of Ch in yolks obviously declined when the addition of AS was 120 mg/kg in the diet (Zhou et al. 2014). Our findings were also consistent with the previous study on laying hens, which were fed using dietary saponins (Sim et al. 1984). This study also suggested 120 mg/kg of AS was effective to lower Ch in the eggs.

The mechanism for this phenomenon may be the liver converts hepatic Ch into bile acids by supplying AS in the diet, thus contributing to emission of Ch from the body, reducing the deposition of Ch in the liver. Song et al. (2012) observed that Korean red ginseng extract and platycodin D promoted bile acid efflux. AS can improve the bile acid enterohepatic circulation, which means bile acid in the body can be converted to Ch and eliminated (Kai et al. 1999). The process of Ch being translated into bile acid is restrained and the expression levels of genes that are related to bile acid reduction are down-regulated.

**AS and genes in livers**

However, whether the metabolism of Ch lowering effects of AS is enhanced by some crucial genes or not is not clear. Therefore, in our study, we examined the Ch pathway on hepatic metabolic and measured the expression of ABCG5 and ABCG8 in the liver. There are two main sources of Ch in hens: one is the dietary supply, the other is synthesis in the liver. To date, multiple studies have shown ABCG5 and ABCG8 are the markers of Ch metabolism.

In the body, ABCG5 and ABCG8 are regulated and expressed in a coordinated manner (Berge et al. 2000; Repa et al. 2002), which is the same with their participation in the transport process. The expression of ABCG5 and ABCG8 is primarily regulated at the transcriptional level. LXR α, a ligand-activated nuclear transcription factor involved in lipid metabolism control, is regarded as the main regulator on mRNA expression of ABCG5 and ABCG8. In support of this conclusion, LXR agonist T0901317 markedly up-regulates mRNA expression of ABCG5 and ABCG8 in the liver of wild type mice, but not in LXR α knockout mice (van der Veen et al. 2007; Calpe-Berdiel et al. 2008). Moreover, two LXR response elements of ABCG5 and ABCG8 have been identified (Back et al. 2013), and ABCG5 and ABCG8 intracellular trafficking depends on their co-expression in cells. Hepatocyte nuclear factor 4α (HNF4α) and GATA-binding protein 4 (GATA4) are also transcription factors, and both can synergistically stimulate ABCG5 and ABCG8 gene transcription in HepG2 cells (Sumi et al. 2007). This suggests that AS may also regulate ABCG5 and ABCG8 genes by regulating the activity of LXR, HNF4α, and GATA4. We will assess the changes in these genes in the next experiment.

### Table 6. Bile acid content of liver and faeces (μmol/g).

| Items         | Groups | AS in diets (mg/kg) |
|---------------|--------|---------------------|
|               | 0      | 60                  | 120                | 240                | 480                |
| Liver         |        |                     |                    |                    |                    |
| Feces         |        |                     |                    |                    |                    |
| Liver         | 54.60 ± 3.91a | 43.91 ± 3.78b | 45.23 ± 1.75ab | 36.79 ± 4.43b | 46.27 ± 5.66ab |
| Liver         | 43.91 ± 3.78b | 45.23 ± 1.75ab | 36.79 ± 4.43b | 46.27 ± 5.66ab |                    |
| Liver         | 45.23 ± 1.75ab | 36.79 ± 4.43b | 46.27 ± 5.66ab |                    |                    |
| Liver         | 36.79 ± 4.43b | 46.27 ± 5.66ab |                    |                    |                    |
| Liver         | 46.27 ± 5.66ab |                    |                    |                    |                    |

Note: The different superscript lowercase letters represent significant differences in the same line (P < .05).

### Table 7. Effect of AS on serum Ch indexes of laying hens.

| Items         | Index | AS in diets (mg/kg) |
|---------------|-------|---------------------|
|               |       |                     |                    |                    |
| TC (mmol/L)   | 2.76 ± 0.02b | 2.96 ± 0.02a | 2.53 ± 0.01c | 2.35 ± 0.03d | 2.55 ± 0.05c |
| TG (mmol/L)   | 11.82 ± 0.52a | 12.54 ± 0.73a | 11.67 ± 0.47a | 10.23 ± 0.96a | 11.27 ± 0.39a |
| HDLC (mmol/L) | 0.83 ± 0.01a  | 0.93 ± 0.01a  | 0.85 ± 0.05a  | 0.92 ± 0.09a  | 0.85 ± 0.07a  |
| LDLC (mmol/L) | 0.87 ± 0.05b  | 1.05 ± 0.05a  | 0.90 ± 0.09b  | 0.76 ± 0.11c  | 0.80 ± 0.04bc |
| VLDLC (mmol/L)| 1.06 ± 0.03a  | 0.98 ± 0.03ab  | 0.78 ± 0.02c  | 0.67 ± 0.03d  | 0.90 ± 0.04ab  |

Note: The different superscript lowercase letters within the same column represent significant differences (P < .05).
Table 8 shows ABCG5 and ABCG8 mRNA expression levels in the liver. Both the 60 and 120 mg/kg AS group, ABCG5 and ABCG8 mRNA expression levels were up-regulated in the liver, while those of the 240 and 480 mg/kg AS groups were down-regulated. The reason may be that the accumulation of Ch in hepatocytes was reduced and Ch cannot be translated into bile acid or converted into small amounts of bile acid when the AS content is too large. Alternatively, ABCG5 and ABCG8 over-expression could reduce regulatory Ch intracellular concentration by excreting the molecules in cells directly, which is harmful to the body. Hence, it may be that excessive AS inhibits the expression of genes, and also may be a mechanism to adjust itself. On the other hand, 3-hydroxyl-3-methylglutaryl-CoA reductase (HMGCR) is one of important rate-limiting enzymes in Ch biosynthesis (Jurevics et al. 2000) and Acyl-CoA: Ch O-acyltransferase 2 (ACAT2) is the key tissue Ch-esterifying enzyme (Cases et al. 1998). The LDL receptor plays an important role in hepatic uptake and plasma Ch clearance (Brown and Goldstein 1986). It is possible that AS affects the expression of these genes, which affected ABCG5 and ABCG8 expression levels, and thus affected the content of Ch. Our previous study showed that AS decreased serum levels of TC, TG, and LDL-C in rats and piglets (Wang et al. 2012; Yuan et al. 2013).

The changes in the concentrations of mRNA encoding multiple enzymes tightly control Ch homeostasis (Matsuyama et al. 2005). And the activity of these enzymes also parallels the observed changes in mRNA levels. Furthermore, our results demonstrated that AS had a remarkable effect on ABCG5 and ABCG8 expression levels in hen’s liver, and the best group was the one with the addition of 120 mg/kg of AS to the diet, which was consistent with the Ch content in eggs in this study. Although previous studies have shown plant sterols could be used as Ch lowering agents (Miettinen et al. 1995; Maki et al. 2001; Richard et al. 2002), these studies require confirmation by assessment of mRNA expression and the activity of key genes. We examined AS effects on production performance and also explored the underlying molecular mechanisms.

ABCG5 and ABCG8 were related in this process when a genetic defect was shown to cause sitosterolemia (Berge et al. 2000; Lee, Lu, Patel 2001), a disorder in which Ch secretion of biliary decreased fivefold and the excretion of plant sterols observed in normal individuals was abolished (Gould et al. 1969; Salen et al. 1989). Interestingly, several studies of genome-wide association have also shown the association between ABCG5 and ABCG8 variants and plasma LDL-C levels (Aulchenko et al. 2009; Kathiresan et al. 2009; Ma et al. 2010; Teupser et al. 2010).

A new finding in our study was that the expression level of ABCG5 was higher than that of ABCG8. This may be due to ABCG5 being easier to regulate by AS supplied in diets. In this study, ABCG5 and ABCG8 expression levels were the highest and the content of Ch was the lowest in eggs when the AS content was 120 mg/kg. Yu et al. (2002) found that ABCG5 and ABCG8 disruption in mice could result in a profound reduction in biliary Ch levels selectively and an apparent accumulation of Ch in the liver after Ch feeding. This indicated ABCG5 and ABCG8 may play a key role in the flux of Ch into the bile and they are the major hepato-biliary transporters of dietary and endogenously synthesized Ch.

This study showed that ABCG5 and ABCG8 expression levels in the liver of laying hens were associated with the concentration of Ch and bile acid in the body. Consequently, we concluded that ABCG5 and ABCG8 play a key role in the enterohepatic trafficking of Ch in poultry and that the expression levels of both genes directly affect bile acid and Ch excretion. AS may inhibit the absorption of Ch and increase Ch excretion, however, whether AS has an impact on other genes in the Ch metabolism pathways, beside ABCG5 and ABCG8, remains to be studied further.

Conclusions

Our study found that AS had Ch lowering effects. The potential mechanism could be attached to the up-regulation of ABCG5 and ABCG8 in the livers of laying hens, AS having an impact on the genes in liver and promotion of Ch into bile acid. Under normal physiological conditions, the discharge of Ch in the liver may be regulated by ABCG5 and ABCG8 in hens with additional dietary AS.

Disclosure statement

No potential conflict of interest was reported by the authors.

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References

Afrose S, Hossain MS, Tsuji H. 2010. Effect of dietary karaya saponin on serum and egg yolk cholesterol in laying hens. Br Poult Sci. 51:797–804.
Afrose S, Hossain MS, Tsuji H. 2011. Hypocholesterolemic effect of karaya saponin in Japanese laying quails (Coturnix coturnix japonica). J Animal Physiol Animal Nutr. 95:693–700.
Aulchenko YS, Ripatti S, Lindqvist I, Boomsma AC, Wilson JF, Spector T, et al. 2009. Loci influencing lipid levels and coronary heart disease risk in 16 European population cohorts. Nat Genet. 41:47–55.
Avato P, Bucci R, Tava A, Vitali C, Rosato A, Bialy Z, Jurzysta M. 2006. Antimicrobial activity of saponins from Medicago sp.: structure-activity relationship. Phytother Res. 20:454–457.
Back SS, Kim J, Choi D, Lee ES, Choi SY, Han K. 2013. Cooperative transcriptional activation of ATP-binding cassette sterol transporters ABCGS and ABCG8 genes by nuclear receptors including liver-X-receptor. BMB Rep. 46:322–327.

Berge KE, Tian H, Graf GA, Yu L, Grishin NV, Schultz J, Kwiterovich P, Shan B, Barnes R, Hobbs HH. 2000. Accumulation of dietary cholesterol in sitosterolemia caused by mutations in identical ABC transporters. Science. 290:1771–1775.

Brown MS, Goldstein JL. 1986. A receptor-mediated pathway for cholesterol homeostasis. Science. 232:24–47.

Calpe-Berdies L, Rotllan N, Fi Vet C, Roig R, Blanco-Vaca F, Escol-Gil JC. 2008. Liver X receptor-mediated activation of reverse cholesterol transport from macrophages to feces in vivo requires ABCGS/GB. J Lipid Res. 49:1904–1911.

Cases S, Novak S, Zheng Y-W, Myers HM, Lear SR, Sande E, Welch CB, Lusis AJ, Spencer TA, Krause BR, et al. 1998. ACAT-2, a second mammalian acyl-coa:cholesterol acyltransferase: its cloning, expression, and characterization. J Biol Chem. 273:26755–26764.

Chawla A, Reppa JJ, Evans RM, Mangelsdorff DJ. 2001. Nuclear receptors and lipid physiology: opening the X-files. Science. 294:1866–1870.

Chowdhury SR, Chowdhry SD, Smith T. 2002. Effects of dietary garlic on cholesterol metabolism in laying hens. Poult Sci. 81:1856–1862.

Cohn JS, Kamili A, Wat E, Chung RWS, Tandy S. 2010. Dietary phospholipids and intestinal cholesterol absorption. Nutrients. 2:116.

Deng W, Dong XF, Tong JM, Xie TH, Zhang Q. 2012. Effects of an aqueous extract of alfalfa on cholesterol absorption in vitro. J Nutr. 142:1021–1027.

Dimitrova-Karamfilova A, Patokova Y, Solarova T, Petrova I, Natchev G. 2012. Effect of alfalfa saponins and flavonoids on gut permeability and active nutrient transport in vitro. J Nutr. 142:1021–1027.

Flavonoids, plant sterol-rich matrixes and their effects on the gut permeability of cholesterol in humans. World Nutr. 44:17–29.

Hou Y, Chen H, Huang R, Pan D, Wang F, Wang H. 2009. Effect of alfaa saponin on production performance, yolk cholesterol and egg traits of layers. China Poult.

Johnson IT, Gee JM, Price K, Curl C, Fenwick GR. 1986. Influence of saponins on gut permeability and active nutrient transport in vivo. J Nutr. 116:2270–2277.

Jurevics H, Hostettler J, Barrett C, Morell P, Toews AD. 2000. Diurnal and positional changes. Poult Sci. 88:152–158.

Kathiresan S, Willer CJ, Peloso GM, Demissie S, Musunuru K, Schadt E, Kaplan L, Bennett D, Li Y, Tanaka T, et al. 2009. Common variants at 30 loci contribute to polygenic dyslipidemia. Nat Genet. 41:56–65.

Kim JH, Hong ST, Lee SH, Kim HJ. 2004. Oral administration of pravastatin reduces egg cholesterol but not plasma cholesterol in laying hens. Vol. 83, p. 5. Champaign, IL: ETATS-UNIS, Poultry Science Association.

Klita PT, Mathison GW, Fenton TW, Hardin RT. 1996. Effects of alfaa root saponins on digestive function in sheep. J Animal Sci. 74:1144–1156.

Lee M-H, Lu K, Hazard S, Yu H, Shulenin S, Hidaka H, Kojima H, Allikmets R, Sakuma N, Pegoraro R, et al. 2001. Identification of a gene, ABCGS, important in the regulation of dietary cholesterol absorption. Nat Genet. 27:79–90.

Lee MH, Lu K, Patel SB. 2001. Genetic basis of sitosterolemia. Curr Opin Lipidol. 12:141–149.

Lichtenstein AH, Appel LJ, Brands M, Carnethon M, Daniels S, Franch HA, Franklin B, Kris-Etherton P, Harris WS, Howard B, et al. 2006. Diet and lifestyle recommendations revision 2006: a scientific statement from the American Heart Association Nutrition Committee. Circulation. 114:82–96.

Liu X, Zhao H, Thiessen S, House JD, Jones PJJH. 2010. Effect of plant sterol-enriched diets on plasma and egg yolk cholesterol concentrations and cholesterol metabolism in laying hens. Poult Sci. 89:270–275.

Lu CD, Jorgensen NA. 1987. Alfalfa saponins affect site and extent of nutrient digestion in ruminants. J Nutr. 117:919–927.

Ma L, Yang J, Runesha B, Tanaka T, Ferrucci L, Bandinelli S, Da Y. 2010. Genome-wide association analysis of total cholesterol and high-density lipoprotein cholesterol levels using the Framingham Heart Study data. BMC Med Genet. 11:11–11.

Maki KC, Davidson MH, Umporowicz DM, Schaefer EJ, Dicklin MR, Ingram KA, Chen S, Mccamara JR, Gebhart BW, Ribaya-Mercado JD, et al. 2001. Lipid responses to plant-sterol-enriched reduced-fat spreads incorporated into a National Cholesterol Education Program Step I diet. Am J Clin Nutr. 74:33–43.

Malinov MR, Connor WE, Mclaughlin P, Stafford C, Lin DS, Livingston AL, Kohler GO, Mnculty WP. 1981. Cholesterol and bile acid balance in Macaca fascicularis. Effects of alfalfa saponins. J Clin Invest. 67:156–162.

Malinov MR, Mclaughlin P, Papworth L, Stafford C, Kohler GO, Livingston AL, Cheeke PR. 1977. Effect of alfalfa saponins on intestinal cholesterol absorption in rats. Am J Clin Nutr. 30:2061–2067.

Matsuura H. 2001. Saponins in garlic as modifiers of the risk of cardiovascular disease. J Nutr. 111:1006S–11006S.

Matsuyama H, Sato K, Nakamura Y, Suzuki K, Akiba Y. 2005. Modulation of beta-sitosterol deposition. MRS Proceed. 564:263.

Mayer R. 1996. Saponins. Von K Hostettmann und A Marston, Cambridge University Press, Cambridge, 1995 (engl.). 40 sw. Abb., 38 Tab., 548 Seiten. 3 Anhange in Tab. form. Hardcover. S 120. / DM 200-240. ISBN 0-521-32970-7. Pharm Unserer Zeit. 25:219–220.

McConigle K, McCracken K. 2002. Study on the effects of Annatto (Bixa orellana) and Yucaidaeidera on cholesterol content and yolk color. Br Poult Sci. 43:41–42.

Miettinen TA, Klett EL, Gylling H, Iioniemi H, Patel SB. 2006. Liver transplantation in a patient with sitosterolemia and cirirosis. Gastroenterology. 130:542–547.

Miettinen TA, Puska P, Gylling H, Vanhanen H, Vartiainen E. 1995. Reduction of serum cholesterol with sitostanol-ester margarine in a mildly hypercholesterolemic population. New Engl J Med. 333:1308–1312.

NRC. 1994. Nutrient requirements of poultry: ninth revised edition. Washington (DC): National Academy Press.

Oleszk W. 1993. Allolepatich potentials of alfalfa (Medicago sativa) saponins: their relation to antifungal and hemolytic activities. J Chem Ecol. 19:1063–1074.

Peet DJ, Turley SD, Ma W, Janowski BA, Lobaccaro JM, Hammer RE, Mangelsdorff DJ. 1998. Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXr. Cell. 93:693–704.

Pesti GM, Bakalli R. 1998. Studies on the effect of feeding cupric sulfate pentahydrate to laying hens on egg cholesterol content. Poult Sci. 77:1540–1545.

Pottor SM, Jimenez-Flores R, Pollack J, Lone T, Berber-Jimenez MD. 1993. Protein-saponin interaction and its influence on blood lipids. J Agricult Food Chem. 41:1287–1291.

Repa JJ, Berge KE, Pomazaj C, Richardson JA, Hobbs H, Mangelsdorff DJ. 2002. Regulation of ATP-binding cassette sterol transporters ABCGS and ABCG8 by the liver X receptors a and B. J Biol Chem. 277:18793–18800.

Richard EO, Racette SB, Okeke A, Stenson WF. 2002. Phytosterols that are naturally present in commercial corn oil significantly reduce cholesterol absorption in humans. Am J Clin Nutr. 75:1000–1004.
Riguera R. 1997. Isolating bioactive compounds from organisms. J Mar Biotechnol. 5:187–193.

Salen G, Shore V, Tint GS, Forte T, Shefer S, Horak I, Horak E, Dayal B, Nguyen L, Batra AK. 1989. Increased sitosterol absorption, decreased removal, and expanded body pools compensate for reduced cholesterol synthesis in sitosterolemia with xanthomatosis. J Lipid Res. 30:1319–1330.

Schoenheimer R. 1931. New contributions in sterol metabolism. Science. 74:579–584.

Sim JS, Kitts WD, Bregg DB. 1984. Effect of dietary saponin on egg cholesterol level and laying hen performance. Can J Animal Sci. 64:977–984.

Song Y-B, An YR, Kim SJ, Park H-W, Jung J-W, Hwang SY, Kim Y-S. 2012. Lipid metabolic effect of Korean red ginseng extract in mice fed on a high-fat diet. J Sci Food Agricult. 92:388–396.

Spence JD, Jenkins DJA, Davignon J. 2012. Egg yolk consumption and carotid plaque. Atherosclerosis. 224:469–473.

Sumi K, Tanaka T, Uchida A, Magooi K, Urashima Y, Ohashi R, Ohguchi H, Okamura M, Kudo H, Daigo K, et al. 2007. Cooperative interaction between hepatocyte nuclear factor 4α and GATA transcription factors regulates ATP-binding cassette sterol transporters ABCG5 and ABCG8. Mol Cell Biol. 27:4248–4260.

Teupser D, Baber R, Ceglarek U, Scholz M, Illig T, Gieger C, Holdt LM, Leichtle A, Greiser KH, Huster D, et al. 2010. Genetic regulation of serum phytosterol levels and risk of coronary artery disease. Circ Cardiovasc Genet. 3:331–339.

Van Der Veen JN, Havinga R, Bloks VW, Groen AK, Kuipers F. 2007. Cholesterol feeding strongly reduces hepatic VLDL-triglyceride production in mice lacking the liver X receptor α. J Lipid Res. 48:337–347.

Wang XX, Shi YH, Wang CZ, Chen TH, Chen ML. 2012. Comparative study on the modelling of hyperlipidemia rat by different high-fat diets. Jiangsu Agricul Sci. 1:182–184.

Washburn KW, Nix DF. 1974. Genetic basis of yolk cholesterol content. Poult Sci. 53:109–115.

Whitney EJ, Krasuski RA, Personius BE, Michalek JE, Maranian AM, Kolasa MW, Monick E, Brown BG, Gotto JAM. 2005. A randomized trial of a strategy for increasing high-density lipoprotein cholesterol levels: effects on progression of coronary heart disease and clinical events. Ann Intern Med. 142:95–104.

Yoshiki Y, Kudou S, Okubo K. 1998. Relationship between chemical structures and biological activities of triterpenoid saponins from soybean. Biosci Biotechnol Biochem. 62:2291–2299.

Yu L, Hammer RE, Li-Hawkins J, Von Bergmann K, Lutjohann D, Cohen JC, Hobbs HH. 2002. Disruption of Abcg5 and Abcg8 in mice reveals their crucial role in biliary cholesterol secretion. Proceed Nat Acad Sci. 99:16237–16242.

Yu C-H, Xie G, He R-R, Zhai Y-J, Li Y-F, Tsai B, Kurihara H, Yang D-P. 2011. Effects of a purified saponin mixture from alfalfa on plasma lipid metabolism in hyperlipidemic mice. J Health Sci. 57:401–405.

Yuan DD, Shi YH, Wang CZ, Guo R, Wang J. 2013. Effect of alfalfa saponins on cholesterol metabolism and its molecular mechanism. Acta Pratacult Sinica. 5:294–301.

Zhang R-Z, Li L, Liu S-T, Chen R-M, Rao P-F. 1999. An improved method of cholesterol determination in egg yolk by HPLC. J Food Biochem. 23:351–361.

Zhou L, Shi Y, Guo R, Liang M, Zhu X, Wang C. 2014. Digital gene-expression profiling analysis of the cholesterol-lowering effects of alfalfa saponin extract on laying hens. PLoS ONE. 9:e98578.