Supplementing cholamine to diet lowers laying rate by promoting liver fat deposition and altering intestinal microflora in laying hens

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ABSTRACT The effects of cholamine, a raw material for synthesis of some active lipids, are unknown in poultry. To address this, 180 52-wk-old Hyline laying hens were randomly divided into 3 groups (20 replicates per group with three hens per replicate). The control group and the treatment groups (treatment 1 and 2) were fed basal diet and the diet supplemented with 500 or 1,000 mg of cholamine per kilogram of the diet for 35 d, respectively. The data showed that supplementary cholamine significantly lowered egg production, daily feed intake, serum high-density lipoprotein cholesterol level, liver index, and the percentages of C15:0 and C20:0 in fatty acid composition of liver, significantly elevated hepatic triglyceride content, the ratio of villus height to crypt depth ($P < 0.05$), and the percentage of C18:2n−6 and the ratio of n−6 to n−3 polyunsaturated fatty acids in liver fat ($P < 0.10$). Moreover, supplementary cholamine altered the relative abundance of some intestinal bacteria with a decrease in the alpha biodiversity ($P < 0.10$). Additionally, transcriptome analysis on the livers of the treatment vs. the control groups identified 1,151 up- and 914 down-regulated differentially expressed genes (**DEGs**), and pathway analysis revealed that the suppressed Notch signaling pathway and the enhanced Oxidative phosphorylation pathway were enriched with DEGs. Particularly, fat absorption, transport and oxidative phosphorylation-related DEGs (e.g., *FABP1, APOA4*, and *PCK1*) were significantly induced, but fatty acid synthesis, and lipid package and secretion-related DEGs (e.g., *FASN, SCD*, and *MTTP*) were not. In conclusion, supplementary cholamine may lower egg production by promoting hepatic lipid deposition and reducing abundances of beneficial intestinal bacteria and microfloral biodiversity in laying hens.

Key words: cholamine, egg production, intestinal morphology, lipid metabolism, microbiota

2022 Poultry Science 101:102084
https://doi.org/10.1016/j.psj.2022.102084

INTRODUCTION

Cholamine, also known as ethanolamine, is generated by the decarboxylation of serine and usually appears as the head group of phosphatidyl ethanolamine (**PE**). PE, as the second most abundant phospholipid in mammalian cells (Patel and Witt, 2017), is involved in a variety of cellular activities and biological processes, including protein synthesis and activity, oxidative phosphorylation, autophagy, membrane fusion, mitochondrial stability, and synthesis of other lipids (Fullerton et al., 2009). Moreover, cholamine can be converted into choline or phosphatidyl choline through PE (Yartseva et al., 2014), and also can participate in sphingolipid metabolism through synthesis of CDP-choline. Sphingolipids, mainly including ceramide, ceramide-1-phosphate, glucosylceramide, sphingomyelin, sphingosine and sphingosine-1-phosphate (Bryan et al., 2016), are biologically active lipids that widely exist in plants and animals (Hannun and Obeid, 2008). As constituents of cell membrane and active lipids, sphingolipids are involved in many cellular activities, including cell growth, cell cycle, cell death, cell senescence, inflammation, immune response, cell adhesion and migration, angiogenesis, nutrient uptake, metabolism, stress response, and autophagy. The disorders of sphingolipid metabolism are closely associated with human diseases such as diabetes, cardiovascular diseases, and fatty liver (Hannun and Obeid, 2018). Through these derivatives, cholamine may play an import role in many biological activities. For example, cholamine can regulate the mTOR signaling pathway and mitochondrial function, thereby affecting the proliferation of intestinal epithelial cells (Yang et al., 2016b).

Cholamine also can regulate the composition of intestinal microbiota, thus affecting the growth and development, productive performance and health of animals (Garsin, 2010; Yang et al., 2016a; Zhou et al., 2018). For example, cholamine can increase the relative abundance of *Bacterioides* in the colon of weaning rats while reduce

Received January 21, 2022.
Accepted July 19, 2022.

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the relative abundances of *Proteobacteria* and *Cyanobacteria* (Zhou et al., 2018). By directly regulating the composition of intestinal microbiota, cholamine may affect digestion and absorption of nutrients (Wang and Gu, 2010; Ciorba, 2012; Neveling and Dicks, 2021), synthesis of bioactive substances (LeBlanc et al., 2013; Khan and Chousalkar, 2020), and maintenance of intestinal health (Peng et al., 2009; Bischoff et al., 2014) in animals. In addition, cholamine also may affect intestinal structure and immune functions via some metabolites produced by intestinal bacteria have the effect of enhancing, thus contributing to intestinal resistance to pathogens and maintenance of intestinal health. For example, short-chain fatty acids produced by intestinal bacteria can promote the expression of tight junction proteins and enhance structural integrity of intestinal mucosa (Walugembe et al., 2015; Borrelli et al., 2017).

Animals do not synthesize cholamine on their own, but both blood and milk contain high level of cholamine. Studies indicate that the concentrations of cholamine in human blood and milk are 2 μM and 46 μM, respectively (Patel and Witt, 2017). Cholamine is mainly of dietary origin, its level vary from feed to feed (Gibellini and Smith, 2010; Yunus et al., 2016) and can be changed by adjusting feed formulation. Intestinal bacteria play an essential role in the metabolism and transformation of cholamine. Although cholamine may affect animal physiology and performance through several pathways, there are few studies on the effects of cholamine addition to feed on animal physiology and production performance. It has been reported that adding cholamine to feed can increase the weights of stomach and small intestine, the length of small intestine, and the ratio of small intestine to body weights, and significantly increase average daily gain (**ADG**) and average daily feed intake (**ADFI**) in weaned piglets (Yang et al., 2016a). To date, no relevant studies however are reported in poultry.

Considering the role of PE and sphingolipids, the derivatives of cholamine in cell growth and death, inflammation and immune responses, and lipid metabolism, and the effects of cholamine on intestinal microbiota and health, we hypothesize that cholamine may influence lipid metabolism in the liver, the composition of intestinal microbiota, the structure and health of intestine, and laying rate of hens. To address the influence, this study determined the effects of supplementary cholamine on tissue growth, blood biochemistry, production performance, uterine and intestinal physiology, the composition of intestinal microbiota, and liver transcriptome in laying hens. The results may provide new ideas for improving laying performance of hens and lay a foundation for revealing the biological role of cholamine and related mechanism.

MATERIALS AND METHODS

The animal protocol was complied with the animal welfare and use guidelines, and was approved by the Animal Welfare and Use Committee of Yangzhou University with permission number SYXK(Su)2016-0020.

Experimental Animals

The experiment was conducted in Jurong Haoyuan Co., Ltd (Zhenjiang, Jiangsu, China). One hundred and eighty 52-wk-old Hyline brown egg-producing hens were selected and randomly divided into 3 groups with 20 replicates each group and 3 hens each replicate. The control group was fed the basal diet, and the treatment groups were fed the basal diet supplemented with 500 and 1,000 mg/kg of low and high levels of cholamine, respectively. The composition and nutritional level of the basal diet are shown in Table 1. Cholamine was purchased from Merck KGaA (Cat. No. E9508-500ML, Darmstadt, Germany) with a purity of ≥98%. To make the treatment diets, certain amount of cholamine was first mixed with about 1 Kg of basal diet, followed by mixing the initial mixture with a large amount of basal diet to make sure the supplemented cholamine was evenly distributed in the diet. The experimental period was 35 d. The birds were raised in cages with free access to feed and water under a lighting programme of 16 h light: 8 h dark. The routine management was carried out. Eggs were collected daily at 14:00.

Data Collection of Animal Production Performance

The number of eggs, the total weight of eggs and feed intake were recorded daily for each replicate during the experimental period. Average daily feed intake, egg production rate, average egg weight, and feed-to-egg ratio were calculated.

Determination of Egg Quality

On the last day of the experiment, 20 eggs (one per replicate) were randomly selected from each group. The

Table 1. The ingredient composition and the nutritonal level of basal diet used in this study (air-dried basis).

Ingredients	Content (%)
Corn	60.00
Soybean meal	24.00
Soybean oil	3.00
Stone powder	8.00
Premix	5.00
Total	100.00
Nutritional level	
ME (MJ/kg)	11.44
Crude protein	16.00
Methionine	0.26
Lysine	0.80
Tryptophane	0.18
Calcium	3.45
Arginine	1.04
Total phosphorus	0.56
Available phosphorus	0.46

Note: the premix per kilogram of feed contains vitamin A 9750 IU, vitamin D_1 3750 IU, vitamin E 27.5 IU, vitamin K_3 3 mg, vitamin B_1 2.2 mg, vitamin B_3 36.75 mg, vitamin B_6 3.75 mg,vitamin B_{12} 0.022 mg, nicotinamide 44 mg, pantothenic acid 16.5 mg, folic acid 1.5 mg, biotin 0.33 mg, choline 325 mg, Zn 85 mg, Fe 80 mg, Mn 120 mg, Cu 12.5 mg, I 1.1 mg, and Se 0.3 mg.

indices of egg quality, including egg weight, egg shape index, shell strength, shell thickness, shell color, albumen height, Haugh units and yolk color, were the determined. Eggshell strength was determined using an Egg Force Reader apparatus (Orka Technology Ltd., Ramat Hasharon, Israel), eggshell thickness was determined using an Egg Force Shell Thickness apparatus (Orka Technology Ltd., Ramat Hasharon, Israel). Egg shape index was determined using a vernier calliper (Minette, 0−200 mm), and yolk color, albumen height and Haugh unit were determined using a SONOVA Egg Analyzer (Orka Technology Ltd., Ramat Hasharon, Israel).

Collection of Blood and Tissue Samples

On the day before the end of the experiment (i.e., the 34th day), 10 hens per group (one per replicate at most) were randomly selected from the control group and the 500 mg/Kg and 1,000 mg/Kg cholamine groups, fasted overnight, and weighed. Blood was then collected from wing vein of the birds, followed by plasma separation and storage at −20°C. After sacrificing these birds by cervical dislocation, the weights of liver, spleen, intestine and abdominal fat was measured. For histomorphological analysis, the middle part of ileum (about 0.5 cm) and uterus (about 2 cm) were collected, followed by rinsing with saline and fixing in 10% formalin. The middle part of the liver was taken for triglyceride content determination and gene expression analysis. The liver samples were stored at −70°C. The ileum and cecum content samples were collected for 16S rDNA analysis of intestinal microflora, which was performed by Personal Biotechnology, Shanghai, China. Blood biochemistry was analyzed using an automated biochemistry instrument (3100 Automatic Analyzer, Tokyo, Japan).

Determination of Triglyceride Content

The triglyceride content in liver samples were determined using a triglyceride assay kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instruction. For sample preparation, a small amount of tissue samples (about 0.1 g) were accurately weighed, homogenized in an ice-water bath with ten times volume of saline by weight, centrifuged at 2,500 r/min for 10 min, collected and diluted the supernatant with 10 times volume of saline before taking 50 μL of the diluted supernatant for determination of triglyceride content.

Determination of Liver Antioxidant Capacity

The antioxidant capacity of liver was determined using the superoxide dismutase (**SOD**) and malondialdehyde (**MDA**) assay kits (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instruction. For sample preparation, a small amount (about 1 g) of tissue samples were accurately weighed, which was followed by adding precooled saline in the ratio of weight (g) to volume (mL) = 1:9, homogenizing in an ice-water bath, and centrifuging at 3,500 r/min for 10 min at 4°C. The supernatant was collected and used for the assays.

Analysis of Fatty Acid Composition

Five liver samples per group were randomly selected for fatty acid composition analysis. According to China national standardized method (GB/T 5413.27-2010), liver lipids were extracted using acetyl chloride-methanol methylation method. About 2 g of liver tissue was accurately weighed, minced, mixed with sea sand, dried in oven at 100°C for 30 min, ground into powder, and put into Soxhlet extractor, followed by adding petroleum ether to extract lipids from liver sample. After drying, 5 mL toluene and 6 mL methanol solution containing 10% (v/v) acetyl chloride were added, and the vessel was filled with nitrogen before closing. The solution was mixed by shaking and incubated in 80°C ± 1 water bath for 2 h of derivatization reaction with shaking once every 20 min. After cooling to room temperature, the reaction solution was transferred to a 50 mL centrifuge tube and centrifuged at 5000 r/min for 5 min. The supernatant was collected and used for fatty acid composition analysis by gas chromatography. The chromatographic column was DB-23 (60 m × 0.25 mm × 0.15 μm), the vaporization chamber was programmed to ramp up from the starting temperature of 50°C to 250°C at a rate of 15°C/min, and maintained for 15 min, the detector was a hydrogen flame ionization detector and set at 300°C, the carrier gas was nitrogen with 186 KPa of pressure, the injection volume was 1 μL, and the inlet port temperature was 280°C. By comparing with the standards (Sigma Chemical Co., St. Louis, MO) based on residence time and peak area, different fatty acids in liver sample were determined qualitatively and quantitatively.

Histomorphometric Analysis

Histomorphometric analysis of the intestine and uterus samples was performed as described previously (Khogali et al., 2021).

16S rDNA Sequencing Analysis of Intestinal Microflora

The ileal and cecal contents of 5 birds per group were randomly selected for microbial composition analysis. DNA was extracted from ileal and cecal content samples using Tiangen DNA Extraction Kit (Tiangen Biotechnology, Beijing, China). The DNA concentration and purity were determined using a NanoDrop instrument (Thermo Fisher Scientific, Waltham, MA), followed by assessing the integrity of DNA samples by 1% agarose gel electrophoresis. Only the qualified samples were subjected to amplification of the 16S rDNA V3-V4 region with the primers: 5′-ACTCCTACGGGAGGCAGCA-3′

and 5′-CGGACTACHVGGGTWTCTAAT-3′. Thermal cycling was composed of initial denaturation at 98°C for 2 min, followed by 25 cycles of denaturation at 98°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, with a final extension of 5 min at 72°C. The sequencing adapters were attached to the ends of the primers. The PCR products were then purified, quantified and homogenized to build a sequencing library, which was subjected to quality control check. Paired-end sequencing of the DNA fragments from the library was performed using a Illumina Hiseq sequencing platform. The sequence quality of the raw data was checked, and re-sequencing was performed for the samples with low sequencing quality. The raw reads that passed the quality screening were partitioned into different libraries and samples according to index and barcode information, and barcode sequences were then removed. Using QIIME2 Dada2 and Vsearch software, sequencing noise was removed, and operational taxonomic unit (**OTU**) clustering analysis (based on 97% sequence similarity) was performed. Alpha diversity metrics (Chao1, Observed species, Shannon, Simpson, Faith's PD, Pielou's evenness) and beta diversity metrics (Bray-Curtis dissimilarity) of intestinal microflora was subsequently were estimated and plotted using QIIME2 (2019.4), R language and ggplot2 package. The composition and distribution of intestinal bacteria was compared for each experimental group using QIIME2 (2019.4). The sequencing analysis was conducted by Personal Biotechnology, Shanghai, China.

Transcriptome Sequencing Analysis

The group of laying hens fed the basal diet was the control group, and the group of laying hens fed the basal diet supplemented with high concentration of cholamine was the treatment group. Liver samples from these 2 groups were transferred on dry ice to Novogene Technology Co., Ltd. (Beijing, China). Total RNA was extracted from liver tissue samples using TRIzol reagent, and RNA integrity was checked by Agilent 2100 bioanalyzer. Using oligo(dT) magnetic beads, mRNA with polyA was enriched, and the first-strand cDNA was synthesized in M-MuLV reverse transcription system using fragmented mRNA as a template and random oligonucleotides as primers, which was followed by degradation of the RNA strands with RNase H. The second-strand cDNA was then synthesized using DNA polymerase I system and dNTPs. The purified double-stranded cDNA was end-repaired, A-tailed and connected with sequencing adapters. The cDNA fragments with the length of about 250 to 300 bp were screened out with AMPure XP beads, which was followed by PCR amplification. The PCR products were purified once again using AMPure XP beads to construct cDNA libraries. The libraries were constructed using the NEBNext Ultra RNA Library Prep Kit for Illumina. The constructed libraries were initially quantified using a Qubit 2.0 Fluorometer and diluted to 1.5 ng/μL. The inserts in the libraries were checked for their sizes using an Agilent 2100 Bioanalyzer. When the insert sizes met the expectation, the effective concentrations of the libraries (which should be higher than 2 nM) were accurately determined by qRT-PCR to ensure the quality of the constructed libraries. After passing the quality control check, the libraries were sequenced using Illumina sequencing platform.

The sequencing images of the DNA fragments were converted into sequence data (reads) using CASAVA base calling software. The raw reads were then filtered to remove adapters and low-quality reads (Qphred $\leq$ 20), etc. The sequencing error rate and GC content distribution were then checked, which was followed by calculation of the Q20 and Q30 values and GC content of the clean reads. The clean reads were annotated by mapping to the reference genome using HISAT2 software. The reads for each gene were counted through the FeatureCounts program, and the differentially expressed genes (DEGs) were then identified using DESeq software with the following criteria: $|\log_2^{\text{Fold change}}| > 0$ and adjusted $P < 0.05$. Finally, the DEGs were used for Gene Ontology (**GO**) and KEGG pathway enrichment analyses by ClusterProfiler software.

Quantitative PCR Analysis

Total RNA purification, cDNA synthesis and quantitative PCR were performed according to the methods described previously (Osman et al., 2016). Ten DEGs were randomly selected to validate the transcriptome sequencing analysis. The primer sequence information is shown in Supplementary Table 1. The specificity of the primers was confirmed by melting curves. The internal reference gene was *GAPDH* gene. The relative expression of the target genes was calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

All data are expressed as the mean ± SEM. One-way analysis of variance (ANOVA) in SPSS software (version 19.0) was used to evaluate the statistical significance of the differences among multiple groups, followed by pairwise comparisons using Tukey's post-hoc test. For the 2-group comparisons in the microbial analysis, the statistical significance between the control and the 500 mg/Kg cholamine group was determined by *t*-test. The significance level was set at $P < 0.05$.

RESULTS

Effect of Supplemented Cholamine on Production Performance and Egg Quality of Laying Hens

Compared to the control group, supplementing 500 mg/kg of cholamine to the basal diet led to significant reduction of average daily egg production rate ($P <$

Table 2. Effects of dietary cholamine on production performance of laying hens.

					P-value	
	0 mg/kg	500 mg/kg	1,000 mg/kg	SEM	Cholamine	Linear
Average egg weight, g	62.59	63.17	62.54	0.193	0.277	0.781
Average daily egg rate, %	82.49[a]	75.62[b]	79.46[ab]	0.711	<0.001	0.065
ADFI, g	96.97[a]	89.17[b]	90.54[b]	1.059	0.005	0.011
F/E	1.93	2.00	1.97	0.022	0.466	0.253
Body weight, Kg	2.01	2.02	1.93	0.026	0.301	0.207

Note: [a,b]Mean values with different superscript letters in the same row are significantly different ($P < 0.05$). Data are presented as the means ± SEM. n = 20.

ADFI, average daily feed intake; F/E, the ratio of feed weight to egg weight.

0.001), and supplementing 500 or 1,000 mg/kg of cholamine led to significant reduction of average daily feed intake ($P < 0.01$) (Table 2). There was no significant effect of supplementing cholamine on average egg weight, the feed-to-egg ratio, eggshell color, egg shape index, egg weight, albumen height, yolk color, Haugh units, yolk weight, eggshell strength, and eggshell thickness (Supplementary Table 2).

Effect of Supplemented Cholamine on Blood Biochemistry

Compared to the control group, supplementing 500 and 1,000 mg/kg of cholamine to the basal diet significantly lowered high-density lipoprotein cholesterol (**HDL-C**) level ($P < 0.05$), supplementing 1,000 mg/kg of cholamine lowered blood glucose level in a tendency ($P = 0.05$), but had no significant effect on other indexes, including alanine aminotransferase (**ALT**) activity, aspartate transaminase (**AST**) activity,the ratio of aspartate aminotransferase to the activity of alanine aminotransferase (**AST/ALT**), Total protein (**TP**), Albumin, Globulin, the ratio of albumin level to globulin level (**ALB/GLB**), total cholesterol (**TCH**), Triacylglycerol and low density lipoprotein-cholesterol (**LDL-C**) (Table 3).

Effect of Supplemented Cholamine on Organ Weight and Abdominal Fat Weight

Compared to the control group, supplementing 500 mg/kg of cholamine to the basal diet significantly reduced the absolute weight of the liver and the relative weight to body weight ($P < 0.05$), but supplementing 500 or 1,000 mg/kg of cholamine had no significant effect on the absolute or relative weights of the spleen, jejunum, and abdominal fat to body weight (Table 4).

Effect of Supplemented Cholamine on Antioxidant Properties, Triglyceride Content, and Fatty Acid Composition in the Liver

Compared to the control group, supplementing 500 mg/kg or 1,000 mg/kg of cholamine to the basal diet had no significant effect on to total SOD (**T-SOD**) activity and MDA content in the liver (Table 4). Supplementing 1,000 mg/kg of cholamine significantly increased triglyceride content in the liver of laying hens ($P < 0.05$) (Table 4). Supplementing 1,000 mg/kg of cholamine significantly reduced the relative content of C15:0 and C20:0 in liver fat, but tended to increase the relative content of C18:2n−6 and the ratio of n−6 polyunsaturated fatty acids (PUFA) to n−3 PUFA ($P < 0.10$) (Table 5).

Table 3. Effect of dietary cholamine on blood biochemistry of laying hens.

					P-value	
	0 mg/kg	500 mg/kg	1,000 mg/kg	SEM	Cholamine	Linear
ALT, (U/L)	7.75	8.30	9.10	0.428	0.451	0.216
AST, (U/L)	212.36	220.78	224.40	8.036	0.839	0.562
AST/ALT	27.82	26.79	26.29	1.010	0.836	0.557
Total protein, (g/L)	50.01	53.84	54.71	1.834	0.573	0.317
Albumin, (g/L)	15.36	16.82	16.98	0.486	0.341	0.177
Globulin, (g/L)	34.69	37.02	37.73	1.434	0.692	0.409
ALB/GLB	0.45	0.46	0.46	0.010	0.798	0.543
Glucose, (mmol/L)	11.15	10.52	10.34	0.141	0.050	0.019
TCH, (mmol/L)	3.28	2.46	3.32	0.196	0.130	0.930
Triacylglycerol, (mmol/L)	9.07	10.85	10.85	0.858	0.655	0.417
HDL-C, (mmol/L)	0.37[a]	0.27[b]	0.28[b]	0.015	0.005	0.005
LDL-C, (U/L)	1.28	1.17	1.31	0.107	0.856	0.889

Note: [a,b]Mean values with different superscript letters in the same row are significantly different ($P < 0.05$). Data are presented as the means ± SEM. n = 10.

ALT, the activity of alanine aminotransferase; AST, the activity of aspartate aminotransferase; AST/ALT, the ratio of aspartate aminotransferase to the activity of alanine aminotransferase; ALB/GLB, the ratio of albumin level to globulin level; TCH, total cholesterol; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol.

Table 4. Effects of dietary cholamine on organ weight, abdominal fat weight, and the antioxidant capacity and the triacylglyceride content in the liver of laying hens.

					P-value	
	0 mg/kg	500 mg/kg	1,000 mg/kg	SEM	Cholamine	Linear
Absolute weight						
Spleen, g	1.77	2.17	1.78	0.142	0.446	0.278
Liver, g	31.30[a]	26.28[b]	27.62[ab]	0.817	0.028	0.467
Jejunum, g	14.67	15.27	16.66	0.608	0.405	0.360
Abdominal fat, g	60.62	75.86	69.47	5.499	0.541	0.643
Relative weight						
Spleen, %	0.09	0.11	0.09	0.007	0.553	0.407
Liver, %	1.56[a]	1.31[b]	1.43[ab]	0.041	0.046	0.214
Jejunum, %	0.73	0.76	0.87	0.033	0.226	0.188
Abdominal fat, %	2.94	3.70	3.56	0.243	0.409	0.819
T-SOD (U/mgprot)	104.65	85.93	77.17	10.46	0.567	0.302
MDA (nmol/mgprot)	0.59	1.44	0.70	0.197	0.163	0.826
TG (mmol/g prot)	0.57[b]	0.72[ab]	0.88[a]	0.041	0.012	0.002

Note: [a,b]Mean values with different superscript letters in the same row are significantly different ($P < 0.05$). Data are presented as the means ± SEM. n = 10. The relative weight denotes the percentage of the weight of a given organ or tissue to body weight.

TG: triacylglyceride; SOD, superoxide dismutase; MDA, malondialdehyde.

Effect of Supplemented Cholamine on Intestinal and Uterine Morphological Structures

Compared to the control group, supplementing 500 mg/kg of cholamine to the basal diet significantly reduced the crypt depth of the ileum and significantly increased the ratio of villus height to crypt depth ($P < 0.05$), but had no significant effect on villus height (Table 6). Supplementing 500 mg/kg of cholamine had no significant impact on the thickness of uterine muscular and mucosal layers (Table 6).

Table 5. Effect of dietary cholamine on hepatic fatty acid composition of laying hens (%)

					P-value	
Fatty acid	0 mg/kg	500 mg/kg	1,000 mg/kg	SEM	Cholamine	Linear
C14:0	0.38	0.37	0.32	0.023	0.509	0.297
C15:0	0.22[a]	0.10[ab]	0.09[b]	0.025	0.034	0.017
C16:0	26.89	26.71	24.98	0.025	0.819	0.577
C17:0	1.17	0.25	0.15	0.313	0.365	0.205
C18:0	9.09	10.76	12.01	0.886	0.436	0.208
C20:0	0.13[ab]	0.15[b]	0.08[a]	0.013	0.053	0.093
C21:0	0.23	0.27	0.29	0.033	0.774	0.493
C22:0	0.39	0.35	0.38	0.048	0.954	0.926
C23:0	0.32	0.36	0.49	0.054	0.453	0.234
C24:0	0.12	0.13	0.20	0.037	0.654	0.414
C14:1n−5	0.17	0.07	0.11	0.025	0.239	0.259
C16:1n−7	3.36	2.65	1.92	0.526	0.564	0.295
C17:1n−7	0.12	0.21	0.09	0.031	0.290	0.657
C18:1n−9	39.58	36.39	38.07	1.149	0.564	0.610
C20:1	0.20	0.14	0.07	0.026	0.133	0.049
C24:1n−9	0.30	0.28	0.24	0.027	0.657	0.383
C18:3n−3	0.15	0.12	0.21	0.023	0.288	0.267
C20:3n−3	0.24	0.27	0.19	0.032	0.574	0.500
C20:5n−3	0.11	0.09	0.07	0.013	0.554	0.289
C22:6n−3	1.12	0.98	0.91	0.092	0.660	0.382
C18:2n−6	11.70	15.55	15.19	0.755	0.057	0.047
C18:3n−6	0.29	0.22	0.20	0.029	0.437	0.236
C20:3n−6	3.59	3.40	3.68	0.293	0.933	0.903
C22:2n−6	0.10	0.12	0.08	0.018	0.673	0.734
SFA	38.96	39.49	38.98	1.004	0.975	0.993
MUFA	43.76	39.76	40.49	1.004	0.550	0.407
n−3 PUFA	1.62	1.46	1.38	0.123	0.744	0.457
n−6 PUFA	15.67	19.29	19.15	0.757	0.205	0.060
PUFA	17.29	20.75	20.53	1.509	0.307	0.202
UFA	61.04	60.51	61.02	1.000	0.975	0.993
SFA/UFA	0.65	0.66	0.64	0.028	0.964	0.921
n−6/n−3 PUFA	10.03	13.99	14.89	0.877	0.066	0.033

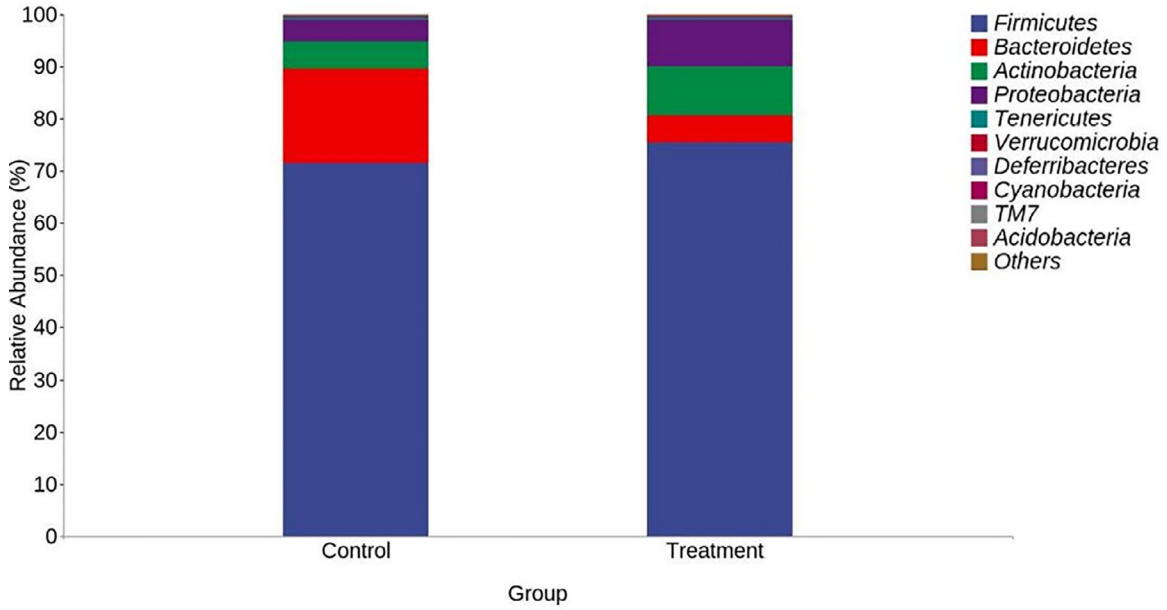
Note: [a,b]Mean values with different superscript letters in the same row are significantly different ($P < 0.05$). Data are presented as the means ± SEM. N = 5.

SFA, saturated fatty acid; UFA, unsaturated fatty acids; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

Table 6. Effect of dietary cholamine on the intestinal and unterine morphology in laying hens.

					P-value	
	0 mg/kg	500 mg/kg	1000 mg/kg	SEM	Cholamine	Linear
Ileum						
Villus height, μm	960.00	1009.47	957.65	12.56	0.146	0.941
Crypt depth, μm	213.82^{a}	192.00^{b}	216.62^{a}	3.988	0.016	0.780
V/C	4.84^{b}	5.57^{a}	4.61^{b}	0.105	<0.001	0.372
Uterus						
Mucosal height, μm	892.33	855.96	845.22	20.36	0.685	0.496
Muscular thickness, μm	311.25	376.78	385.75	12.94	0.101	0.059

Note: ^{a,b}Mean values with different superscript letters in the same row are significantly different ($P < 0.05$). Data are presented as the means ± SEM. N = 10. V/C, the ratio of villus height to crypt depth.



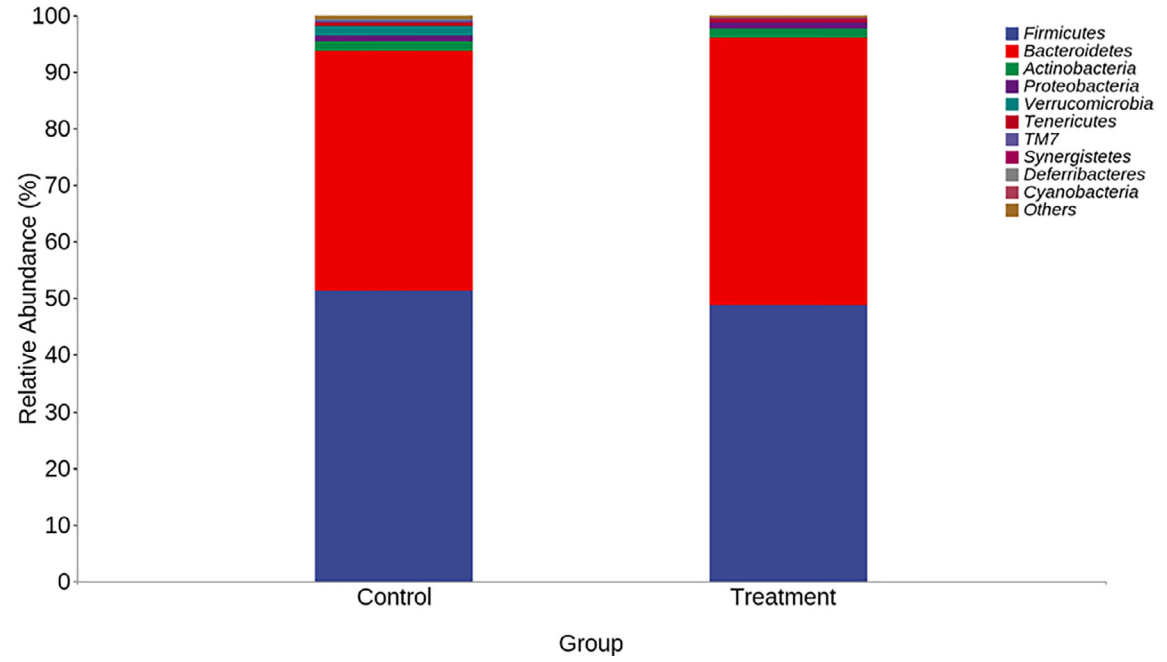


Figure 1. The relative abundance distribution of ileal and cecal microflora at phylum level (A, B) and genus level (C, D). Note: The laying hens in control group were fed basal diet, and those in the treatment group were fed basal diet plus 500 mg/Kg. n = 5.

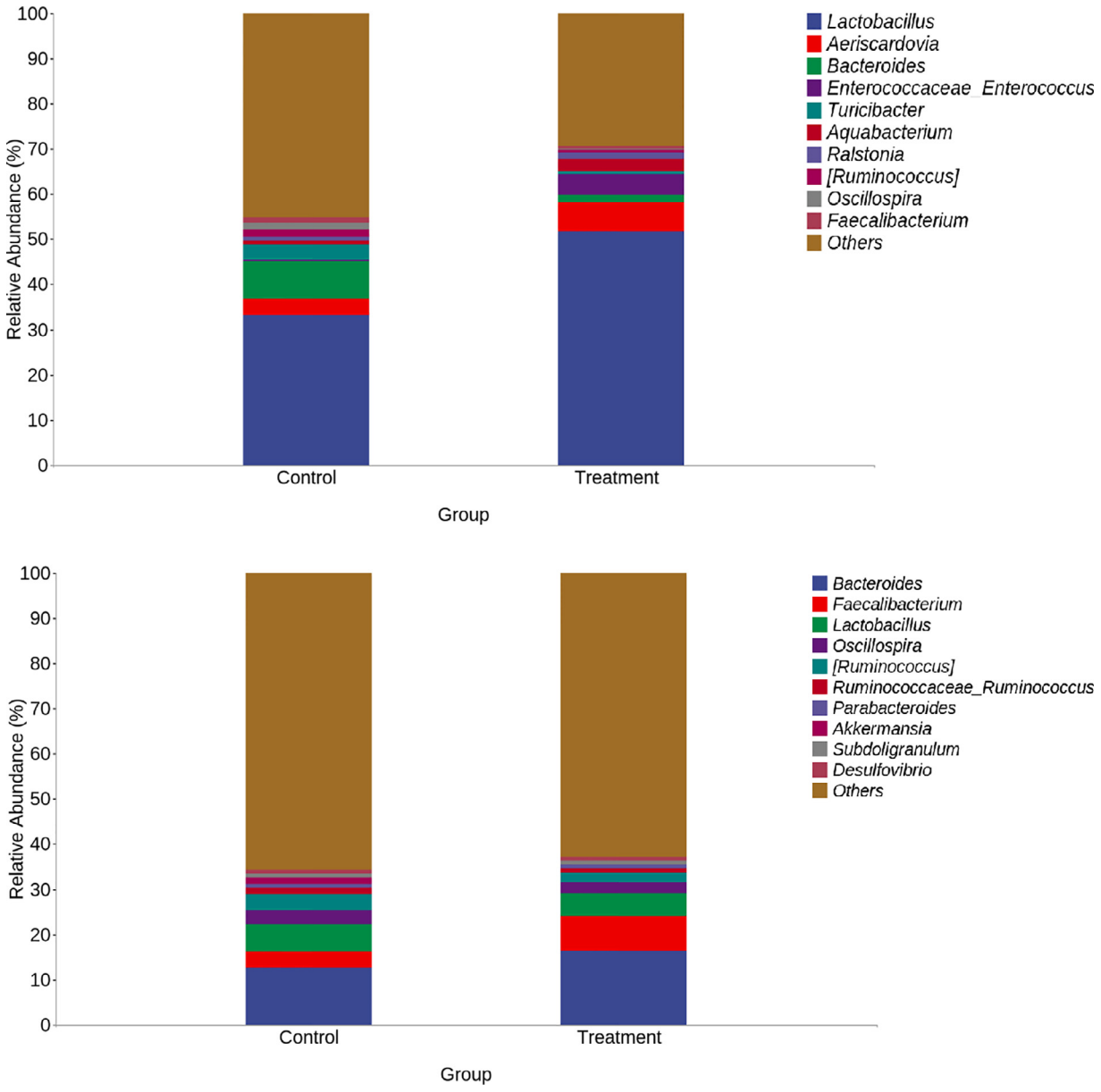


Figure 1 Continued.

Effect of Supplemented Cholamine on Gut Microbiota

Compared to the control group, supplementing 500 mg/kg of cholamine to the basal diet reduced the α-biodiversity of ileal microflora, which is indicated by the Chao1 index (from 1563 to 872, $P = 0.081$) and the Observed_species index (from 1100 to 671, $P = 0.12$). Other indexes including Faith_pd, Pielou_e, Shannon and Simpson also decreased but not significantly (Supplementary Table 3). Supplementing 500 mg/kg of cholamine had no obvious effect on the α-biodiversity of cecal microflora (Supplementary Table 3). The compositional differences were identified between treatment and control groups by principal coordinate analysis using the Bray Curtis distance parameter (Supplementary Figure 1A, B). In addition, beta diversity analysis indicated that most of the samples from each group were aggregated into the same group, and the samples between the groups could be differentiated (Supplementary Figure 1A, B).

The analysis on the relative abundance of intestinal microflora showed that the dominant phyla in the ileum of the control and cholamine-treated groups were *Firmicutes* (71.4%, 75.4%), *Bacteroidetes* (18.2%, 5.2%), *Actinobacteria* (5.2%, 9.3%), and *Proteobacteria* (4.1%, 9.0%), the dominant genera were *Lactobacillus* (33.2%, 51.7%), *Aeriscardovia,* (3.7%, 6.5%), *Bacteroides* (8.3%, 1.7%) and *Enterococcaceae_Enterococcus* (0.36%, 4.5%), while the dominant phyla in the cecum were *Firmicutes* (51.4%, 48.8%), *Bacteroidetes* (42.5%, 47.3%), *Actinobacteria* (1.6%, 1.5%) and *Proteobacteria* (1.1%, 1.0%), and the dominant genera were *Bacteroides* (12.6%, 16.4%), *Faecalibacterium* (3.7%, 7.7%), *Lactobacillus* (6.0%, 4.9%), and *Oscillospira* (3.0%, 2.7%) (Supplementary Table 4, Supplementary Table 5, Figure 1A–D).

Compared to the control group, supplementing 500 mg/kg of cholamine significantly altered the relative abundance of some intestinal bacteria, that is, supplemented cholamine decreased the relative abundance of the major phylum *Bacteroidetes* (from 18.2% to 5.2%, $P = 0.13$), the major genus *Bacteroides* (from 8.3% to 1.7%, $P = 0.13$) in the ileum and *Ruminococcaceae_Ruminococcus* in the cecum (from 1.4% to 0.93%, $P < 0.10$), but increased the relative abundance of *Cyanobacteria* phylum in the ileum (from 0.051% to 0.15%, $P < 0.05$) and *Faecalibacterium* genus in the cecum (from 3.7% to 7.7%, $P = 0.096$) (Supplementary Table 4, Supplementary Table 5, Figure 1A−D).

Effect of Supplemented Cholamine on Liver Transcriptome

Transcriptome sequencing analysis showed that supplementing 1,000 mg/kg of cholamine significantly affected the mRNA expression levels of 2,065 genes: 1,151 differentially expressed genes (**DEG**s) were induced whereas 914 DEGs were repressed compared to the control group. The top 10 up- or down-regulated DEGs with the lowest *P*-value and more than 2-fold change are listed in Table 7. The KEGG pathway analysis revealed 6 pathways were significantly enriched with the up-regulated DEGs, including Ribosome, Oxidative phosphorylation, Proteasome, Protein export, Cardiac muscle contraction, and Terpenoid backbone biosynthesis, and the KEGG pathways significantly enriched with the down-regulated DEGs were mainly the Notch signaling pathway (Figure 2A, B).

Ten DEGs were selected for validation, including apolipoprotein A4 (*APOA4*), phosphoenolpyruvate carboxykinase 1 (*PCK1*), metallothionein 3 (*MT3*), metallothionein 4-like (*MT4L*), lactate dehydrogenase B (*LDHB*), coiled-coil-helix-coiled-coil-helix domain containing 10 (*CHCHD10*), 3-hydroxy-3-methylglutaryl-CoA synthase 2 (*HMGCS2*), hydroxy acyl-CoA dehydrogenase trifunctional multienzyme complex subunit beta (*HADHB*), pyrophosphatase 1 (*PPA1*), and avian beta-defensin 9 (*AvBD9*). The quantitative PCR analysis indicated all the selected genes except for *MT4L* were validated (Figure 3), suggesting that the transcriptome sequencing analysis was reliable.

Effect of Supplemented Cholamine on the Expression of Genes Related to fat Deposition in the Liver

A search for genes related to fat deposition in liver transcriptome data revealed that the expression levels of genes related to fat synthesis, packaging and secretion (*FASN, SCD1*, and *MTTP*) in the liver were not significantly affected by supplemented cholamine whereas the expression levels of genes related to fat absorption, transport and oxidative phosphorylation were obviously affected, that is, the expression of *FABP1, APOA4*, and *PCK1* genes were induced by supplemented cholamine (Figure 4).

Table 7. Top 10 up- and down-regulated differentially expressed genes in the liver of laying hens fed cholamine supplemented diet vs. those fed basal diet.

Gene	Log2fold change	*P*-value
Up-regulated genes		
APOA4	2.02	5.19E−09
PPA1	1.10	8.38E−07
HADHB	1.03	1.00E−06
ABHD3	1.23	1.15E−06
EIF2A	1.06	2.06E−06
FABP1	1.24	3.66E−06
CFAP97D1	1.46	3.33E−05
LDHB	1.56	6.86E−05
ZNF226L	1.99	7.09E−05
HMGCS2	1.16	8.58E−05
Down-regulated genes		
SUN2	−1.87	1.57E−12
CHKA	−2.11	1.18E−07
DNAJC6	−1.89	4.29E−07
MVB12B	−1.04	1.53E−06
GGACT	−1.93	2.03E−06
AKR1B1	−2.88	3.79E−06
SMC2	−2.44	9.67E−06
PRKCB	−1.88	1.49E−05
CBX2	−1.43	2.56E−05
SH2B3	−1.39	2.75E−05

Note: The differentially expressed genes (DEGs) were identified from transcriptome analysis of the livers from the laying hens fed basal diet plus 1,000 mg/Kg of cholamine vs. those fed basal diet as control. These DEGs shown in this table are those with the lowest p values and |Log2fold change| > 1 among the upregulated or downregulated DEGs.

APOA4, apolipoprotein A-IV precursor; *PPA1*, Inorganic Pyrophosphatase 1; *HADHB*, Hydroxyacyl-CoA Dehydrogenase Trifunctional Multienzyme Complex Subunit Beta; *ABHD3*, Abhydrolase Domain Containing 3, Phospholipase; *EIF2A*, Eukaryotic Translation Initiation Factor 2A; *FABP1*, Fatty Acid Binding Protein 1; *CFAP97D1*, CFAP97 Domain Containing 1; *LDHB*, Lactate Dehydrogenase B; *ZNF226L*, Zinc Finger Protein 226; *HMGCS2*, 3-Hydroxy-3-Methylglutaryl-CoA Synthase 2; *SUN2*, Sad1 And UNC84 Domain Containing 2; *CHKA*, Choline Kinase Alpha; *DNAJC6*, DnaJ Heat Shock Protein Family (Hsp40) Member C6; *MVB12B*, Multivesicular Body Subunit 12B; *GGACT*, Gamma-Glutamylamine Cyclotransferase; *AKR1B1*, Aldo-Keto Reductase Family 1 Member B; *SMC2*, Structural Maintenance of Chromosomes 2; *PRKCB*, Protein Kinase C Beta; *CBX2*, Chromobox 2; *SH2B3*, SH2B Adaptor Protein 3. The *P*-values are displayed in scientific notation or E-notation.

DISCUSSION

In this study, supplemented cholamine significantly reduced egg production but did not significantly alter egg quality, suggesting that cholamine may have a significant effect on follicular growth, differentiation and maturation without substantial effect on the functions of the oviduct and uterus such as albumen secretion and eggshell formation. Indeed, supplemented cholamine did not significantly change the thickness of the mucosal and muscular layers of the uterus. The uterus is the site of eggshell formation, mucosal cells are responsible for secreting the eggshell components, and changes in the structure and function of mucosal layer can affect eggshell quality (Nii et al., 2018; Wang et al., 2021). Therefore, in this study, no significant effect of supplemented cholamine on the thickness of the mucosal layer is consistent with no significant change of egg quality. On the

other hand, cholamine is known to be involved in the regulation on the function of EGF receptor in the cell through the mTOR pathway (Yang et al., 2016b), so cholamine may regulate the growth, differentiation and maturation of follicles in laying hens by affecting the mTOR pathway, leading to change of egg production rate. This speculation needs to be verified in the future.

In this study, supplemented cholamine significantly reduced liver weight and liver growth index, which is consistent with the inhibition of follicular growth mentioned above. Previous studies, however, indicate that supplemented cholamine can increase body weight, stomach weight and small intestine weight in weaned piglets (Yang et al., 2016a). This inconsistency may be related to epigenetic regulation of different tissue types. Although liver growth was inhibited by supplemented cholamine, liver fat was significantly increased, that is, supplemented cholamine promoted liver fat deposition. To date, it is unclear the mechanism by which cholamine promotes fat deposition in the liver. In general, liver fat deposition depends on the rate of exogenous fat transport to the liver, the rate of de novo fatty acid synthesis, the rates of fat packaging in the liver and fat transporting to peripheral tissues such as adipose and muscle tissues for storage and utilization, and the rate of fatty acid oxidation in the liver (Nguyen et al., 2008). This study showed that supplemented cholamine did not significantly affect the expression of *SCD*, *FASN*, and

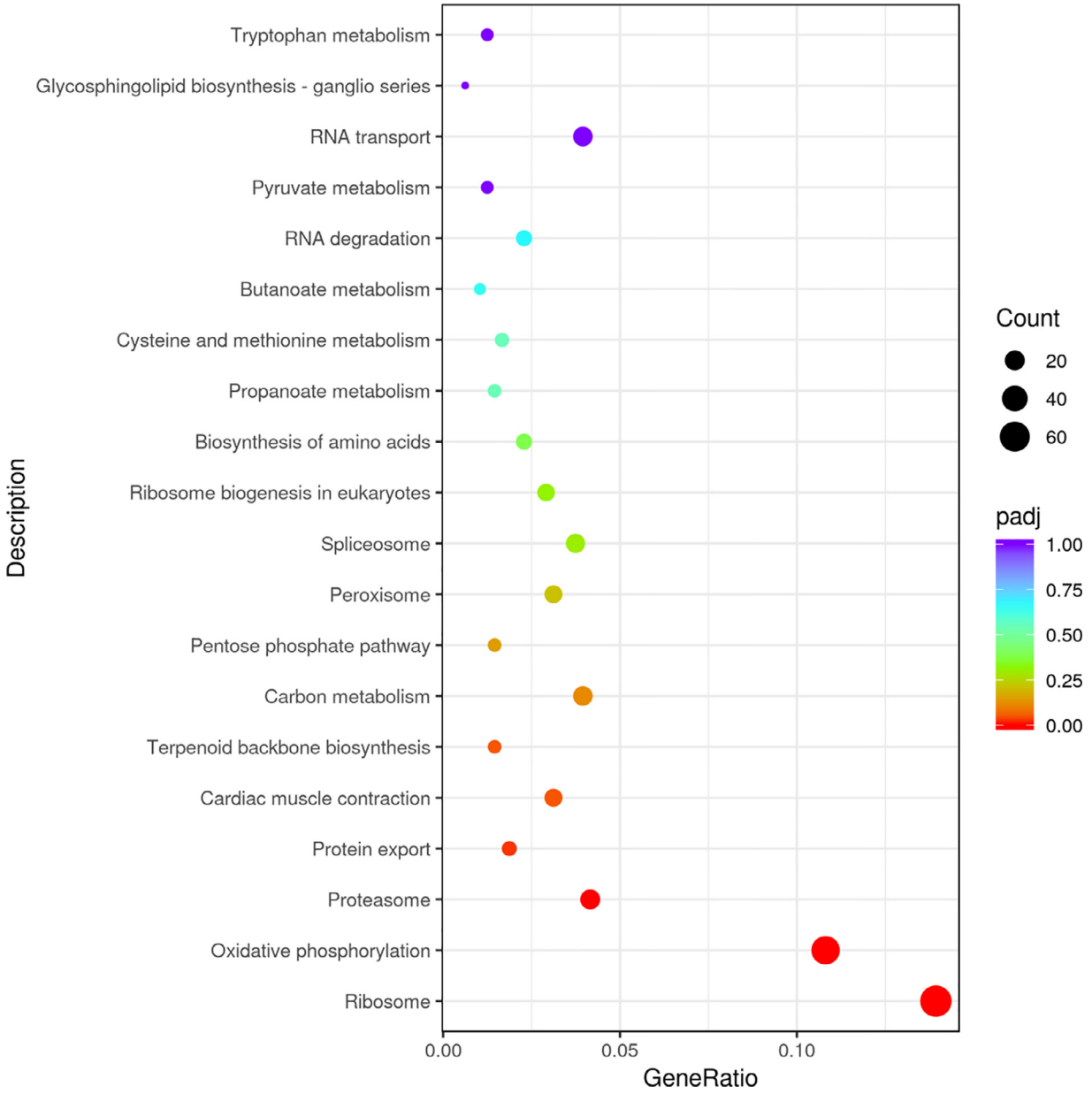


Figure 2. The top 20 KEGG pathway enriched with the up-regulated (A) and down-regulated (B) differentially expressed genes (DEGs) identified by transcriptome analysis of the livers from the laying hens fed basal diet plus 1,000 mg/Kg of cholamine vs. those fed basal diet as control. Note: the x-axis is the ratio of the number of the annotated DEGs to the number of all the annotated genes in a specified KEGG pathway, and y-axis is the KEGG pathway. The sizes of the dot indicate the numbers of the DEGs, while the colors of the dot indicate the values of adjusted *P*-values (padj).

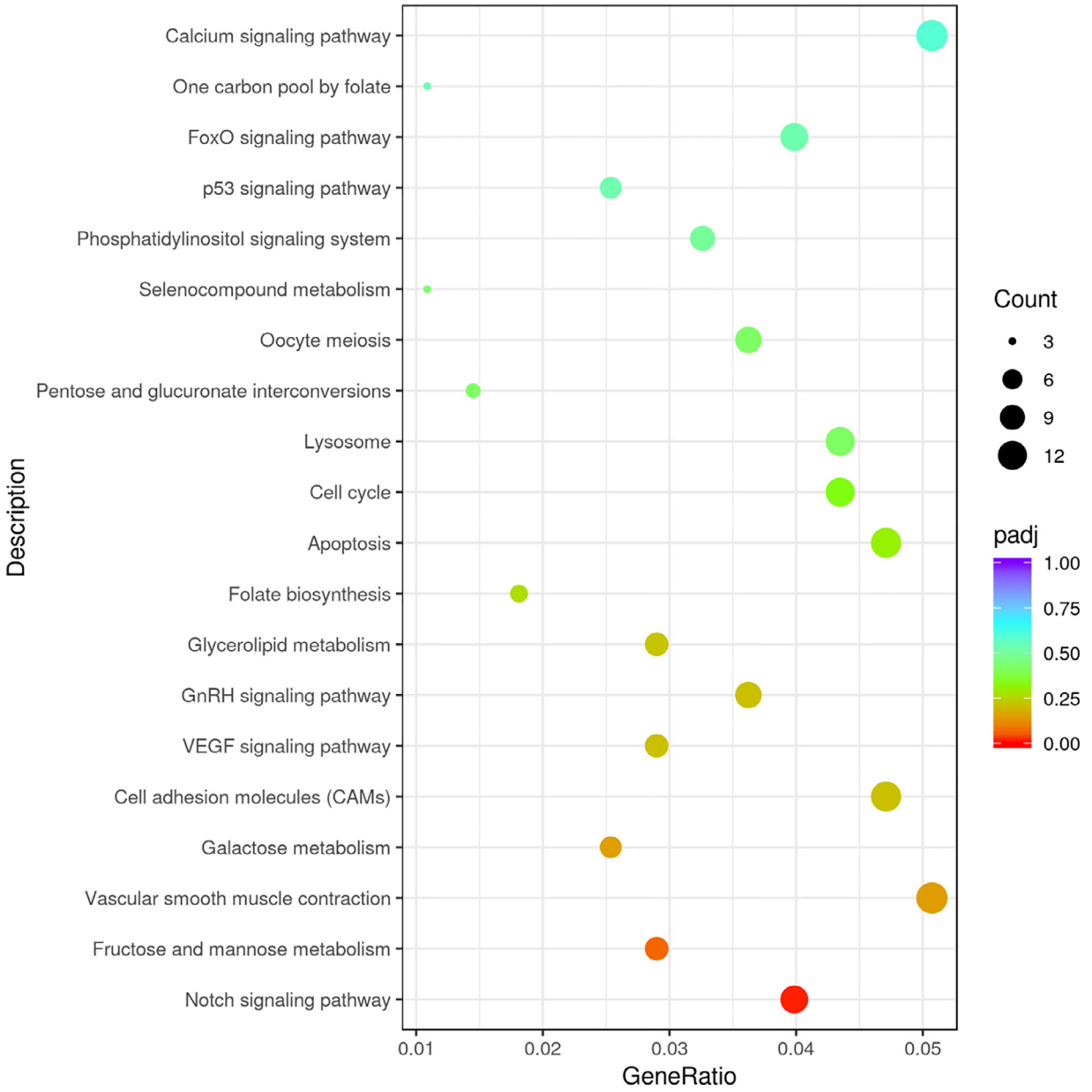


Figure 2 Continued.

MTTP genes in the liver, but significantly induced the expression of *FABP1, APOA4*, and *PCK1* genes. *SCD* and *FASN* are key genes involved in fatty acid synthesis, *MTTP* plays an important role in lipid packaging and secretion in liver cells, and *APOA4* is a key component of chylomicron and HDL, *FABP1* as a fatty acid-binding protein participates in fatty acid uptake, transport and metabolism, and *PCK1, COX4*, and *ACOX1* are the genes involved in oxidative phosphorylation. Therefore, the promoting role of supplemented cholamine in liver fat deposition may be related to fat absorption, transport and oxidation rather than lipid synthesis. Furthermore, HDL is the transporter of cholesterol from peripheral tissues and plasma to the liver for metabolism, low-density lipoprotein (**LDL**) is the primary transporter of endogenous cholesterol from the liver to peripheral tissues (Nguyen et al., 2008). In mammals, low HDL-C level in the blood is usually accompanied by metabolic diseases such as obesity and fatty liver (Girona et al., 2019). In this study, the supplemented cholamine caused a significant reduction of serum HDL-C level in laying hens, which is in line with a substantial increase of liver fat. In addition, although the abdominal fat index of laying hens was not significantly affected by supplemented cholamine, both low and high levels of supplemented cholamine led to an increase of the abdominal fat index, which is also in line with the increased liver fat.

In addition to the direct effects of supplemented cholamine and its derivatives, cholamine may play a role indirectly in laying hens. It is reported that cholamine can change the competitiveness of certain bacteria with other bacteria, which may in turn change the composition of the entire intestinal microflora and their metabolites (Thiennimitr et al., 2011). Previous studies have shown that changes in gut microflora and their

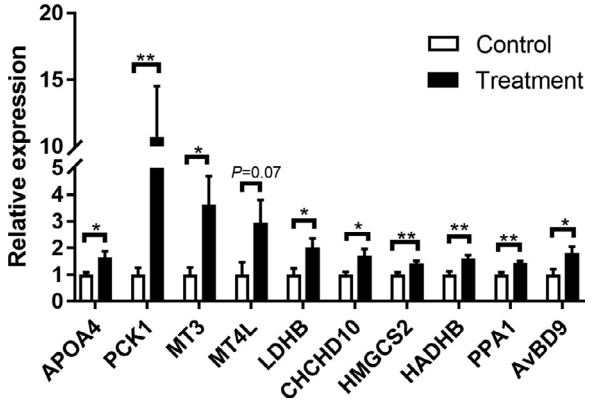


Figure 3. Validation of some differentially expressed genes (DEGs) in the livers of the laying hens fed basal diet plus 1000 mg/Kg of cholamine vs. those fed basal diet as control using quantitative PCR. The relative mRNA transcript abundance is presented as fold change as compared with the control group. n = 6. *, ** indicate $P < 0.05$ and 0.01 vs. the control group, respectively. The data are expressed as the means ± SEM.

metabolites may affect intestinal health, morphological structure, and digestive and absorptive functions by altering the biological barrier, the tight junction and permeability, and inflammatory and immune responses of the intestine, and may also affect feeding behavior and endocrine system through the gut-brain axis (Clark and Mach, 2016; Zhou et al., 2017; Schoultz and Keita, 2020). For example, supplementing probiotics such as *Lactobacillus* and *bifidobacterium* to diet can reduce the abundance of harmful bacteria, increase biodiversity, and promote intestinal health and digestive and absorptive functions (Servin, 2004). In this study, supplemented cholamine reduced the biodiversity of intestinal microflora and altered the structure of the community. Data showed that supplemented cholamine led to an increase in the relative abundance of *Cyanobacteria* phylum and *Faecalibacterium* genus, and a decrease in the relative abundance of *Bacteroides* phylum, *Bacteroides* genus, and *Ruminococcaceae_Ruminococcus* genus. It is possible that such changes in the composition of intestinal microflora promote fatty acid synthesis, increase liver fat deposition and even alter the composition of liver fat by increasing the amount of exogenous fatty acids into the liver through enhanced intestinal absorption. Indeed, the results show that supplemented cholamine significantly reduced crypt depth and increased the ratio of villus height to crypt depth of intestine, thereby enhancing intestinal digestive and absorptive functions. Supplemented cholamine also altered the fatty acid composition of liver fat by reducing the relative percentages of C15:0 and C20:0 but increasing the percentage of C18:2n−6 and the n−6 to n−3 PUFA ratio. Previous studies indicate that increased n−3 polyunsaturated fatty acids have a protective effect on animal health (Tvrzicka et al., 2011), while increased n−6 polyunsaturated fatty acids contribute to the production of proinflammatory molecules and promote the differentiation of preadipocytes (Ailhaud et al., 1992). It has been reported that during adipocyte formation (in utero and early infancy), exposure of animals to excessive n−6 fatty acids may promote fat deposition early in life (Gibson et al., 2011). The n−6 to n−3 PUFA ratio is also associated with obesity in animals (Su et al., 2020). In addition, many studies have shown that obesity and obesity-related diseases such as nonalcoholic fatty liver disease (**NAFLD**) are closely associated with a decrease in the biodiversity of intestinal microflora (Arslan, 2014; Panasevich et al., 2016), and this association is also present in this study, that is, a reduction in the α-biodiversity of intestinal microflora due to supplemented cholamine was associated with an increase in liver fat.

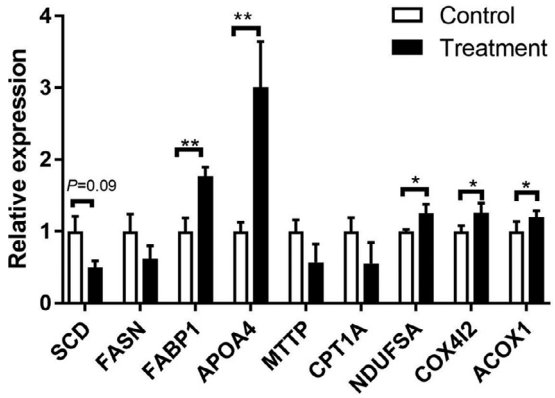


Figure 4. Effect of dietary ethanolamine on the mRNA expression of genes related to lipid metabolism in the livers of the laying hens fed basal diet plus 1,000 mg/Kg of ethanolamine (the treatment group) vs. those fed basal diet (the control group). The relative mRNA expression level is retrieved from transcriptome analysis. N = 4. *, ** indicate $P <$ 0.05 and 0.01 vs. the control group, respectively. The data are expressed as the means ± SEM.

In this study, KEGG pathway enrichment analysis indicated that the following pathways were enriched DEGs, including short-chain volatile lipid metabolism (Propanoate metabolism, Butanoate metabolism, Glycosphingolipid biosynthesis, and Glycerolipid metabolism pathways, etc.), energy metabolism-related pathway (Oxidative phosphorylation, Pyruvate metabolism, Fructose and mannose metabolism, Peroxisom, etc.), the pathways related to protein metabolism, the pathways related to cell growth, differentiation, apoptosis (GnRH signaling pathway, Cell cycle, Apoptosis, Phosphatidylinositol signaling system), and some pathways related to important biological processes (Notch signaling pathway, FoxO signaling pathway, Oocyte meiosis, and Cell adhesion molecules). These pathways laid a foundation for revealing the broad biological effects of cholamine.

CONCLUSION

Supplementing cholamine to the diet of laying hens decreased blood HDL-C level, the biodiversity of intestinal microflora, and the relative abundance of *Bacteroidetes* phylum, *Bacteroides* genus, and *Ruminococcaceae_Ruminococcus* genus, increased the percentage of C18:2n−6 in liver fat, the n−6 to n−3 PUFA ratio, and the villus height to crypt depth ratio of the intestine, induced the expression of *FABP1* and *APOA4* genes in the liver, and

significantly affected the pathways such as oxidative phosphorylation and Notch signaling pathway. These effects may all contribute to liver fat deposition and reduction of egg production. However, the related mechanism needs to be further investigated.

ACKNOWLEDGMENTS

This work was supported by Jiangsu Province Major Agricultural New Varieties Creation Project (PZCZ201731), Joint International Research Laboratory of Agriculture and Agri-Product Safety of the Ministry of Education of China, and the Priority Academic Program Development of Jiangsu Higher Education Institutions.

DISCLOSURES

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.102084.

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