Animal Nutrition 7 (2021) 1258–1270

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

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

ARTICLE INFO

Article history:
Received 21 July 2020
Received in revised form
29 January 2021
Accepted 23 February 2021
Available online 14 September 2021

Keywords:
Sinapine
Expeller rapeseed meal
Trimethylamine N-oxide
Liver lipid
Cecum microbiota
Laying hen

ABSTRACT

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-α*, $P = 0.01$), 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-α* and *SREBP-2* expressions.

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.,

* Corresponding author.
E-mail address: zkeying@sicau.edu.cn (K. Zhang).
Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

https://doi.org/10.1016/j.aninu.2021.02.008

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 TMA and lipid metabolism were linked to the genetic variant in flavin-containing monooxygenase-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 RSM could produce TMA by intestinal microbiota in laying hens (Fenwick and Pearson, 1981; Hobsonâ Frohock et al., 1977; Long et al., 2017). Given the close association among TMAO levels, gut microbiota, and BA metabolism, we hypothesized that RSM-induced fatty liver has a correlation with the TMAO-induced liver cholesterol metabolism disorder mediated by intestinal microbiota. Ethanolic extracts from broccoli sprouts rich in Gl, particularly glucoraphanin, reduced body weight gain from 5.88 to 4.74 g/d and mesenteric adipose tissue weight from 4.97 to 2.91 g/100 g body weight in rats fed high-fat diets (Lee et al., 2009). Also, EA increased cholesterol deposition (Carroll, 1962). There were a lot of anti-nutritional factors in RSM mainly containing Gl and EA. Therefore, 4 kinds of RSM with high or low Gl and EA content was chosen to investigate their effects on liver lipids deposition. The objective of this study was to investigate whether fatty liver induced by RSM is due to TMAO via altering microbiota composition and diversity.

2. Materials and methods

The Institutional Animal Care and Use Committee of Sichuan Agricultural University approved all procedures used in this study (Chengdu, Sichuan, China).

2.1. Hens, diets, and management

At 33 weeks of age, a total of 600 laying hens (Lohmann white, Sun Daily Inc., Chengdu, Sichuan, China) were randomly divided into 5 treatment groups including a control group without RSM (a corn-soybean diet) and 4 varieties of 14% RSM (DY5, DY6, MB1, and XH3 RSM). The 4 varieties of RSM were made from 4 varieties of Chinese *Brassica napus* rapeseeds by a hot expeller (Processing temperature: 70 to 87 °C [10 min], 87 to 130 °C [10 min], 130 °C [2 to 3 min]). The trial lasted for 8 weeks. The analyzed nutrients and antinutritional factor concentrations of 4 varieties of RSM are shown in Table 1. There were 8 replicates per treatment with 15 hens per replicate. Hens were housed individually in stainless steel cages (38.2 cm × 50.1 cm × 40.0 cm) and room environment was controlled at 22 °C by a daily lighting schedule of 16 h light and 8 h dark per day. Hens were allowed ad libitum access to experimental diets and water. The hens were fed diets in mash form (Table 2), which were formulated to meet or exceed energy and nutrient requirements according to NRC (1994) and a published management guide (Lohmann Tierzucht GmbH, Cuxhaven, Lower Saxony, Germany). The analyzed nutrients and antinutritional factors of diets are shown in Table 3.

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 cm^3 of liver was fixed in 4% paraformaldehyde for oil red O staining. Another liver sample about 1 cm^3 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 Spray 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.

Table 1
Analyzed nutrients and antinutritional factors components of expeller rapeseed meal (as-fed basis).[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	9.98×10^3	6.89×10^3	8.77×10^3	7.61×10^3
Crude fiber, %	19.02	19.69	18.65	20.79
Erucic acid, %	16.20	3.50	0.70	44.6
Total Gl[2], 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
Isothiocyanates[2], mg/g	2.09	0.49	0.13	2.63
Oxazolidine thione[2], mg/g	1.11	0.61	0.13	1.24

DY5 = Deyou No.5; DY6 = Deyou No.6; MB1 = Mianbangyou No.1; XH3 = Xiheyou No.3; Gl = glucosinolate.
[1] Erucic acid content is relative to total fatty acid content.
[2] Those analyzed value were measured from rapeseed, and Gl content was calculated on the basis of expeller rapeseed meal.

Table 2
Ingredient composition and energy and nutrient content of experimental diets (as-fed basis, %).

Item	RSM	
	0	14%
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
Corn gluten meal	0.02	0.35
Soybean oil	0.98	2.26
Calcium carbonate	7.91	7.82
Calcium phosphate	1.17	1.10
NaCl	0.40	0.40
Mineral premix [1]	0.50	0.50
Vitamin premix [2]	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] Provided per kilogram of diet: 60 mg Fe ($FeSO_4 \cdot 7H_2O$), 8 mg Cu ($CuSO_4 \cdot 5H_2O$), 60 mg Mn ($MnSO_4 \cdot H_2O$), 80 mg Zn ($ZnSO_4 \cdot 7H_2O$), 0.3 mg Se ($NaSeO_3$), and 0.35 mg I (KI).
[2] Provided per kilogram of diet: 8,000 IU vitamin A, 1,600 IU vitamin D3, 5 IU vitamin E, 0.8 mg vitamin B_1, 2.5 mg vitamin B_2, 1.5 mg vitamin B_6, 0.004 mg vitamin B_{12}, 2.2 mg D-pantothenic acid, 0.25 mg folic acid, 20 mg nicotinic acid, and 0.1 mg biotin.

Table 3
Analyzed nutrients and antinutritional factors components of experiment diets (as-fed basis).

Item	0 RSM	14% RSM			
	Control	DY6	MB1	DY5	XH3
Gross energy, cal/g	3,641	3,699	3,695	3,694	3,681
Dry matter, %	89.22	89.95	89.61	89.40	89.74
Crude protein, %	16.43	16.90	16.86	16.41	17.02
Crude fat, %	4.21	6.01	6.24	5.87	6.21
Crude fiber, %	2.99	8.03	9.79	7.04	10.70
Gl, μmol/g	N.D	2.98	3.53	6.13	7.98
5-VOT, mg/kg	N.D	4.46	33.69	93.04	117.48
SCN^-, mg/kg	N.D	30.20	29.80	28.60	33.20
Erucic acid[1], %	N.D	0.20	1.34	3.70	11.23

N.D = not detected; 5-VOT = 5-vinyl-1,3-oxazolidine-2-thione; SCN^- = thiocyanate; Gl = glucosinolate; DY5 = Deyou No.5; DY6 = Deyou No.6; MB1 = Mianbangyou No.1; XH3 = Xiheyou No.3.
[1] Erucic acid is relative to total fatty acids.

2.5. Analysis of relative gene expression using real-time PCR

Total RNA was isolated from the liver using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and further purified by Qiagen 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. Expressions of targeted genes in the liver were analyzed by real-time PCR using SYBR Premix Ex Taq reagents (TaKaRa Biotechnology, Dalian, China). The qRT-PCR were performed in a 384-well plate on QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the following programs: 95 °C for 30 s, 40 cycles at 95 °C for 5 s and 60 °C for 34 s, 95 °C for 15 s, and a dissociation stage of 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s. Expression levels were normalized to beta-actin and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and gene expression was calculated as $2^{-\Delta\Delta Ct}$ 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.

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

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:	CCTGAACCTCTCATTGCCA		
GAPDH	F:	ATGGCATCCAAGGAGTGA	141	NM 204,305.1
	R:	GGGAGACAGAAGGGAACAG		
CYP7A1	F:	GAT CTT CCC AGC CCT TGT GG	82	AY700578
	R:	AGC CTC TCC CAG CTT CTC AC		
LXR-α	F:	GAC CTG AGC TAT AAT CGG GAT G	255	AF492498
	R:	TCA GGT GAT CAT TTG GTC TGT TG		
FXR	F:	AGT AGA AGC CAT GTT CCT CCG TT	182	AF492497
	R:	GCA GTG CAT ATT CCT CCT GTG TC		
SREBP-1	F:	CAT TGG GTC ACC GCT TCT TCG TG	236	AY029224
	R:	CGT TGA GCA GCT GAA GGT ACT CC		
SREBP-2	F:	ACA GAC GCC AAG ATG CAC AAG TC	339	AJ414379
	R:	CCA CAG GAG GAG AGT 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.

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

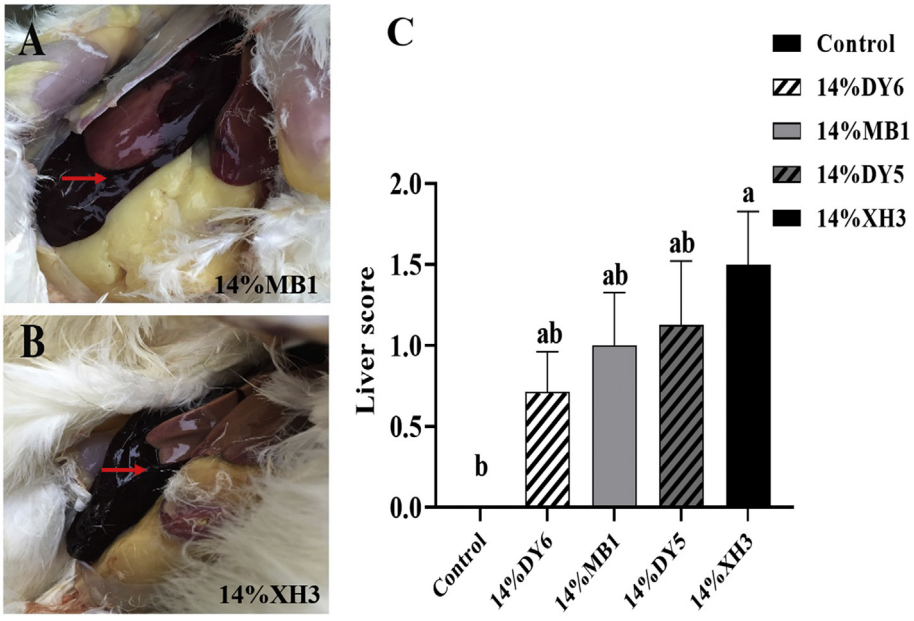

Fig. 1. Hen livers died from liver hemorrhage during week 1 to 8. (A) 14% MB1 rapeseed meal (RSM), (B) 14% XH3 RSM, (C) Liver score of fatty liver hemorrhagic syndrome. *P* contrast < 0.01, *P* ANOVA = 0.01. DY5 = Deyou No.5; DY6 = Deyou No.6; MB1 = Mianbangyou No.1; XH3 = Xiheyou No.3.

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 ($P = 0.76$, $P = 0.54$, and $P = 0.34$, 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 ($P < 0.01$, and $P < 0.01$, 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 ($P < 0.01$).

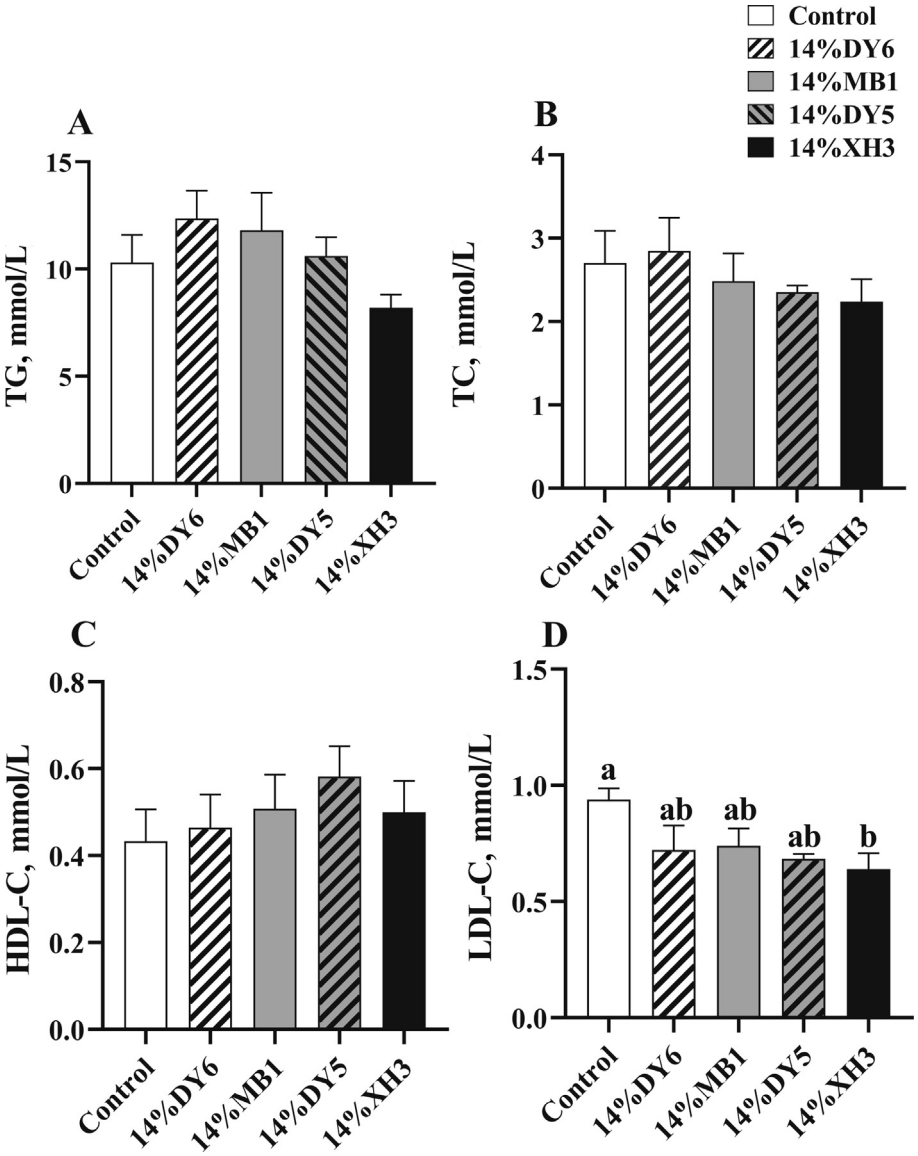

Fig. 2. The effects of rapeseed expeller meal on serum lipids profile at week 8 ($n = 8$). (A) P contrast = 0.76, P ANOVA = 0.17; (B) P contrast = 0.54, P ANOVA = 0.65; (C) P contrast = 0.34, P ANOVA = 0.69; (D) P contrast <0.01, P ANOVA = 0.04.

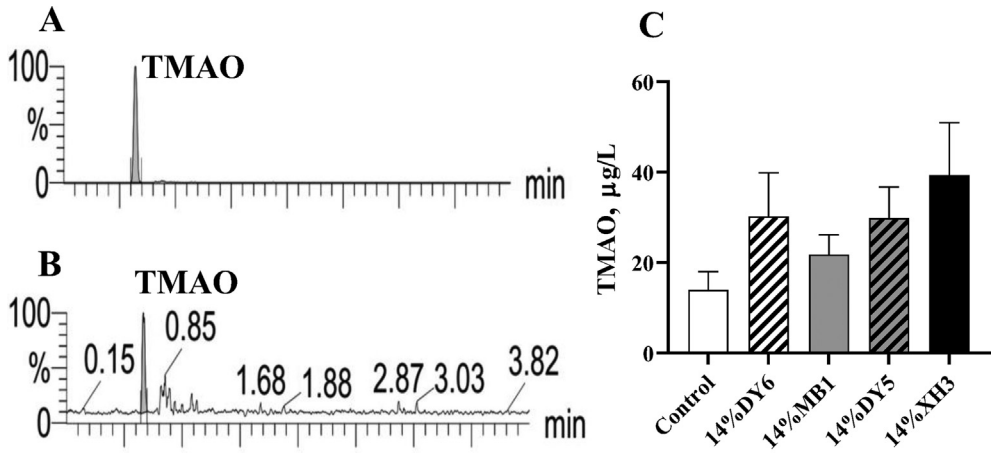

Fig. 3. The effects of rapeseed expeller meal on serum trimethylamine-N-oxide (TMAO) content of laying hens at week 8 ($n = 8$). (A) 133.97 ng/mL TMAO standard, (B) 2.354 ng/mL TMAO of serum sample, (C) Serum TMAO content comparison of control and rapeseed meal groups, P contrast = 0.08, P ANOVA = 0.25.

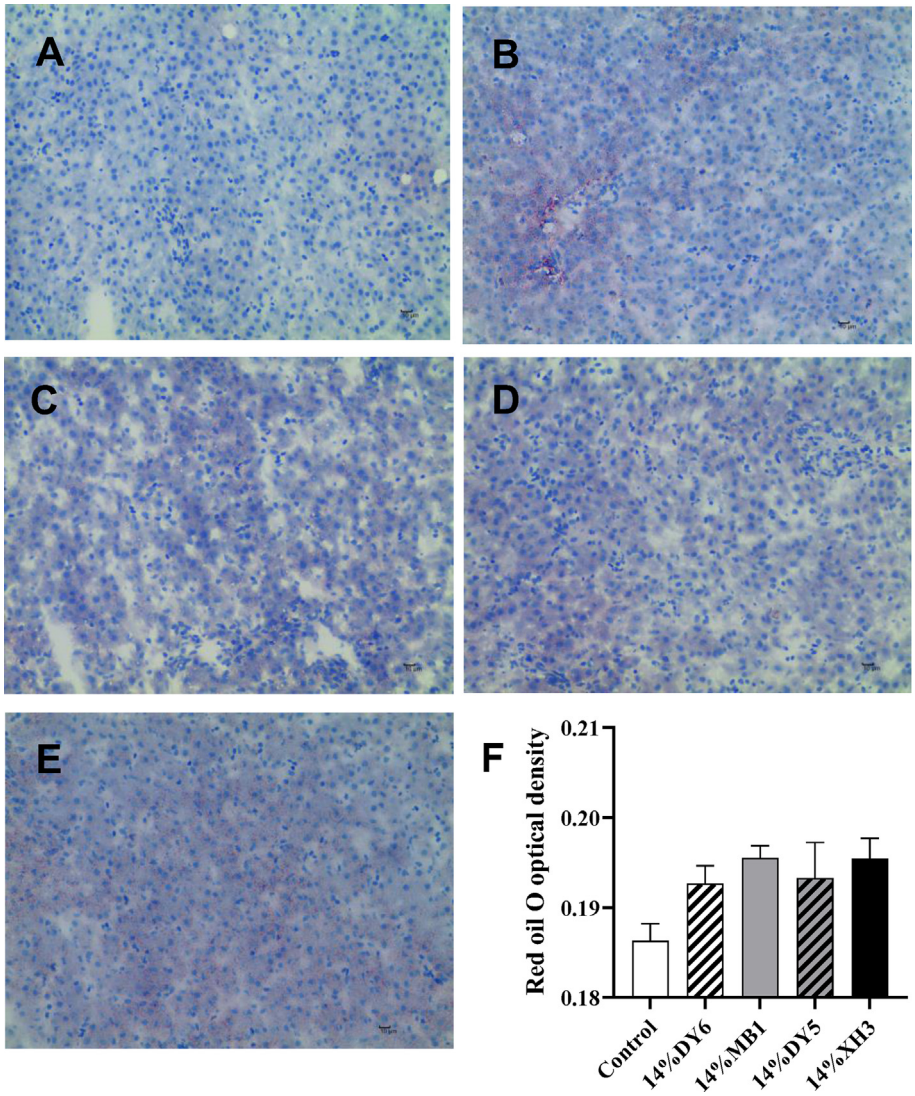

Fig. 4. Microscopic examination of oil red O stained liver from hens fed with control and 14% rapeseed meal (RSM) diets at week 8 ($n = 8$). (A) Control diet, (B) 14% DY6 RSM diet, (C) 14% MB1 RSM diet, (D) 14% DY5 RSM diet, (E) 14% XH3 RSM diet, (F) comparison of red oil O optical density of liver,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

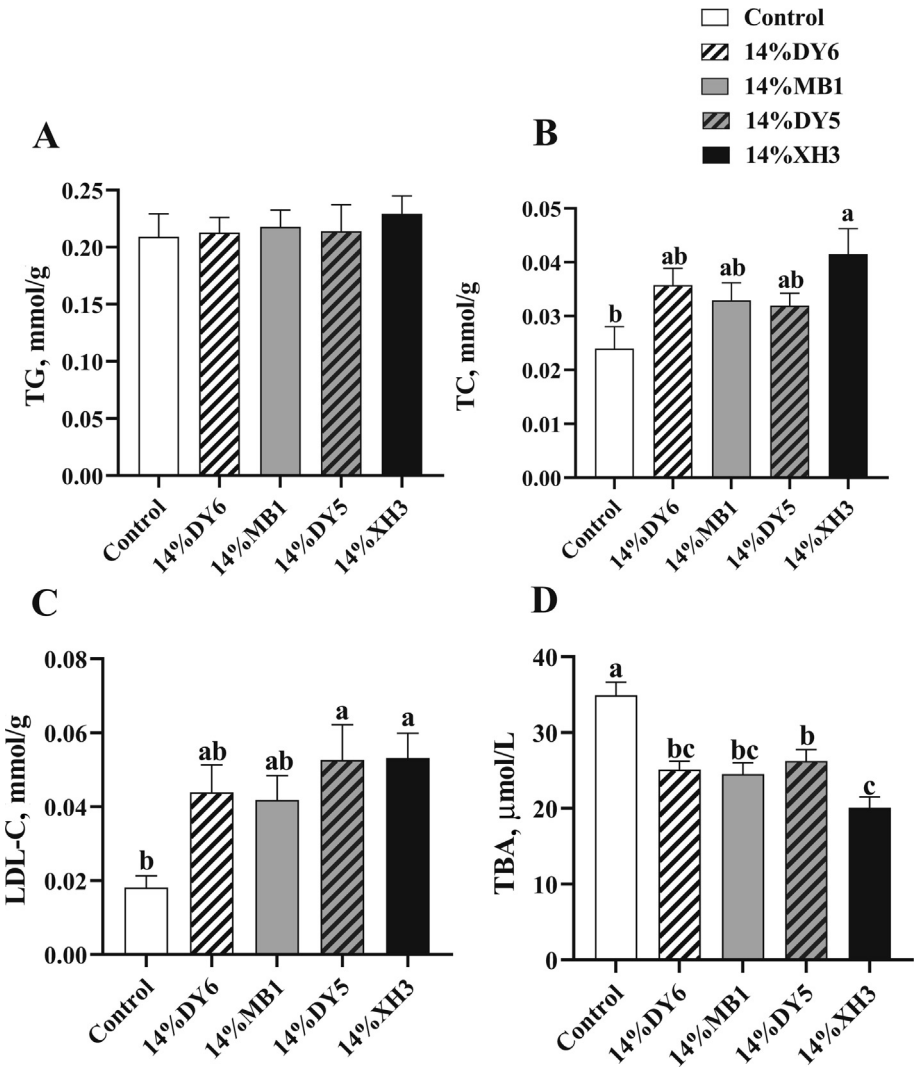

Fig. 5. Lipids content of livers from hens fed with control and 14% rapeseed meal diets at week 8 ($n = 8$). (A) Liver total triglyceride (TG) content (mmol/g): P contrast = 0.64, P ANOVA = 0.95. (B) Liver total cholesterol (TC) content (mmol/g): P contrast = 0.01, P ANOVA = 0.03. (C) Liver low density 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.

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 Chao1 analyses ($P = 0.01$), and decreased microbiota diversity revealed by the Observed-species ($P = 0.02$) 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 chyme 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, $P = 0.01$).

The LEfSe analysis revealed that there were 6 families where the relative abundance of cecum microbiota was higher in the control group than in the RSM group: Bacteroidaceae, Ruminococcaceae, Lachnospiraceae, Christensenellaceae, Peptococcaceae, and Marinibiaceae (Fig. 11A). At the genus level, there were 17 genera where the relative abundance of cecal microbiota was higher in the control

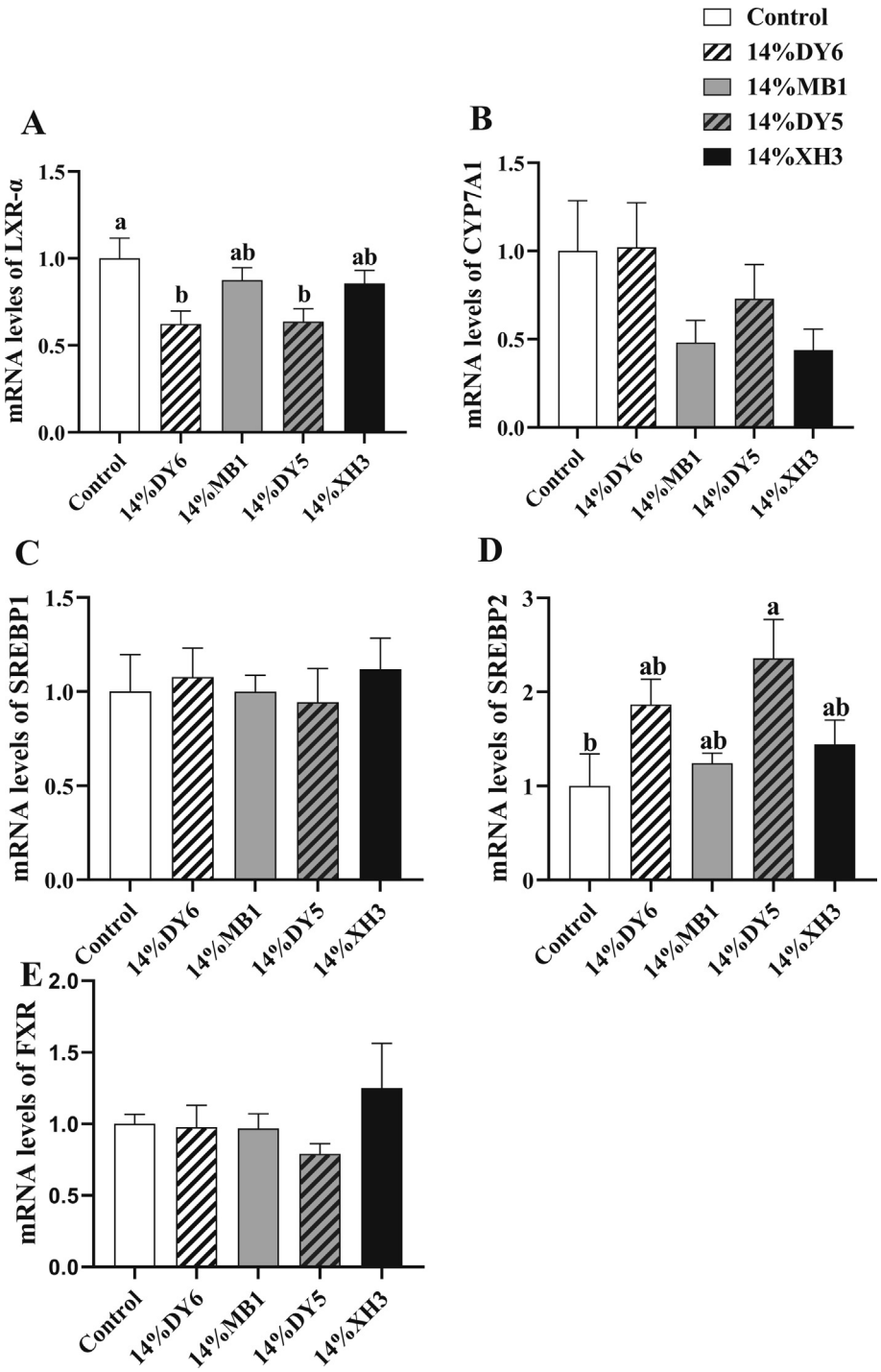

Fig. 6. Gene expression related to cholesterol metabolism in the liver from hens fed with control and rapeseed meal diets at week 8 ($n = 8$). (A) Liver mRNA levels of liver X receptor α (*LXR-α*): *P* contrast = 0.01, *P* ANOVA = 0.01. (B) Liver mRNA levels of cholesterol 7-alpha hydroxylase (*CYP7A1*): *P* contrast = 0.16, *P* ANOVA = 0.16. (C) Liver mRNA levels of sterol regulatory element binding protein 1 (*SREBP-1*): *P* contrast = 0.85, *P* ANOVA = 0.94. (D) Liver mRNA levels of *SREBP-2*: *P* contrast = 0.04, *P* ANOVA = 0.03. (E) Liver mRNA levels of farnesoid X receptor (*FXR*): *P* contrast = 0.99, *P* ANOVA = 0.45.

group compared to RSM: *Bacteroides, Christensenellaceae-R-7-group, Alistipes, Peptococcus, unidentified-Ruminococcaceae, Parabacteroides, Ruminococcaceae-UCG-005, Shuttleworthia, Mangroviflexus, Lachnospiraceae-AC2044-group, Anaerosporobacter, Pseudarthrobacter, Erysipelatoclostridium, Ruminococcaceae-NK4A214-group, Sellimonas, Coprococcus-1,* and *Oscilldspira*. 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 coprocola*, *Bacteroides vaginalis*, *Lactobacillus ingluviei*, *Collinsella aerofaciens*, *Clostridiales bacterium-77-5d*, *Pseudarthrobacter oxydans*, *Ruminococcaceae bacterium AM2*, *bacterium-ic1391*, *Bacteroides eggerthii*, *Ruminococcus flavefaciens*, and *iron reducing bacterium enrichment culture*. Birds fed the RSM diet showed a higher abundance of *Ruminococcaceae-UCG-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

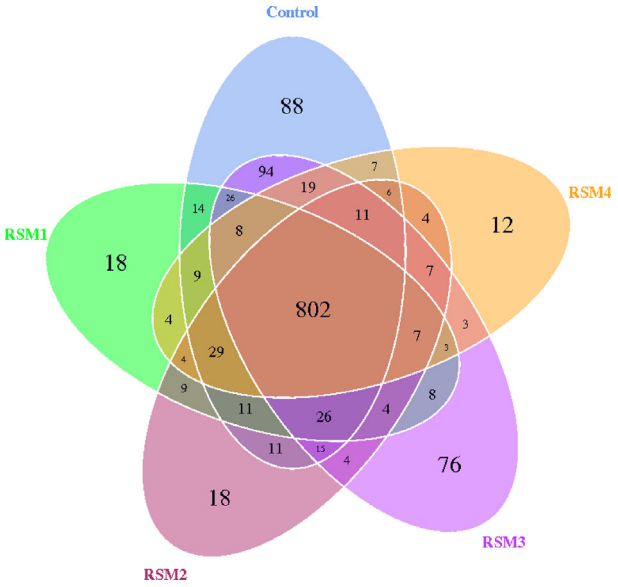

Fig. 7. Venn diagrams showing percentage of shared operational taxonomic units (OTU). RSM1: 14% DY6 RSM. RSM2: 14% MB1 RSM. RSM3: 14% DY5 RSM. RSM4: 14% XH3 RSM. DY5 = Deyou No.5; DY6 = Deyou No.6; MB1 = Mianbangyou No.1; XH3 = Xiheyou No.3; RSM = rapeseed meal.

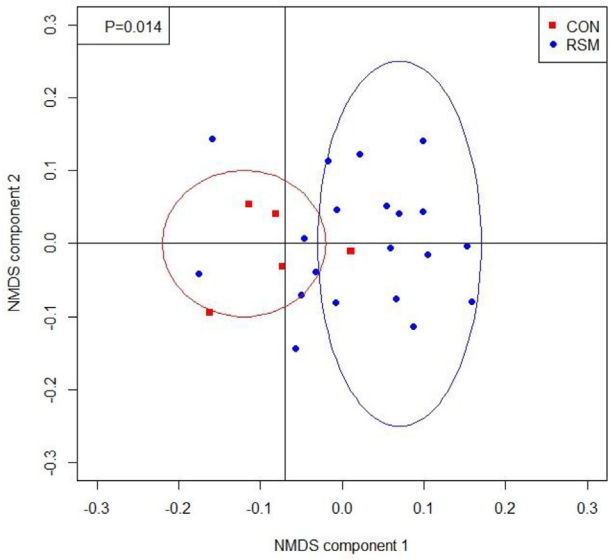

Fig. 8. The distinct clusters based on β diversity of microbiota from control and RSM group. CON: Control, RSM: 14% DY6, MB1, DY5, and XH3 RSM. RSM = rapeseed meal.

comparison was performed to determine the differences among the microbiota of the control and RSM groups (Fig. 11B: phylum and Fig. 11C: genus). At the genus level, RSM decreased the abundance of *Bacteroides* ($P = 0.03$).

Variation-partitioning analysis (VPA) was performed to identify individual and interactive contributions of GI, thiocyanate (SCN^-), sinapine, and EA to the variances of microbial community structure (Fig. 12). Dietary GI, SCN^-, and sinapine content, and EA content explained 33.16% and 7.84% of the total variance, and the interactions between the two environmental factors contributed to 59% of the total variance. At the phylum level (Table 5), the hepatic TC and LDL-C content and serum TMAO showed a negative relationship with Proteobacteria and Actinobacteria ($P < 0.01$, $P = 0.04$, $P = 0.04$).

4. Discussion

Mortality attributable to haemorrhagic liver was evident only among hens receiving the high GI 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 lipids 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 (Mosbach, 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). FMO_3 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). Cai 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

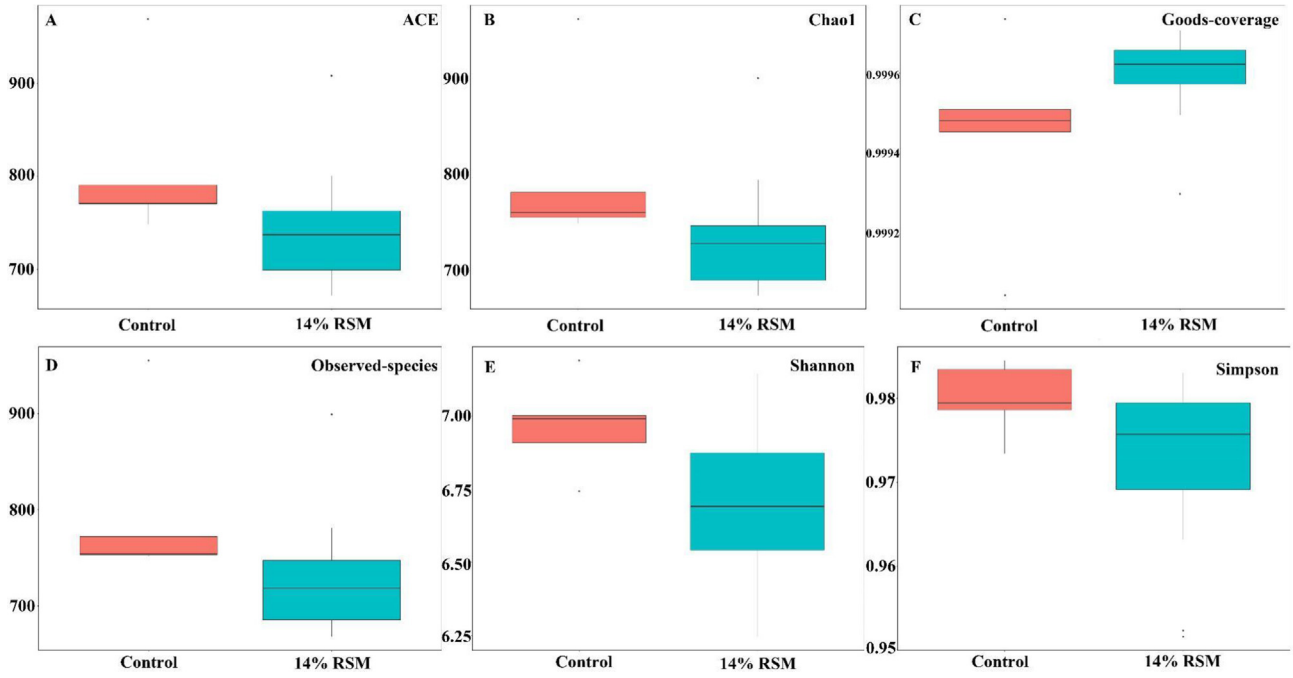

Fig. 9. Alpha diversity of cecum microbiota is significantly different between Control and 14% RSM diet. (A) ACE index, $P = 0.02$. (B) Chao1 index, P value = 0.01. (C) Goods-coverage, P value = 0.09. (D) Observed-species, P value = 0.02. (E) Shannon index, P value = 0.02. (F) Simpson index, P value = 0.07. RSM = rapeseed meal; DY5 = Deyou No.5; DY6 = Deyou No.6; MB1 = Mianbangyou No.1; XH3 = Xiheyou No.3.

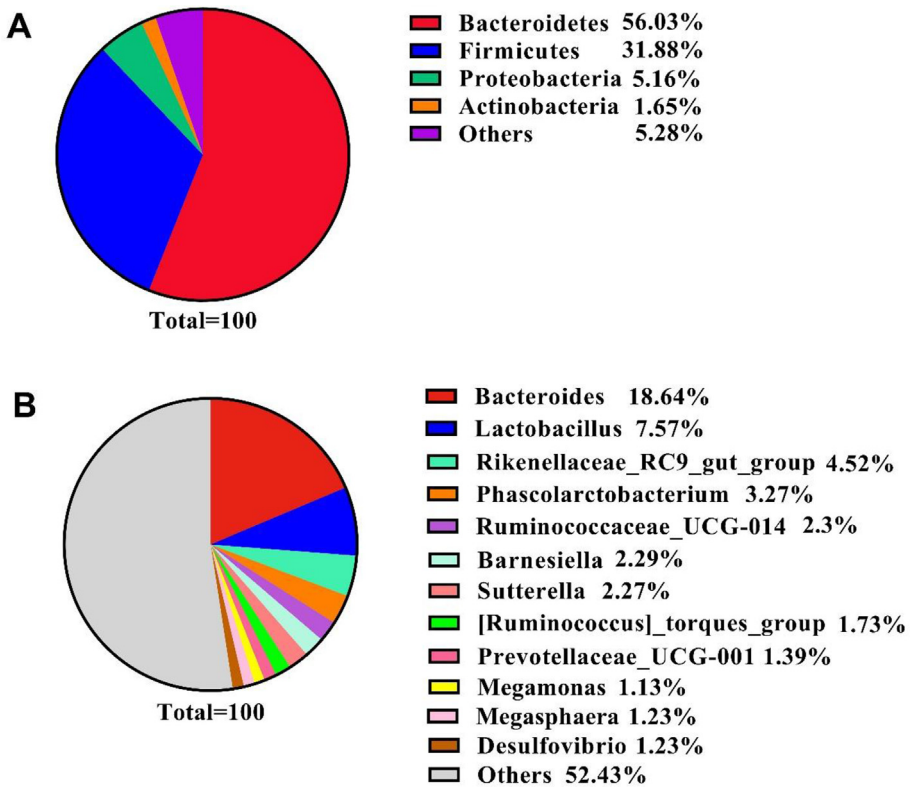

Fig. 10. Phylum and genus level assignments of the sequences in cecum microbiota, averaged across all samples. (A) Phylum level. (B) Genus level.

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

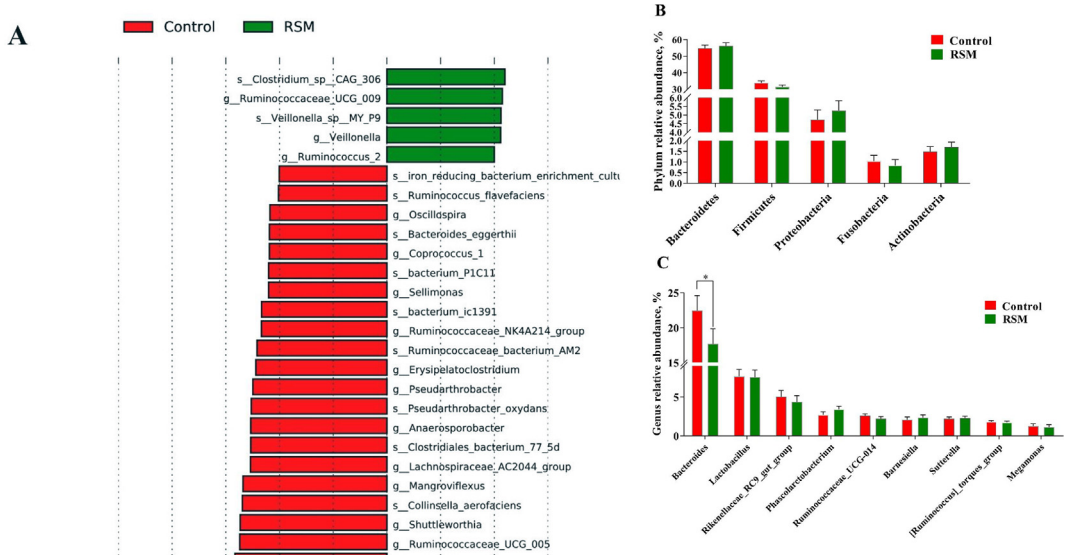

Fig. 11. Taxonomic differences of cecum microbiota between Control and 14% RSM groups. (A) RSM-enriched taxa are indicated with a positive LDA score (green), and taxa enriched in Control have a negative score (red). Only taxa meeting an LDA significant threshold > 2 are shown. (B) and (C) Comparison 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.

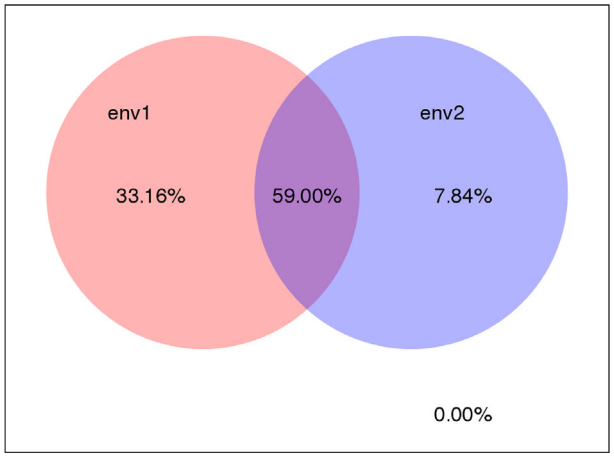

Fig. 12. Variation partitioning analysis (VPA) of the effects of antinutritional factors on bacterial community structure. env1: contain Gl, SCN, and sinapine; env2: contain EA. Gl = glucosinolates; SCN = thiocyanate; EA = erucic acid.

with a previous study which reported broccoli and carrot extracts with different levels and varieties of Gl inhibited phylum Firmicutes abundance in the vitro experiment (Reiner et al., 2011). EA had a slight contribution on microbiota composition changes in this

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

Item	Proteobacteria	Actinobacteria	Verrucomicrobia	Ignavibacteriae
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.

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

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 FMO_3 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 *LXR-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 **Qiufeng 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.

Acknowledgments

This project was fanatically supported by National Science and Technology Project (2014BAD13B04) and the Sichuan Provincial Science and Technology project (2018NZ20009, 2014NZ0043). The authors wish to thank Drs. Greg and Susan Fraley, Ms. Brooke Van Wyk, and MS. Amanda Bentley (Hope College, Holland, MI USA) for their critical comments on this manuscript.

References

Arika W, Nyamai D, Osano K, Ngugi M, Njagi E. Biochemical markers of in vivo hepatotoxicity. J Clin Toxicol 2016;6:297.
Boas EP, Parets AD, Adlesberg D. Hereditary disturbance of cholesterol metabolism; a factor in the genesis of atherosclerosis. Am Heart J 1948;35:611–22.
Butler EJ, Pearson AW, Fenwick GR. Problems which limit the use of rapeseed meal as a protein source in poultry diets. J Sci Food Agric 2010;33:866–75.
Cai C, Song L, Wang Y, Wu P, Ye Y, Zhang Z, Yang C. Assessment of the feasibility of including high levels of rapeseed meal and peanut meal in diets of juvenile crucian carp (Carassius auratus gibelio ♀× Cyprinus carpio ♂): growth, immunity, intestinal morphology, and microflora. Aquaculture 2013;410–411:203–15.
Campbell LD. Incidence of liver hemorrhage among White Leghorn strains fed on diets containing different types of rapeseed meals. Br Poultry Sci 1979;20:239–46.
Carroll KK. Studies on the mechanisms by which erucic acid affects cholesterol metabolism: distribution of erucic acid in adrenal and plasma lipids. Can J Physiol Biochem 1962;40(8):1115–22.
Chen ML, Yi L, Zhang Y, Zhou X, Ran L, Yang J, Zhu JD, Zhang QY, Mi MT. Resveratrol attenuates Trimethylamine-N-Oxide (TMAO)-induced atherosclerosis by regulating TMAO synthesis and bile acid metabolism via remodeling of the gut microbiota. mBio 2016a;7:e02210.
Chen ML, Yi L, Zhang Y, Zhou X, Ran L, Yang J, Zhu JD, Zhang QY, Mi MT. Resveratrol attenuates Trimethylamine-N-Oxide (TMAO)-induced atherosclerosis by regulating TMAO synthesis and bile acid metabolism via remodeling of the gut microbiota. mBio 2016b;7:e02210–2215.
Chiang JY. Bile Acid regulation of gene expression: roles of nuclear hormone receptors. Endocr Rev 2002;23:443–63.
Claesson MJ, Jeffery IB, Conde S, Power SE, O'Connor EM, Cusack S, Harris HM, Coakley M, Lakshminarayanan B, O'Sullivan O. Gut microbiota composition correlates with diet and health in the elderly. Nature 2012;488:178–84.
Ding L, Chang MR, Guo Y, Zhang LY, Xue CH, Yanagita T, Zhang TT, Wang YM. Trimethylamine-N-oxide (TMAO)-induced atherosclerosis is associated with bile acid metabolism. Lipids Health Dis 2018;17(1):286.
Donova MV. Transformation of steroids by *Actinobacteria*: a review. Prikl Biokhim Mikrobiol 2007;43(1):1–14.
Fennema D, Phillips IR, Shephard EA. Trimethylamine and trimethylamine N-oxide, a flavin-containing monooxygenase 3 (FMO3)-Mediated host-microbiome metabolic Axis implicated in health and disease. Drug Metab Dispos 2016;44(11):1839–50.
Fenwick GR, Pearson AW. Rapeseed meal tannins and egg taint. Anim Feed Sci Technol 1981;6:421–31.
Hargis PS. Modifying egg yolk cholesterol in the domestic fowl-a review. World Poultry Sci J 1988;44:17–29.
Hobsonà Frohock A, Fenwick GR, Heaney RK, Land DG, Curtis RF. Rapeseed meal and egg taint: association with sinapine. Br Poultry Sci 1977;18:539–41.
König B, Koch A, Spielmann J, Hilgenfeld C, Stangl GI, Eder K. Activation of PPARα lowers synthesis and concentration of cholesterol by reduction of nuclear *SREBP*-2. Biochem Pharmacol 2007;73:574–85.
Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, Britt EB, Fu X, Wu Y, Li L, Smith JD, DiDonato JA, Chen J, Li H, Wu GD, Lewis JD, Warrier M, Brown JM, Krauss RM, Tang WH, Bushman FD, Lusis AJ, Hazen SL. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. Nat Med 2013;19:576–85.
Lee JJ, Shin HD, Lee YM, Kim AR, Lee MY. Effect of broccoli sprouts on cholesterol-lowering and anti-obesity effects in rats fed high fat diet. J Korean Soc Food Sci Nutr 2009;38(3):309–18.
Li T, Kong X, Owsley E, Ellis E, Strom S, Chiang J, L Y. Insulin regulation of cholesterol 7α-Hydroxylase expression in human hepatocytes roles of forkhead box o1 and sterol regulatory element-binding protein 1c. J Biol Chem 2006;281:28745–54.
Lin SM, Mai KS, Tan BP, Liu WS. Effects of four vegetable protein supplementation on growth, digestive enzyme activities, and liver functions of juvenile tilapia, Oreochromis niloticus × Oreochromis aureus. J World Aquacult Soc 2010;41:583–93.
Liu J, Yan H, Cao S, Liu J, Li Z, Zhang H. The response of performance in grower and finisher pigs to diets formulated to different tryptophan to lysine ratios. Livest Sci 2019a;222:25–30.
Liu J, Zhang Y, Li Y, Yan H, Zhang H. L-tryptophan enhances intestinal integrity in diquat-challenged piglets associated with improvement of redox status and mitochondrial function. Animals 2019b;9:266.
Liu X, Wang Y, Hoeflinger JL, Neme BP, Jeffery EH, Miller MJ. Dietary broccoli alters rat cecal microbiota to improve glucoraphanin hydrolysis to bioactive isothiocyanates. Nutrients 2017;9:262.
Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif.) 2001;25:402–8.
Long C, Wang J, Zhang HJ, Wu SG, Qi GH. Effects of dietary rapeseed meal supplementation on cecal microbiota in laying hens with different flavin-containing monooxygenase 3 genotypes. Poultry Sci 2017;96.
Martland MF, Butler EJ, Fenwick GR. Rapeseed induced liver haemorrhage, reticulolysis and biochemical changes in laying hens: the effects of feeding high and low glucosinolate meals. Res Vet Sci 1984;36:298–309.
Ministry of Agriculture of the PRC. High erucic acid rapeseed (NY/T 1990–2011). Beijing, China: Agricultural Press of China; 2011.
Ministry of Health of the PRC. Rapeseed oil (GB/T 1536–2004). Beijing, China: Standards press of china; 2004.
Mosbach EH. Regulation of bile acid synthesis. 1974.
Murray CJ, Lopez AD. Alternative projections of mortality and disability by cause 1990-2020: global burden of disease study. Lancet (London, England) 1997;349:1498–504.
NRC. Nutrient requirements of poultry. 9th rev. Natl. Acad. Press; 1994.
Pearson AW, Butler EJ. The oestrogenised chick as an experimental model for fatty liver-haemorrhagic syndrome in the fowl. Res Vet Sci 1978;24:82–6.
Pearson AW, Butler EJ, Curtis RF, Fenwick GR, Hobson-Frohock A, Land DG, Hall SA. Effects of rapeseed meal on laying hens (Gallus domesticus) in relation to fatty liver-haemorrhagic syndrome and egg taint. Res Vet Sci 1978;25:307.
Reiner J, Duc Son L, Turnbaugh PJ, Cathy T, Clifton B, Gordon JI, Jonathan K. Energy-balance studies reveal associations between gut microbes, caloric load, and nutrient absorption in humans. Am J Clin Nutr 2011;94:58–65.
Romano KA, Vivas EI, Daniel AN, Rey FE. Intestinal microbiota composition modulates choline bioavailability from diet and accumulation of the proatherogenic metabolite trimethylamine-N-oxide. mBio 2015;6:e02481.
Savary RK, Macisaac JL, Rathgeber BM, Mclean NL, Anderson DM. Evaluating Brassica napus and Brassica juncea meals with supplemental enzymes for use in laying hen diets: production performance and egg quality factors. Can J Anim Sci 2017;97.
Schugar RC, Shih DM, Warrier M, Helsley RN, Burrows A, Ferguson D, Brown AL, Gromovsky AD, Heine M, Chatterjee A. The TMAO-producing enzyme flavin-containing monooxygenase 3 regulates obesity and the beiging of white adipose tissue. Cell Rep 2017;19:2451–61.

Siperstein MD, Murray AW. Cholesterol metabolism in man. West J Med 1955;128:13–25.
Tang WHW, Zeneng W, Levison BS, Koeth RA, Britt EB, Xiaoming F, Yuping W, Hazen SL. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. N Engl J Med 2013;368:1575–84.
Tripathi MK, Mishra AS. Glucosinolates in animal nutrition: a review. Anim Feed Sci 2007;132:1–27. Technol.
Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002;3. Research0034.
Wei S, Morrison M, Yu Z. Bacterial census of poultry intestinal microbiome. Poultry Sci 2013;92:671–83.
Wang J, Long C, Zhang HJ, Zhang YN, Wang H, Yue HY, Wang XC, Wu SG, Qi GH. Genetic variant in flavin-containing monooxygenase 3 alters lipid metabolism in laying hens in a diet-specific manner. Int J Biol Sci 2016;12(11):1382–93.
Wieland A, Frank DN, Harnke B, Bambha K. Systematic review: microbial dysbiosis and nonalcoholic fatty liver disease. Aliment Pharmacol Ther 2015;42:1051–63.
Yan H, Zhang L, Guo Z, Zhang H, Liu J. Production phase affects the bioaerosol microbial composition and functional potential in swine confinement buildings. Animals 2019;9:90.
Yan H, Zhou P, Zhang Y, Zhang Z, Liu J, Zhang H. Short-chain fructo-oligosaccharides alleviates oxidized oil-induced intestinal dysfunction in piglets associated with the modulation of gut microbiota. J Funct Foods 2020;64:103661.
Zeneng W, Elizabeth K, Bennett BJ, Robert K, Levison BS, Brandon D, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature 2011;472(7341):57–63.
Zhang L, Xie C, Nichols RG, Chan SH, Jiang C, Hao R, et al. Farnesoid X receptor signaling shapes the gut microbiota and controls hepatic lipid metabolism. mSystems 2016;1(5):e00070-16.
Zhu L, Baker SS, Gill C, Liu W, Alkhouri R, Baker RD, Gill SR. Characterization of gut microbiomes in nonalcoholic steatohepatitis (NASH) patients: a connection between endogenous alcohol and NASH. Hepatology 2013;57(2):601–9.