Original Research Article

Serum trimethylamine-N-oxide and gut microbiome alterations are associated with cholesterol deposition in the liver of laying hens fed with rapeseed meal

Liping Zhu a, Jianping Wang a, Xuemei Ding a, Shiping Bai a, Qiufeng Zeng a, Yue Xuan a, Gregory S. Fraley b, Keying Zhang a, *

a Animal Nutrition Institute, Key Laboratory for Animal Disease-Resistance Nutrition of China, Ministry of Education, Sichuan Agricultural University, Chengdu, Sichuan, 611130, China
b Department of Biology, Hope College, Holland, MI, 49423, USA

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A B S T R A C T
Sinapine derived from cruciferous plants could be converted into trimethylamine by intestinal microbiota. Its metabolite, trimethylamine N-oxide (TMAO), is closely linked to increased risk of cardiovascular disease and fat deposition in mammals. Hens fed with rapeseed meal (RSM) suffered from fatty liver hemorrhage syndrome (FLHS). This study was conducted to investigate whether RSM-induced fatty liver is due to TMAO via altering microbiota composition and diversity. At 33 weeks of age, 600 laying hens were randomly divided into 5 treatment groups, namely control and 14% RSM treatment groups (DY5, with 16.2% erucic acid [EA] and 74.66% glucosinolate [Gl] contents; MB1, with 3.50% EA and 43.23% Gl contents; DY6, with 6.7% EA and 22.67% Gl contents; XH3, with 44.60% EA and 132.83% Gl contents) for 8 weeks. Results revealed that 3 hens died due to liver hemorrhage after ingesting 14% RSM diet. The 14% RSM decreased serum low-density lipoprotein cholesterol (LDL-C) content ($P < 0.01$) while tended to increase serum TMAO content compared to the control group ($P = 0.08$). The 14% RSM diet increased red oil O optical density ($P < 0.01$), and increased total cholesterol (TC) and LDL-C content in the liver ($P < 0.01$, and $P < 0.01$, respectively). The 14% RSM decreased liver total bile acid (TBA) content compared to the control ($P < 0.01$). The DY6 had a higher TBA content in the liver than the XH3 ($P < 0.01$). The 14% RSM decreased mRNA abundance of liver X receptors alpha ($LXR-\alpha$, $P = 0.01$), and increased mRNA abundance of sterol response element binding protein 2 ($SREBP-2$, $P = 0.04$). Results revealed that the in-feed RSM could alter richness and diversity of cecal microbiota compared to the control ($P < 0.05$). Liver TC content and serum TMAO showed a negative relationship with Proteobacteria and Actinobacteria ($P = 0.04$). In conclusion, 14% RSM increased liver TC and induced high liver score of FLHS, which was possibly associated with the altered cecal microbiota composition, increased serum TMAO levels and $LXR-\alpha$ and $SREBP-2$ expressions.

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1. Introduction

The incidence of cardiovascular diseases (CVD), such as atherosclerosis (AS), is increasing globally and has become an expensive public health issue (Murray and Lopez, 1997). The hereditary disturbance of cholesterol metabolism is a factor in the genesis of AS (Boas et al., 1948). Recent metabolomic approaches have identified that plasma trimethylamine-N-oxide (TMAO), a choline metabolite, is a novel and independent risk factor for promoting AS both in humans and mice (Tang et al., 2013; Zeneng et al.,
2011). TMAO generation is dependent on gut microbiota by metabolizing dietary choline to trimethylamine (TMA) (Koeth et al., 2013). Previous studies have shown that choline increased serum TMAO content in humans and rats (Koeth et al., 2013; Romano et al., 2015). L-carnitine-induced TMAO (a nutrient in red meat) has been shown to induce AS by affecting cholesterol metabolism through inhibiting hepatic bile acids (BA) synthesis in female mice (Koeth et al., 2013). It was found that antibiotics and resveratrol could attenuate TMAO-induced AS by reducing TMAO synthesis via altering the gut microbiota in humans and mice (Chen et al., 2016b; Koeth et al., 2013). Resveratrol attenuated TMAO-induced AS by decreasing TMAO levels while increasing hepatic BA synthesis (Chen et al., 2016b). There was little research on the relationship between TMAO and hens’ health. However, Wang’s study solely revealed that variations in TMAO and lipid metabolism were linked to the genetic variant in flavin-containing monoxygenase-3 (FMO3) in a diet-specific manner, and mutant hens fed with RSM had a higher plasma TMAO than normal (Wang et al., 2016).

Rapeseed is an important oilseed crop with 7.41 million tonnes of rapeseed oil and 11.3 million tonnes of rapeseed meal (RSM) produced in 2018 (https://www.indexmundi.com/agriculture). RSM is an important feedstuff for laying hens, but the use of RSM is limited because of its low available energy and protein for animals as well as the presence of antinutritional factors. The RSM is sorted into 4 categories by the glucosinolates (Gl) content, including very low Gl RSM (1 to 5 μmol Gl/g), low Gl RSM (10 to 30 μmol Gl/g), moderate Gl RSM (30 to 60 μmol Gl/g), and high Gl RSM (≥ 60 μmol Gl/g) (Tripathi and Mishra, 2007). According to the erucic acid (EA) content in oil, RSM is sorted into 2 categories, containing high-EA RSM/REC (≥ 43%, NY/T 1990–2011) and low-EA RSM/REC (< 3%, GB/T 1536–2004). Several studies revealed that hens fed with RSM suffered from fatty liver hemorrhage syndrome (FLHS) (Butler et al., 2010; Martland et al., 1984; Pearson et al., 1978). FLHS has characteristics that include brittle blood vessel walls that cause blood vessels to rupture. At the same time, sinapine derived from rapeseeds by a hot expeller (Processing springs Instrument Co., Inc., Yellow Springs, OH, USA) was used for further analysis. Cecum chyme was removed to enzyme-free EP tubes from different individuals and preserved in liquid nitrogen before storing at −80 °C, and subsequently used for DNA extraction and PCR amplification.

2.2. Sampling procedure

At the end of the feeding trial, one hen was chosen from each replicate for blood collection via the jugular vein following an overnight fast. The blood was then centrifuged (3,000 × g for 10 min) at 4 °C to obtain serum (Liu et al., 2019a). Serum was stored at −20 °C for later analysis (Yan et al., 2020). After blood collection, birds were euthanized by cervical dislocation. Liver was scored subjectively as follows according to Pearson and Butler (1978): 0 = no hemorrhages; 1 = a few hemorrhagic spots; 2 = a lot of hemorrhagic spots; 3 = massive hemorrhages. About 1 cm³ of liver was fixed in 4% paraformaldehyde for oil red O staining. Another liver sample about 1 cm² was frozen in liquid nitrogen and stored at −80 °C for gene expression analysis (Liu et al., 2019b), and the remaining liver was stored at −20 °C for further analysis. Cecum chyme was removed to enzyme-free EP tubes from different individuals and preserved in liquid nitrogen before storing at −80 °C, and subsequently used for DNA extraction and PCR amplification.

2.3. Serum parameters determination

Serum total triglyceride (TG), total cholesterol (TC), and high density lipoprotein- and low density lipoprotein–cholesterol (HDL-C and LDL-C) were determined using an automatic biochemical analyzer that performed quality control when it turned on (Yellow Springs Instrument Co., Inc., Yellow Springs, OH, USA). Reagent kits for TG, TC, HDL-C and LDL-C determination were purchased from Maccura biotechnology Co. Ltd. (Chengdu, China).

The quantification of serum TMAO was performed using a HPLC-MS/MS system, consisting of an Agilent 1100 HPLC coupled to a Waters Acquity TQD (SPA009) mass spectrometer (Massachusetts, USA) equipped with an electrospray ionization source (ESI) according to Yan et al. (2015) which used the d9-TMAO as an internal standard. Ions were acquired in multiple reaction monitoring (MRM) mode. The compounds were isolated with a gradient of 20% acetonitrile and 80% 10 mmol/L ammonium formate (pH 3.0) at a flow of 0.2 mL/min. The column (phenyl, 1.7 μm, 2.1 mm × 100 mm) temperature was set to 33 °C and the injection volume was 10 μL. The selected values for Sprayer Chamber parameters were as follows: gas temperature, 300 °C; drying gas flow, 8 L/min.

2.4. Liver biochemical measurements

Liver TG, TC, LDL-C, and TBA were determined using kits obtained from the Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Livers were stained by oil red O and counterstained with hematoxylin to visualize the lipid droplets. The stained liver sections were photographed using Motic Microscope BA400 at 400× magnification.
2.5. Analysis of relative gene expression using real-time PCR

Total RNA was isolated from the liver and kidneys using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) and further purified using QiaQuick RNeasy Mini kit (Qiagen, Valencia, CA, USA). All the procedures were based on the manufacturer’s protocols. The concentration of RNA was determined using spectrophotometry based on absorbance at 260 nm, and integrity was verified by agarose gel electrophoresis. Reverse transcription using the PrimeScriptRT reagent kit (TaKaRa Biotechnology) was carried out according to the manufacturer’s instructions. Expression of targeted genes in the liver were analyzed by real-time PCR using SYBR Premix Ex Taq reagents (TaKaRa Biotechnology) with the following programs: 95°C for 34 s, 95°C for 1 min, 95°C for 15 s, and a dissociation stage of 95°C for 15 s. Expression levels were normalized to beta-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were normalized to beta-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers used are presented in Table 4.

2.6. DNA extraction and microbiota analysis

Total DNA was isolated and purified using the QIAamp DNA stool Mini kit (Qiagen, GmbH, Hilden, Germany) modified to contain a bead-beating step. The concentration and purity of the extracted genomic DNA were measured using a NanoDrop ND-1000 spectrophotometer.

### Table 1

| Item                        | DY5 | MB1 | DY6 | XH3 |
|-----------------------------|-----|-----|-----|-----|
| Dry matter, %               | 94.84 | 94.21 | 94.30 | 94.84 |
| Gross energy, cal/g         | 4.631 | 4.713 | 4.701 | 4.773 |
| Crude protein, %            | 36.59 | 36.69 | 36.98 | 38.30 |
| Crude fat, %                | 8.76  | 8.47  | 8.32  | 11.10 |
| Sinapine, mg/kg             | 9.98 × 10³ | 8.89 × 10³ | 8.77 × 10³ | 7.61 × 10³ |
| Crude fiber, %              | 19.02 | 19.69 | 18.65 | 20.79 |
| Ercucic acid, %             | 16.20 | 3.50  | 0.70  | 44.6  |
| Total Gl, µmol/g            | 74.66 | 43.23 | 22.67 | 132.83 |
| 2-OH-3-butenyl Gl, µmol/g   | 17.68 | 8.93  | 4.04  | 42.96  |
| 3-Butenyl Gl, µmol/g        | 10.7  | 5.54  | 1.76  | 23.44  |
| 4-OH-3-indolylmethyl Gl, µmol/g | 2.96 | 1.87 | 1.78 | 2.75 |
| Phenethyl Gl, µmol/g        | 2.32  | 1.46  | 1.76  | 1.26  |
| Isothiocyanates², mg/g      | 2.09  | 0.49  | 0.13  | 2.63  |
| Oxazolidine thione², mg/g   | 1.11  | 0.61  | 0.13  | 1.24  |

### Table 2

| Item                        | RSM | 0 RSM | 14% RSM |
|-----------------------------|-----|-------|---------|
| Ingredients                 |     |       |         |
| Corn                        | 61.04 | 58.00 |
| Soybean meal (CP, 43%)      | 26.77 | 14.91 |
| Wheat bran                  | 0.94  | 0.05  |
| RSM                         | 0.0  | 14.00 |
| Soybean oil                 | 0.02  | 0.35  |
| Soybean meal                | 0.98  | 2.26  |
| Calcium carbonate           | 7.91  | 7.82  |
| Calcium phosphate           | 1.17  | 1.10  |
| NaCl                        | 0.40  | 0.40  |
| Mineral premix              | 0.50  | 0.50  |
| Vitamin premix              | 0.03  | 0.03  |
| L-Lys.HCl                   | 0.0   | 0.23  |
| DL-Met                      | 0.14  | 0.13  |
| L-Thr                       | 0.0   | 0.06  |
| L-Trp                       | 0.0   | 0.01  |
| Chloride choline            | 0.10  | 0.10  |
| Rice hull powder            | 0.0   | 0.05  |
| Total                       | 100  | 100   |
| Calculated energy and nutrient contents | | |
| AMEn, kcal/kg               | 2.700 | 2.700 |
| CP                          | 16.50 | 16.50 |
| Ca                          | 3.50  | 3.50  |
| Total P                     | 0.53  | 0.57  |
| Available P                 | 0.32  | 0.32  |
| Digestible Lys              | 0.78  | 0.78  |
| Digestible Met              | 0.37  | 0.37  |
| Digestible Thr              | 0.55  | 0.55  |
| Digestible Trp              | 0.17  | 0.17  |

RSM – rapeseed meal, AMEn – nitrogen-corrected apparent metabolizable energy.

1 Erucic acid is relative to total fatty acids.

2 Provided per kilogram of diet: 8,000 IU vitamin A, 1,600 IU vitamin D3, 5 IU vitamin E, 0.8 mg vitamin B12, 2.5 mg vitamin B1, 1.5 mg vitamin B6, 0.004 mg vitamin B2, 22 mg D-pantothenic acid, 0.25 mg folic acid, 20 mg nicotinic acid, and 0.1 mg biotin.

**Table 3**

| Item                        | 0 RSM | 14% RSM |
|-----------------------------|-------|---------|
| Gross energy, cal/g         | 3.641 | 3.699 |
| Dry matter, %               | 89.22 | 89.95 |
| Crude protein, %            | 16.43 | 16.90 |
| Crude fat, %                | 4.21  | 6.01  |
| Crude fiber, %              | 2.99  | 8.03  |
| Gl, µmol/g                  | N.D   | 3.53  |
| 5-VOT, mg/kg                | N.D   | 33.69 |
| SCN², mg/kg                 | N.D   | 29.80 |
| Ercucic acid¹, %            | N.D   | 1.34  |

N.D – not detected; 5-VOT – 5-vinyl-1,3-oxazolidine-2-thione; SCN² – thiocyanate; Gl – glucosinolate; DY5 – Deyou No.5; DY6 – Deyou No.6; MB1 – Mianbangyou No.1; XH3 – Xiheyou No.3; Gl – glucosinolate.

¹ Erucic acid is relative to total fatty acids.

² Provided per kilogram of diet: 80 mg Fe (FeSO₄·7H₂O), 8 mg Cu (CuSO₄·5H₂O), 60 mg Mn (MnSO₄·H₂O), 80 mg Zn (ZnSO₄·7H₂O), 0.3 mg Se (NaSeO₃), and 0.35 mg I (KI).

The analysis of relative gene expression was performed using the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the following programs: 95°C for 15 s and 60°C for 34 s, 95°C for 15 s, and a dissociation stage of 95°C for 15 s. Expression levels were normalized to beta-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and gene expression was calculated as 2^{-ΔΔCt} and expressed as the relative fold change to the control group, as described by Livak and Schmittgen (2001) and Vandesompele et al. (2002). The primers used are presented in Table 4.
spectrophotometer (NanoDrop, Germany). The integrity of the extracted genomic DNA was determined by electrophoresis on a 1% (wt/vol) agarose gel (Yan et al., 2019). Sequencing and bioinformatics analyses were performed commercially by Novogene Company (Beijing, China). Prior to high-throughput sequencing, a DNA library was prepared. Briefly, the DNA extracted from the cecal chyme samples was used as a template to amplify the hypervariable regions V3 and V4 of 16S ribosome RNA gene. The primers contained a base pair sequence complementary to the V3 and V4 regions and illumine adaptors and molecular barcodes as previously described. The resulting amplicons were gel purified, quantified, pooled and sequenced using the 250-bp paired-end reads strategy on the Illumina HiSeq 2000 platform. The resulting sequences were clustered into operational taxonomic units (OTU) using USEARCH drive5 at 97% sequence similarity. The chimeric OTU were removed using UCHIME v4.2. Representative sequences for each OTU were picked and aligned using QIIME 1.8. The alpha and beta diversity calculations and the taxonomic community assessments were performed using QIIME 1.8.

2.7. Statistical analysis

All data were analyzed as one-way ANOVA using the GLM procedure in SAS 8.1 software (SAS Institute Inc., Cary, NC). To test the effect of RSM in diets, data were analyzed using single df contrast to compare all the RSM diets treatments with the control. When an effect was significant (P < 0.05), arithmetic means were compared by Tukey’s HSD test to determine specific differences. Data were expressed as mean and standard deviation. Sequencing and bioinformatics analyses were performed by Novogene Bioinformatics Technology Co. (Tianjin, China). The beta diversity was produced using Nonmetric Multidimensional Scaling (NMDS). Richness and diversity estimations used the α diversity index including Shannon, Chao1, ACE, and Simpson. Kruskal—Wallis tests were used to compare the relative abundance of cecum microbiota at the phylum and genus levels between the control and RSM groups. A LEfSe analysis with Kruskal—Wallis rank-sum test with a normalized relative abundance matrix was used to detect features with significantly different abundances between assigned taxa and to

Table 4
Primer design for genes analyzed by real-time PCR.1

| Target gene | Nucleotide sequence of primers (5’-3’) | Product length, bp | Accession number |
|-------------|---------------------------------------|--------------------|-----------------|
| Beta-actin  | F: GAGAAATTGTGCGTGACATCA               | 152                | L08165          |
|             | R: CCTGAACCTCTCATTGCAC                 |                    |                 |
| GAPDH       | F: ATGCCATCCAGGAGTCA                   | 141                | NM 204,305.1    |
|             | R: GGGGACAGCAGGAGGAAAGAC              |                    |                 |
| CYP7A1      | F: GAT CCT CCC ACC CCT TCT GG          | 82                 | AY700578        |
|             | R: AGC TTC TCC CAG CTT CTC AC         |                    |                 |
| LXR-α       | F: GAC CTG AGC TAT AAT CGG GAT G       | 255                | AF492498        |
|             | R: TCA GGT CAT CAT TGG TGC TGT TG      |                    |                 |
| FXR         | F: AGT AGA AGC CAT GTT CCT CCG TT      | 182                | AF492497        |
|             | R: GCA GTC CAT ATT CCT CTC GTC TC      |                    |                 |
| SREBP-1     | F: CAT TGG GTC ACC CCT TCT TCG TG      | 236                | AY029224        |
|             | R: CGT TGA GCA GCT GAA GCT ACT CC      |                    |                 |
| SREBP-2     | F: ACA GAC GCC AAG ATG CAC AAG TC      | 339                | AJ414379        |
|             | R:CCA CAG CAG CAG ACT CAG GTT CA       |                    |                 |

GAPDH = glyceraldehyde-3-phosphate dehydrogenase; CYP7A1 = cholesterol 7-alpha hydroxylase; LXR-α = liver X receptor α; FXR = farnesoid X receptor; SREBP = sterol regulatory element binding protein.

1 Accession number refer to NCBI.

Fig. 1. Hen livers died from liver hemorrhage during week 1 to 8. (A) 14% MB1 rapeseed meal (RSM), (B) 14% XH3 RSM, (C) Liver score of fatty liver hemorrhagic syndrome. P contrast < 0.01, P ANOVA = 0.01. DY5 = Deyou No.5; DY6 = Deyou No.6; MB1 = Mianbangyou No.1; XH3 = Xiheyou No.3.
perform LDA to estimate the effect size of each feature. Spearman correlation analysis was performed to compare serum TMAO content, and liver lipids content with microbiota.

2.8. Data deposition

The raw sequence data for all samples are available at NCBI, under the SRA database with the accession number PRJNA530694.

3. Results

3.1. Serum lipid-related substances and liver score of FLHS

In the present study, 3 hens died due to liver hemorrhage after ingesting 14% RSM diet (Fig. 1). RSM increased the liver score of FLHS compared with the control group at week 8 \((P < 0.01)\), but there was no difference among the 4 varieties of RSM. The effects of RSM on serum lipids content are shown in Fig. 2. Compared with the control group, 14% RSM had no effect on serum TG, TC, and HDL-C content at week 8 \((P = 0.76, P = 0.54, \text{ and } P = 0.34, \text{ respectively})\), but decreased serum LDL-C content at week 8 \((P < 0.01)\). There were no differences in serum lipid levels among the 4 varieties of RSM. The effects of RSM on serum TMAO content is shown in Fig. 3. Compared to the control group, RSM tended to increase serum TMAO content \((P = 0.08)\), and there was no difference in serum TMAO content among the different varieties of RSM.

3.2. Liver lipid-related substances

The effects of RSM on liver red oil O staining at week 8 are illustrated in Fig. 4. Hens fed with 14% RSM accumulated lipids within their livers. The 14% RSM diet had a higher red oil O optical density compared to the control diet \((P < 0.01)\). The effects of RSM on lipids content of the liver at week 8 are shown in Fig. 5. RSM increased the TC and LDL-C content of liver \((P < 0.01, \text{ and } P < 0.01, \text{ respectively})\), and decreased liver TBA content \((P < 0.01)\). However, RSM had no effects on liver TG content compared to the control \((P = 0.64)\). The 14% DY6 RSM had higher TBA levels in the liver than the 14% XH3 RSM group \((P < 0.01)\).

Fig. 2. The effects of rapeseed expeller meal on serum lipids profile at week 8 \((n = 8)\). (A) \(P \text{ contrast} = 0.76, P \text{ ANOVA} = 0.17\); (B) \(P \text{ contrast} = 0.54, P \text{ ANOVA} = 0.65\); (C) \(P \text{ contrast} = 0.34, P \text{ ANOVA} = 0.69\); (D) \(P \text{ contrast} <0.01, P \text{ ANOVA} = 0.04\).
Fig. 3. The effects of rapeseed expeller meal on serum trimethylamine-N-oxide (TMAO) content of laying hens at week 8 (n = 8). (A) 133.97 ng/mL TMAO standard, (B) 2.354 ng/mL TMAO of serum sample, (C) Serum TMAO content comparison of control and rapeseed meal groups, P contrast = 0.08, P ANOVA = 0.25.

Fig. 4. Microscopic examination of oil red O stained liver from hens fed with control and 14% rapeseed meal (RSM) diets at week 8 (n = 8). (A) Control diet, (B) 14% DY6 RSM diet, (C) 14% MB1 RSM diet, (D) 14% DY5 RSM diet, (E) 14% XH3 RSM diet, (F) comparison of red oil O optical density of liver, P contrast = 0.01, P ANOVA = 0.07.
3.3. Gene expression related to cholesterol metabolism in hens

The effects of RSM on gene expression related to cholesterol metabolism in the liver at week 8 are shown in Fig. 6. Treatment with 14% RSM decreased mRNA abundance of liver X receptor α (LXR-α, $P = 0.01$), while increased mRNA abundance of sterol regulatory element binding protein 2 (SREBP-2, $P = 0.04$). There were no differences in mRNA expression of cholesterol 7-alpha hydroxylase (CYP7A1), SREBP-1, and farnesoid X receptor (FXR) among the control and RSM groups ($P = 0.16$, $P = 0.85$, and $P = 0.99$, respectively).

3.4. Composition of cecum microbiota

The sequences were assigned to 1,357 OTU defined at a 97% similarity, with 802 of those observed in all control and experimental groups and identified as the core OTU (Fig. 7). Cecum microbiota of all RSM groups had a lower α diversity than the control group (Fig. 9). RSM diets decreased microbiota richness as shown by ACE ($P = 0.02$) and Shannon index ($P = 0.02$). All of the cecal chyme samples were dominated by four phyla (> 1%) containing Bacteroidetes (56.03%), Firmicutes (31.88%), Proteobacteria (5.16%), and Actinobacteria (1.65%) (Fig. 10A). All of the cecum microbiota samples were dominated by 12 genera (> 1%) including Bacteroides (18.64%), Lactobacillus (7.57%), Rikenellaceae-RC9-gut-group (4.52%), Phascolarctobacterium (3.27%), Ruminococcaceae-UCG-014 (2.3%), Barnesiella (2.29%), Sutterella (2.27%), Ruminococcus-torques-group (1.73%), Prevotellaceae-UCG-001 (1.39%), Megamonas (1.13%), Megasphaera (1.23%), and Desulfovibrio (1.23%). In addition, the samples had unidentified taxa that comprised 52.43% of the sequences (Fig. 10B). Beta-diversity was calculated using the weighted UniFrac metric and revealed that the cecum microbiota from hens fed the control and RSM diets could be divided into 2 different clusters by NMDS (Fig. 8, $P = 0.01$).

Fig. 5. Lipids content of livers from hens fed with control and 14% rapeseed meal diets at week 8 ($n = 8$). (A) Liver total triglyceride (TG) content (mmol/g): $P$ contrast = 0.64, $P$ ANOVA = 0.95. (B) Liver total cholesterol (TC) content (mmol/g): $P$ contrast = 0.01, $P$ ANOVA = 0.03. (C) Liver low density lipoprotein cholesterol (LDL-C) content (mmol/g): $P$ contrast < 0.01, $P$ ANOVA = 0.01. (D) Liver total bile acid (TBA) content (μmol/L): $P$ contrast < 0.01, $P$ ANOVA < 0.01.
group compared to RSM: Bacteroides, Christensenellaceae-R-7-group, Alistipes, Peptococcus, unidentiﬁed-Ruminococcaceae, Parabacteroides, Ruminococcaceae-UGC-005, Shuttleworthia, Mangroviflexus, Lachnospiraceae-AC2044-group, Anaeroproporobacter, Pseudarthrobacter, Erysipelotrichaceae, Ruminococcaceae-NC4A214-group, Sellimonas, Coprococcus-I, and Oscilidspira. At the species level, there were 13 species where the relative abundance of cecum microbiota was higher in the control group: containing Bacteroides barnesiae, Bacteroides coprophilus, Bacteroides coprococola, Bacteroides vaginalis, Lactobacillus ingluviei, Collinsella aerofaciens, Clostridiales bacterium-77-5d, Pseudarthrobacter oxydans, Ruminococcaceae bacterium AM2, bacterium-1391, Bacteroides eggerthii, Ruminococcus ﬂavefaciens, and iron reducing bacterium enrichment culture. Birds fed the RSM diet showed a higher abundance of Ruminococcaceae-UGC-009, Veillonella, and Ruminococcus-2 at the genus level, and Clostridium-sp-CAG-306 and Veillonella-sp-MY-P9 at the species level. A taxonomic-based
individual and interactive contributions of Gl, thiocyanate (SCN) to the variance of microbial community structure between soybean- and RSM-fed hens. The 3 dominant microbes at phylum level contained Bacteroidetes, Firmicutes, and Proteobacteria in the layers of cecum. Our study showed liver fat of 14% MB1 RSM and was signiﬁcantly related to reticulolysis (Campbell, 1979; Martland et al., 1984). In the present study, large hematomas covering a major portion of the liver were observed in hens fed with 14% MB1 RSM and 14% XH3 RSM after 8 weeks of feeding. High fat content in the liver could lead to liver cirrhosis (Savary et al., 2017). In the present study, the 14% RSM increased oil red O option density, consistent with previous studies that showed liver fat of fish fed with RSM diet was higher than that of a soybean meal diet (Lin et al., 2010). We found that RSM increased liver lipids content including TC and LDL-C and decreased serum LDL-C content compared to the control. The observation that supplementation with RSM led to liver TC deposition could be due to its inhibition of liver LDL-C transport into the blood. SREBP-2, which is highly expressed in the liver, controls the transcription of various target genes such as hydroxy-methylglutaryl-CoA reductase (HMG-CoA) and the LDL receptor involved in cholesterol synthesis and uptake (König et al., 2007). In the present study, RSM diet increased SREBP-2 expression in the liver which might have contributed to the liver lipid accumulation. There are two main pathways for the metabolism of liver cholesterol in layers. First, cholesterol binds vitellogenin and very low-density lipoprotein to participate in the composition of the cell membrane (Arika et al., 2016), or transports into egg yolk (Hargis, 1988). Second, cholesterol, which is mainly absorbed by the ileum, forms TBA in the liver (Moshbach, 1974). A small percentage of cholesterol is discharged directly into the intestines through the biliary system (Siperstein and Murray, 1955). Conversion of cholesterol to TBA requires 15 different enzymatic steps. CYP7A1 is a liver-speciﬁc enzyme that catalyzes the ﬁrst and rate-limiting step in the classical bile acid synthesis pathway (Chiang, 2002). LXR-α and FXR are positive and negative regulators of CYP7A1 transcription, respectively. In addition, SREBP-1c, which interacts with LXR-α, regulates the expression of CYP7A1 (Li et al., 2006). In the present study, RSM downregulated the abundance of LXR-α expression in the liver which might induce the liver TBA content decrease, thus increasing liver cholesterol deposition. High serum TMAO in the diets with RSM could have inhibited the cholesterol from being converted to bile acids. This was in line with Romano et al. who reported that 1% wt/wt choline increased serum TMAO compared with choline deﬁcient diets, and that colonization with TMA-producing bacteria resulted in higher cecum TMA content and serum TMAO content compared to the control (Romano et al., 2015; Ding et al., 2018). FMO3 gene knockout mice had a low serum TMAO and low-fat mass compared to normal mice (Schugar et al., 2017), and resveratrol inhibited liver cholesterol by inhibiting serum TMAO which in turn inhibited bile acid synthesis in the liver by down-regulating CYP7A1 expression (Chen et al., 2016a). In the present study, the RSM diet group had a lower CYP7A1 expression than the control, which further induced low TBA in the liver of RSM group.

Diet composition may inﬂuence gut microbiota composition that is correlated to health parameters (Claesson et al., 2012). Caï et al. (2013) showed that RSM inhibited total aerobic bacteria including E. coli and Aeromonas and increased total anaerobic bacteria. Using NMDS, we showed a signiﬁcant difference in the gut microbial community structure between soybean- and RSM-fed hens. The 3 dominant microbes at phylum level contained Bacteroidetes, Firmicutes, and Proteobacteria in the layers’ cecum. Our observations are in agreement with a previous study that also reported that the 2 dominant microbes in the cecum were Bacteroides and Lactobacillus (Wei et al., 2013). In our study, RSM decreased the diversity and richness of the cecal microbiome. Liu et al. also

4. Discussion

Mortality attributable to haemorrhagic liver was evident only among hens receiving the high Gl RSM and was signiﬁcantly related to reticulolysis (Campbell, 1979; Martland et al., 1984). In the present study, large hematomas covering a major portion of the liver were observed in hens fed with 14% MB1 RSM and 14% XH3 RSM after 8 weeks of feeding. High fat content in the liver could induce vascular friability and breakdown, eventually induce liver haemorrhage (Savary et al., 2017). In the present study, the 14% RSM increased oil red O option density, consistent with previous studies that showed liver fat of fish fed with RSM diet was higher than that of a soybean meal diet (Lin et al., 2010). We found that RSM increased liver lipids content including TC and LDL-C and decreased serum LDL-C content compared to the control. The observation that supplementation with RSM led to liver TC deposition could be due to its inhibition of liver LDL-C transport into the blood. SREBP-2, which is highly expressed in the liver, controls the transcription of various target genes such as hydroxy-methylglutaryl-CoA reductase (HMG-CoA) and the LDL receptor involved in cholesterol synthesis and uptake (König et al., 2007). In the present study, RSM diet increased SREBP-2 expression in the liver which might have contributed to the liver lipid accumulation. There are two main pathways for the metabolism of liver cholesterol in layers. First, cholesterol binds vitellogenin and very low-density lipoprotein to participate in the composition of the cell membrane (Arika et al., 2016), or transports into egg yolk (Hargis, 1988). Second, cholesterol, which is mainly absorbed by the ileum, forms TBA in the liver (Moshbach, 1974). A small percentage of cholesterol is discharged directly into the intestines through the biliary system (Siperstein and Murray, 1955). Conversion of cholesterol to TBA requires 15 different enzymatic steps. CYP7A1 is a liver-specific enzyme that catalyzes the first and rate-limiting step in the classical bile acid synthesis pathway (Chiang, 2002). LXR-α and FXR are positive and negative regulators of CYP7A1 transcription, respectively. In addition, SREBP-1c, which interacts with LXR-α, regulates the expression of CYP7A1 (Li et al., 2006). In the present study, RSM downregulated the abundance of LXR-α expression in the liver which might induce the liver TBA content decrease, thus increasing liver cholesterol deposition. High serum TMAO in the diets with RSM could have inhibited the cholesterol from being converted to bile acids. This was in line with Romano et al. who reported that 1% wt/wt choline increased serum TMAO compared with choline deficient diets, and that colonization with TMA-producing bacteria resulted in higher cecum TMA content and serum TMAO content compared to the control (Romano et al., 2015; Ding et al., 2018). FMO3 gene knockout mice had a low serum TMAO and low-fat mass compared to normal mice (Schugar et al., 2017), and resveratrol inhibited liver cholesterol by inhibiting serum TMAO which in turn inhibited bile acid synthesis in the liver by down-regulating CYP7A1 expression (Chen et al., 2016a). In the present study, the RSM diet group had a lower CYP7A1 expression than the control, which further induced low TBA in the liver of RSM group.

Diet composition may influence gut microbiota composition that is related to health parameters (Claesson et al., 2012). Caï et al. (2013) showed that RSM inhibited total aerobic bacteria including E. coli and Aeromonas and increased total anaerobic bacteria. Using NMDS, we showed a significant difference in the gut microbial community structure between soybean- and RSM-fed hens. The 3 dominant microbes at phylum level contained Bacteroidetes, Firmicutes, and Proteobacteria in the layers’ cecum. Our observations are in agreement with a previous study that also reported that the 2 dominant microbes in the cecum were Bacteroides and Lactobacillus (Wei et al., 2013). In our study, RSM decreased the diversity and richness of the cecal microbiome. Liu et al. also
showed that Chao1 and OTU in the cecal microbiome of rats fed with Gl from broccoli were lower than that of rats fed with Gl-free broccoli (Liu et al., 2017). In the present study, the family of Ruminococcaceae, Lachnospiraceae, Christensenellaceae, and Peptococcaceae within the phylum Firmicutes was lower in the cecal microbiome of the RSM group. These observations are in agreement...
with a previous study which reported broccoli and carrot extracts with different levels and varieties of Gl inhibited phylum Firmicutes abundance in the in vitro experiment (Reiner et al., 2011). EA had a slight contribution on microbiota composition changes in this study, but a search of literature found few investigations on the effect of dietary EA on gut microbiota. So, it is hard to explain how the EA affect gut microbiota, and more research is needed to investigate it. A statistically significant and drastic decrease in Firmicutes and increase in Proteobacteria was apparent in the obese and Nonalcoholic steatohepatitis (NASH) groups (Zhu et al., 2013). An increase in Actinobacteria abundance had positive correlation to adipogenesis (Wieland et al., 2015). Gly-MCA inhibited the progression of fatty liver that was induced by a high-fat diet by inhibiting Actinobacteria abundance (Zhang et al., 2016). Similarly, we verified that liver lipid content including TC and LDL-C had a positive correlation with Actinobacteria in this research. Actinobacteria are efficient biocatalysts of many processes involving steroid bioconversion which can oxidate alcohols to ketones or

![Fig. 11. Taxonomic differences of cecum microbiota between Control and 14% RSM groups. (A) RSM-enriched taxa are indicated with a positive LDA score (green), and taxa enriched in Control have a negative score (red). Only taxa meeting an LDA significant threshold > 2 are shown. (B) and (C) Comparison of relative abundance of the top 10 bacterial phylum and top 20 genus between Control and 14% RSM group, 0.01 < *P < 0.05. RSM = rapeseed meal.](image)

![Fig. 12. Variation partitioning analysis (VPA) of the effects of antinutritional factors on bacterial community structure. env1: contain Gl, SCN, and sinapine; env2: contain EA. Gl = glucosinolates; SCN = thiocyanate; EA = erucic acid.](image)

**Table 5**
The correlation between microbiota phylum and content of liver TC, LDL-C, and serum TMAO content.

| Item   | Proteobacteria | Actinobacteria | Verrucomicrobia | Ignavibacteria |
|--------|----------------|----------------|-----------------|---------------|
| TMAO   | 0.9*           | 0.9*           | −0.6            | −0.67         |
| TC     | 0.9*           | 0.9*           | −0.6            | −0.67         |
| LDL-C  | 1**           | 1**           | −0.3            | −0.67         |

TC = total cholesterol; LDL-C = low-density lipoprotein cholesterol; TMAO = trimethylamine-N-oxide. *P-value < 0.05; **P-value < 0.01.
aldehydes (Donova, 2007), so increased liver lipids in the RSM group might be due to the increased cecal Actinobacteria abundance in this study. Proteobacteria is the main degrading bacteria for TMA from choline (Fennema et al., 2016), and sinapine in RSM could be decomposed into choline. In the liver, TMA could be oxidized to TMAO with FMO3 enzyme, so this indirectly explained how it is possible that Proteobacteria had a positive correlation with serum TMAO increasing in the present study.

5. Conclusion
In conclusion, 14% RSM in diets increased liver TC and LDL-C content in hens and induced high liver score of fatty liver hemorrhagic syndrome, which was possibly associated with the altered cecal microbiota composition and increasing serum TMAO levels. Down-regulating mRNA abundance of LXRs-α which inhibited the conversion of liver TC to bile acids might increase liver TC content. Up-regulation of SREBP-2 expression in the RSM diets could promote TC synthesis.

Author contributions
Liping Zhu designed and performed the trial, and wrote the manuscript. Jianping Wang, Xuemei Ding, Shiping Bai, and Qiu-feng Zeng assisted with all of the data analyses and helped in drafting the manuscript. Yue Xuan assisted with sample collection and detection. Gregory S. Fraley revised the manuscript. Keying Zhang obtained funding and contributed to experimental design. All authors have read and approved the final manuscript.

Conflict of interest
We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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