Dietary betaine improves egg-laying rate in hens through hypomethylation and glucocorticoid receptor–mediated activation of hepatic lipogenesis-related genes

Nagmeldin A. Omer,[*,†,‡] Yun Hu,[*,†] Abdulrahman A. Idriss,[*,†] Halima Abobaker,[*,†] Zhen Hou,[*,†] Shu Yang,[*,†] Wenqiang Ma,[*,†,1] and Ruqian Zhao[*,†]

*MOE Joint International Research Laboratory of Animal Health & Food Safety, College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, P. R. China; †Key Laboratory of Animal Physiology & Biochemistry, Ministry of Agriculture and Rural Affairs, College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, P. R. China; and ‡College of Allied Medical Sciences, University of Nyala, Nyala, Sudan

ABSTRACT In avian species, liver lipid metabolism plays an important role in egg laying performance. Previous studies indicate that betaine supplementation in laying hens improves egg production. However, it remains unclear if betaine improves laying performance by affecting hepatic lipid metabolism and what mechanisms are involved. We fed laying hens a 0.5% betaine-supplemented diet for 4 wks to investigate its effect on hepatic lipids metabolism in vivo and confirmed its mechanism via in vitro experiments using embryonic chicken hepatocytes. Results showed that betaine supplemented diet enhanced laying production by 4.3% compared with normal diet, accompanied with increased liver and plasma triacylglycerol concentrations ($P < 0.05$) in hens. Simultaneously, key genes involved in hepatic lipid synthesis, such as sterol regulatory element binding protein 1 (SREBP-1), fatty acid synthase, acetyl-CoA carboxylase, and stearoyl-CoA desaturase 1 (SCD1) were markedly upregulated at the mRNA level ($P < 0.05$). Western blot results showed that SREBP-1 and SCD1 protein levels were also increased ($P < 0.05$). Moreover, mRNA expression of main apolipoprotein components of yolk-targeted lipoproteins, apolipoprotein B (ApoB) and apolipoprotein-V1 (ApoV1), in addition to microsomal triglyceride transfer proteins, which is closely related to the synthesis and release of very-low density lipoprotein, were also markedly elevated ($P < 0.05$). Methylated DNA immunoprecipitation combined with PCR detects reduction of methylation levels in certain regions of the above gene promoters. Chromatin immunoprecipitation PCR assays showed increased binding of glucocorticoid receptor (GR) to *SREBP1* and *ApoB* gene promoters. Similar results of ApoV1 gene expression were obtained from cultured hepatocytes treated with betaine. Additionally, betaine increased the expression of GR and some genes involved in methionine cycle in vitro. These results suggest that betaine supplementation could alter the expression of liver lipid synthesis and transport-related genes by modifying the methylation status and GR binding on their promoter and hence promote the synthesis and release of yolk precursor substances in the liver.

Key words: betaine, hepatocytes, lipogenesis, hens, methylation

2020 Poultry Science 99:3121–3132
https://doi.org/10.1016/j.psj.2020.01.017

INTRODUCTION

The process of egg production requires a massive increase in triacylglycerol (**TG**) synthesis to support the energy demands of new yolk formation (Wu et al., 2013). In liver, *de novo* synthesized triglycerides are packaged into very-low density lipoprotein (**VLDL**) and used for yolk formation (Cherian, 2015; Li et al., 2017). Hepatic TG homeostasis, the balance between *de novo* lipogenesis and export of VLDL particles, is extremely important for egg production and chicken

Received May 1, 2019.
Received in revised form January 17, 2020.
Accepted January 20, 2020.
Availability of data and materials: The data sets used and/or analysed during the current study are available from the corresponding author upon request.

[1]Corresponding author: wq8110@njau.edu.cn

health (Liu et al., 2016). Nevertheless, the impact of induced hepatic lipogenesis on egg production is debatable.

More than 90% of the *de novo* synthesis of fatty acids (FA) occurs in the liver of poultry. The key FA synthesis-related genes including fatty acid synthase (**FAS**), acetyl-CoA carboxylase (**ACC**), and stearoyl-CoA desaturase 1 (**SCD1**) could be regulated by sterol regulatory element-binding protein-1 (**SREBP1**) (Choi and Ginsberg, 2011). Hepatic SCD1 is of considerable importance to monounsaturated FA synthesis and is upregulated at the onset of egg-laying in hens (Li et al., 2017). Apolipoprotein B (**ApoB**) and VLDL-apolipoprotein II, also named ApoV1, are major components of VLDL-yolk, the specialized avian lipoprotein produced by the liver and function to transport lipids to the ovary for yolk deposition (Chan et al., 1980; Kirchgessner et al., 1987). The expression levels of these 2 apolipoproteins are found to be dramatically elevated in laying hens compared with nonlaying (Li et al., 2017). Microsomal triglyceride transfer protein, an enzyme which is located in the lumen of the endoplasmic reticulum, plays a major role in the synthesis and secretion of VLDL particles from the liver (Wetteru et al., 1997). In sexually mature hens, all these enzymes are basically controlled by sex hormones, mainly estrogen (Speake et al., 1998; Walzem et al., 1999; Riegler et al., 2011). Although, nutritional factors may also play an essential role.

Betaine is a naturally occurring micronutrient, chemically known as trimethylglycine. It was first reported in sugar beets, then later discovered in many products. In cells, betaine plays 2 critical roles, stabilizing osmolyte and donating methyl group to other biomolecules (Craig, 2004). Numerous studies in hens indicated that betaine supplementation improved the laying performance and egg production (Zou and Lu, 2002; Gudev et al., 2011; Xing and Jiang, 2012). Previously, we also reported improved egg production in betaine-supplemented hens which might be attributed to the activation of hepatic VTGII expression (Omer et al., 2018). However, whether the modulation of hepatic lipids metabolism induced by betaine might influence laying performance in hens is yet to be studied. Furthermore, glucocorticoid receptor (**GR**), which is thought to be involved in modulating lipids metabolism and TG homeostasis (Yu et al., 2010; Wang et al., 2012), was induced by dietary betaine in hens (Omer et al., 2018). Nevertheless, the correlation among betaine supplementation, hepatic GR expression, and hepatic lipogenesis is largely uncertain in hens.

Betaine acts as a primary methyl donor for the epigenetic regulation of gene expression through DNA methylation (Andderson et al., 2012; Day and Kempson, 2016). Hypermethylation of the promoter regions leads to gene silencing because of blocking its accessibility by transcription machinery, whereas hypomethylation results in gene activation (Razin, 1998). Wang *et al.* reported decreasing hypermethylation pattern of hepatic MTTP gene promoter induced by a high-fat diet, which increased its mRNA expression, when male mice were fed betaine-supplemented diet (Wang et al., 2014). A study in laying hens abdominal fat revealed that betaine-supplemented diet increases FAS mRNA abundance and may affect the methylation status of its promoter (Xing and Jiang, 2012). Hu et al. reported that *in ovo* betaine injection induced SREBP1 and cholesterol metabolism-related genes expression in liver of newly hatched chicks (Hu et al., 2015). Nevertheless, it seems that betaine effect on lipids metabolism varies with animal species, sex, and physiological condition. Some studies concluded that the inhibition of hepatic FA synthesis as a mechanism by which betaine prevent or improve fatty liver syndrome (Song et al., 2007; Kathirvel et al., 2010; Xing et al., 2011).

Therefore, we investigated the key gene expression involved in hepatic TG homeostasis, DNA methylation status of the promoter regions of the affected genes, and its binding activity of GR to further reveal the relationship between betaine supplementation and laying performance in hens.

MATERIALS AND METHODS

Animals and Treatment

As previously reported (Omer et al., 2018), one hundred and twenty 38-wk Rugao yellow breeders laying hens were randomly divided into 2 groups: control group (basal diet) and betaine group (basal diet supplemented with 0.5% pure betaine) as presented in Table 1. The betaine (75% purity) was purchased from the Skystone Feed Co., Ltd., Jiangsu, China. The laying performance was recorded daily throughout the 4-wk feeding period. Sixteen hens were randomly selected, weighed, and killed by rapid decapitation at the end of experiment. Blood samples were taken, and plasma was separated and stored at −20°C. Liver samples were dissected, snap frozen in liquid nitrogen then stored at −80°C.

Table 1. Composition of the experimental diets.[1]

Ingredient (%)	CON	BET
Corn	65.00	65.00
Soybean meal	24.67	24.67
Shell powder	6.70	6.70
Limestone	2.07	2.07
Salt	0.30	0.30
Dicalcium phosphate	0.83	0.83
Zeolite	0.01	0.01
Choline chloride	0.17	0.17
Methionine	0.12	0.12
Vitamin premix[2]	0.03	0.03
Minerals premix[3]	0.10	0.10
Betaine	0.00	0.50

[1]CON, control group; BET, betaine group.

[2]The vitamins premix contain (per kg): vitamin D_3: 9,000,000 IU; vitamin K: 35,000,000 IU; vitamin B_1: 10 g; vitamin B_2: 28 g; vitamin B_6: 12 g;vitamin B_{12}: 80 mg; vitamin E: 140 g; vitamin K_3: 9 g; D-Biotin: 5.60 g; D-pantothenic acid: 36 g; folic acid: 3.50 g; niacinamide: 100 g; ethoxyquin:1.65 g.

[3]The minerals premix contain (per kg): Cu: 6.4 g; Fe: 72 g; Zn: 64g; Mn: 72 g; Se: 240 mg; I: 480 mg.

Determination of Hepatic and Plasma Triglyceride Concentration

The liver and plasma concentrations of triglyceride were examined by an automatic biochemical analyzer (Beckman coulter, AU2700) using commercial assay kits (E1013 and E1003, Applygen Technologies Inc., Beijing, China) following the manufacturer's instructions.

Histological Analysis of Liver Tissue

Liver samples were fixed in 4% paraformaldehyde, paraffin-embedded, sectioned, and then stained with hematoxylin-eosin. For Oil Red staining frozen sections of liver were used.

In Vitro Experiment

Fertilized chicken eggs at embryonic day 17 (E17) were obtained from a local farm and artificially incubated for an additional 2 D at 37.5°C and 60% relative humidity. Embryos E19 were euthanized by decapitation to collect livers. Livers were pooled, digested, and filtrated to get hepatocytes. Then the cell pellet was suspended in M199 medium (Shanghai Basalmedia Technologies Co., Ltd., Shanghai, China), supplemented with 10% fetal bovine serum. Hepatocytes were seeded onto 6-well plates, 2 mL/well. The cells density was about 5.5×10^6 cells/well. The cells were incubated for 24 h at 37.8°C with 5% CO_2 before dosing. Then, hepatocytes were treated for 24 h with vehicle control, 10 mmol betaine, 100 nM β-estradiol, or combination of 10 mmol betaine with 100 nM β-estradiol, in a basal medium supplemented with insulin-transferrin-selenium mix (ITS 1 mg/mL; Sigma-Aldrich, St Louis, MO) (2 mL/well). After 24 h of treatment, cells were directly harvested and used for the subsequent experiment.

Total RNA Isolation and Real-Time PCR

Total RNA was isolated from frozen hens liver samples and the embryonic hepatocytes using TRIzol reagent (Invitrogen, Carlsbad, CA) and then reverse-transcribed to cDNA by HiScript qRT SuperMix (Vazyme Biotech Co., Ltd., Nanjing, China). Diluted cDNA (1:50, vol/vol) was used for real-time PCR with QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA). β-actin and 18s rRNA were selected as internal controls for normalization. All primers including those used for qRT-PCR (Table 2) were synthesized by Genewiz (Suzhou, China). Data were analyzed using the method of $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001).

Protein Extraction and Western Blotting

Total protein of the hen's liver was extracted from 40 mg frozen tissues as previously described (Cai et al., 2014a). For cell, total protein was extracted using a lysis buffer purchased from Nanjing Sunshine Biotechnology. In brief, the culture dish was washed by gentle immersion in cold PBS for 2 times. The cells were then scratched to 1.5 mL tubes and centrifuged for 5 min at 700 × g to remove the PBS. The pellet was dissolved

Table 2. Nucleotide sequences of primers used for qRT-PCR.

Target genes	GenBank accession	Primer sequences (5′ to 3′)	PCR products (bp)
APOB1	NM_001044633.1	F: GGTTACTCCCACGATGGCAA R: AATGCCCTTCCTTCAGGAGC	113
APOV1	NM_205483.2	F: CTTAGCACCACTGTCCCTGAAGT R: TGCATCAGGGATGACCAGC	130
ACC	NM_205505.1	F: TTGTGGCACAGAAGAGGGAA R: GTTGGCACATGGAATGGCAG	161
FAS	NM_205155.2	F: AAGCAATTCGTCACGGACAG R: GGCACCATCAGGACTAAGCA	116
MTTP	NM_001109784.2	F: TTCTGAAGGACATGCGTGCT R: GTCTAGGCCGTACGTGGATG	116
SCD	NM_204890.1	F: ACCTTAGGGCTCAATGCCAC R: GTTCTCCCGTGGGTTGATGT	93
SREBP1	NM_204126.2	F: GATGCGTTGGAGTACCTTCAG R: GTCACCCTTCAGCCAGTGAAT	168
BHMT	XM_414685.3	F: TCTTCCTGAATTTCCCTT R: TGAACATCCCATCTAGTGA	157
DNMT1	NM_206952.1	F: CGAGTGGGACGGCTTCTT R: AGGCGATAGGTGTCAGGGA	144
GNMT	XM_015283546.1	F: GGAGGAGGGCTTCCAAGTGA R: GCTCCAGCGTCAGCCAGTT	140
GR	NM_001037826.1	F: CTTCCATCCGCCCTTCA R: TCGCATCTGTTTCACCC	203
β-actin	NM_205518.1	F: ATG GCTCCGGTATGTGCAA R: TGTCTTTCTGGCCCATACCAA	120
18S rRNA	MG967540.1	F: ATAACGAACGAGACTCTGGCA R: CGGACATCTAAGGGCATCACA	138

Abbreviations: ACC, acetyl-CoA carboxylase; APOB1, apolipoprotein B1; APOV1, apolipoprotein-V1; BHMT, betaine homocysteine methyltransferase; DNMT1, DNA (cytosine-5-)-methyltransferase 1; FAS, fatty acid synthase; GNMT, glycine-N-methyltransferase; GR, glucocorticoid receptor; MTTP, microsomal triglyceride transfer protein; SCD1, stearoyl-CoA desaturase 1; SREBP1, sterol regulatory element-binding protein-1.

in 200 μL cold lysis buffer supplemented with protease inhibitor cocktail and then subjected to ultrasonication to insure cells destruction. Following centrifugation at 12,000 × g for 10 min, the supernatant was collected in new 1.5 mL tubes. Nuclear protein was extracted by a special kit (Beyotime Biotechnology, Wuhan, China). Protein concentrations were measured using a Pierce BCA Protein Assay kit (No. 23225; Thermo Fisher Scientific, Rockford, IL). Western blot analysis for SREBP1 (14088-1-AP, Proteintech, Rosemont, IL, diluted 1:500), SCD1 (2438, Cell Signaling Technology, Danvers, MA, diluted 1:500), BHMT (15965-1-AP, Proteintech, diluted 1:200), DNMT1 (24206-1-AP, Proteintech, diluted 1:1,000), GNMT (18,790, Proteintech, diluted 1:500), GR and MAT2b (15952-1-AP, Proteintech, diluted 1:1,000) was carried out according to the recommended protocols provided by the manufacturers. β-actin (AP0060, Bioworld, Dublin, OH, diluted 1:10,000), β-tubulin (AP0064, Bioworld, diluted 1:5,000) and histone H1 (BS1655, Bioworld, diluted 1:500) were used as loading control. Images were captured using VersaDoc 4,000 MP system, and the band density was analyzed by Quantity One software (Bio-Rad Laboratories, Hercules, CA).

Methylated DNA Immunoprecipitation Analysis

In brief, liver samples were extracted to obtain high-quality genomic DNA and then sonicated to approximately 500 bp sized fragments. Fragmented DNA was heat-denatured, methylated to obtain the immunoprecipitated methylated DNA fractions using a mouse monoclonal antibody against 5-methyl cytosine (ab10805, Abcam, Cambridge, UK). The immune complexes were treated using the protein G agarose beads (No. Sc-2003, Santa Cruz Biotechnology, Santa Cruz, CA), then washed via digestion buffer containing proteinase K (No. P0021, Nanjing Sunshine Biotechnology Ltd., Nanjing, China) to obtain purified methylated DNA immunoprecipitation (**MeDIP**) DNA. A small aliquot of MeDIP DNA and control input DNA was used to amplify the proximal promoter sequence of

Table 3. Nucleotide sequences of primers used for MeDIP-PCR.

Target genes	Primer sequences (5′ to 3′)	PCR products (bp)
SREBP1 Segment 1	F: AACGGGTCCCGGTGTTAC R: GGCGCAGCCTTTAGGACC	192
SREBP1 Segment 2	F: CTCAGCTCCATCTGACCCAC R: TTGGGGGCAATGAGGTTTGA	190
SREBP1 Segment 3	F: GTGGTCTGGGTCGTTGCC R: GAAGACGCCCCGTAACCCAA	226
SREBP1 Segment 4	F: CAGGACCGCCGTGATGTC R: CTCCCCCAAAAACAGGAGGG	238
ACC Segment 1	F: ATCGGCATCTCCTCATTGGC R: CCTGGTGCAGGGCAGC	272
ACC Segment 2	F: CCACCGTCGGTTGGGTTC R: CCATTGGCTGCTGACGGA	264
ACC Segment 3	F: CAGCTGTGGAGAGGTTTCCC R: CACCGCCTGAACCCTCC	247
ACC Segment 4	F: AGCAATCACTCCTCCAGCAT R: CGTGGGAAACCTCTCCACAG	269
SCD1 Segment 1	F: CACCTCCCCTTCTGGGCA R: CTACCGTGTCCCTGTCCCT	280
SCD1 Segment 3	F: CATTCTTTCCAATTCGCTGGC R: GGCTTGCCCTCGCCATT	205
SCD1 Segment 3	F: ACACGGTGGGGTTAGGAGAG R: CTGGTGGAGGGCAGAAGAAG	220
SCD1 Segment 4	F: TGTGTCTGAGAGCAGGCAAG R: ACAGTGGGGCTGATTCAGTG	239
FAS Segment 1	F: GACTGCGGCGAGGAGTAAAC R: CGTAGCGCGGACGGTTC	216
FAS Segment 2	F: ACCGAAAGGCAGACATCCGA R: AGGGTGTTGCTAGGCGATAAG	253
FAS Segment 3	F: ACAACAAGCGGTAACAACGAT R: TTCCTTGCACAAGCCGGTAG	206
FAS Segment 4	F: GCAACGTGCTGTTAGAAAGGG R: CCTTCCTGGGAAGAGCTGTG	255
MTTP Segment 1	F: GGCTGCATGGGCATTCAAAAAT R: CAAGAAGCGAACTGCTGACAATGG	221
MTTP Segment 2	F: AGGAGCAGATTCCTCCCAGT F: GCACACGTTTCCCCATTTCC	227
MTTP Segment 3	F: ACCGCGGATTAAAAGGAGCA R: CGCTAAGGTCACAAGAGCCA	228
MTTP Segment 4	F: GGGTGTGGTGGTGAATCGTA R: ATTTGGACAGGATGCCAGGG	241

Abbreviations: ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; MTTP, microsomal triglyceride transfer protein; SCD1, stearoyl-CoA desaturase 1; SREBP1, sterol regulatory element-binding protein-1.

Table 4. Nucleotide sequences of primers used for ChIP-PCR.

Target genes	Primer sequences (5′ to 3′)	PCR products (bp)
SREBP1	F: TGATGCGCGATCACCCCA	71
	R: AAACCCCGCCCGTAGAAC	
ACC	F: CCAGCACAGCACTTGGTTTC	76
	R: GCAGAGTTAGGCTTCTGCCA	
SCD1	F: TTGCCAGCCACCTCTGGATA	104
	R: GGCAGCTGTCAAAAGTGAGC	
FAS	F: AGATGGCAAAGAAGGGTGGC	90
	R: GTCGGCTGGAGTCATTGCTT	
MTTP	F: GTAACAGGCATACAACTGGCTG	78
	R: AGAATGTTAGAGGCCAGATCCC	
APOB1	F: ACATGAACACATTCTGAGTTGCC	168
	R: TGTGCGATTTAGGCATCTTCCC	
APOV1	F: CAAATGTTCTGCCTCCTGTTCAA	76
	R: AGCCCATTGGGTCTTCAGTAA	

Abbreviations: ACC, acetyl-CoA carboxylase; APOB1, apolipoprotein B1; APOV1, apolipoprotein-V1; FAS, fatty acid synthase; MTTP, microsomal triglyceride transfer protein; SCD1, stearoyl-CoA desaturase 1; SREBP1, sterol regulatory element-binding protein-1.

chicken SREBP1, ACC, SCD1, FAS, and MTTP genes by real-time PCR with specific primers listed in Table 3. Data were normalized against the input and presented as the fold change in comparison to the average value of the control group.

Chromatin Immunoprecipitation Analysis

The glucocorticoid receptor element (**GRE**) binding sites are predicted on the promoter sequences of target genes (SREBP1, ACC, SCD1, FAS, MTTP, ApoB, and ApoV1) using the online software, PROMO (Messeguer et al., 2002; Farré et al., 2003). Chromatin immunoprecipitation (**ChIP**) analysis was performed as previously described (Cai et al., 2014b). In brief, liver samples were ground, suspended, cross-linked, and sonicated to obtain an average chromatin fragment size of 300–500 bp. Sheard chromatin was precleared, incubated overnight, and treated using protein A/G agarose beads to capture the immunoprecipitated complexes. Finally, DNA fragments were released from immunoprecipitated complexes via reverse cross-linking and then used as template for real-time PCR with specific primers (Table 4).

Statistical Analysis

All data are presented as means ± SEM. Comparisons were performed using independent-samples t test and analysis of variance when applicable, with SPSS18.0 for Windows. The data were considered statistically significant when the P-value was less than 0.05.

RESULTS

Laying Performance

Dietary betaine supplementation significantly enhanced egg-laying rate ($P < 0.05$) but did not affect body weight, average daily feed intake, and egg weight (Table 5).

Hepatic and Plasma Concentration of TG

Oil red staining showed increasing hepatic lipids droplet in hens supplemented with betaine (Figure 1A). Betaine supplementation markedly elevated hepatic and plasma concentration of TG ($P < 0.05$) (Figures 1B and 1C).

Hepatic Expression of Genes Involved in Fatty Acid Synthesis and VLDL Formation and Export

Dietary betaine greatly enhanced ($P < 0.05$) the mRNA and protein level of SREBP-1 and SCD1 in addition to enhancing mRNA expression of *FAS* and *ACC* (Figures 2A and 2C). These genes are key regulators for hepatic lipogenesis. Meanwhile, betaine supplementation upregulated *MTTP, ApoB,* and *ApoV1* expression at the mRNA level ($P < 0.05$), which involved in TG synthesis and export to extrahepatic tissues (Figure 2B). Protein expression of these genes was not carried out because of a lack of chicken specific antibodies.

MeDIP Analysis for DNA Methylation Status on the Promoter of Affected Genes

Schematic diagram of promoter sequences of chicken *SREBP1, ACC, FAS, SCD1,* and *MTTP* are shown in Figures 3A–3E. Four segments (designated S1-S4) from the promoter of each gene were analyzed with MeDIP-PCR. Betaine supplementation markedly

Table 5. Production performance in laying hens.

Parameters	CON	BET	P-value
Initial body weight, kg	1.40 ± 0.01	1.41 ± 0.01	0.33
Final body weight, kg	1.42 ± 0.02	1.43 ± 0.02	0.60
Average daily feed intake, g/D	91.03 ± 1.18	95.95 ± 2.82	0.15
Average daily laying rate, %	80.19 ± 1.12	84.50 ± 0.96	0.03
Egg weight, g	43.09 ± 0.44	42.01 ± 0.41	0.31

Abbreviations: BET, betaine group; CON, control group.

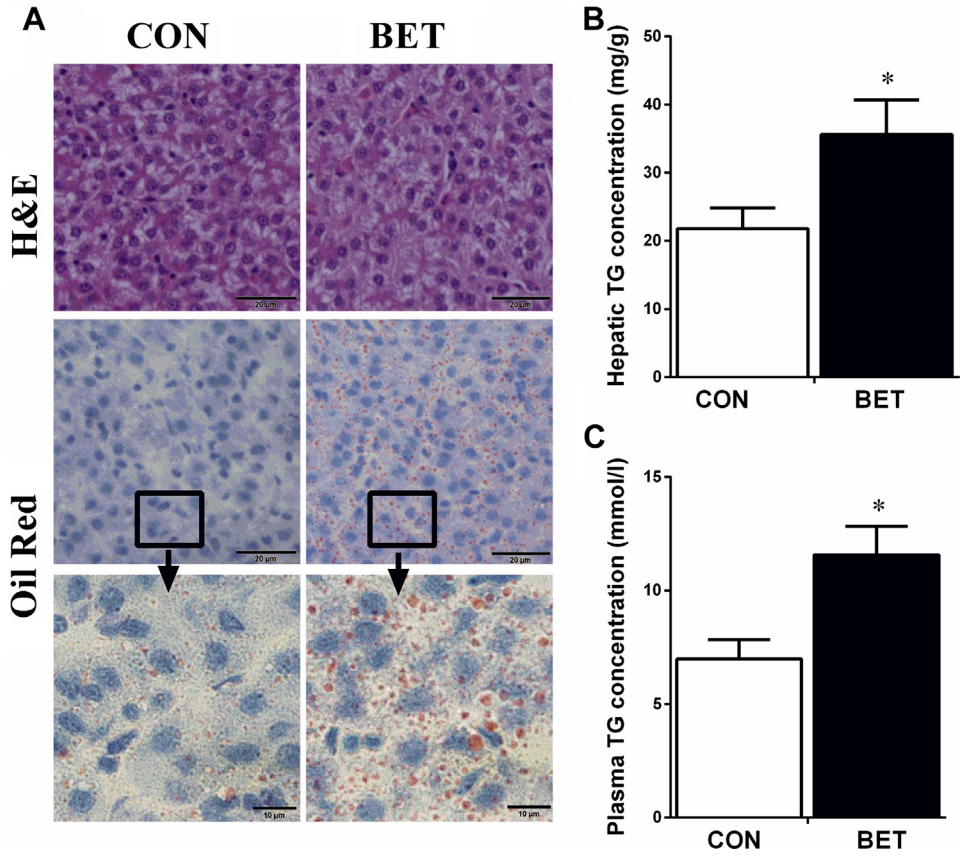

Figure 1. Plasma and liver concentration of TG and histological analysis of liver tissue. (A) Top: Hematoxylin and eosin (H&E) staining; middle and bottom: oil red staining. (B) Hepatic TG concentration. (C) Plasma TG concentration. Values are means ± SEM, $^*P < 0.05$, compared with control (n = 8). Abbreviation: TG, triacylglycerol.

($P < 0.05$) induced hypermethylation of S1 and S4 of *SREBP1* promoter (Figure 3A), S1 and S2 of *ACC* promoter (Figure 3B), S1 and S2 of *FAS* promoter (Figure 3C), S1 and S2 of SCD1 promoter (Figure 3D), and S3 of *MTTP* promoter (Figure 3E). MeDIP for *ApoB* and *ApoV1* genes was not performed because their promoter sequences contain few CpGs, which make them difficult to construct primers for MeDIP-PCR.

ChIP Analysis for GR Binding on the Promoter of Lipogenic Genes

Schematic diagram of promoter sequences of *SREBP1* and *ApoB* are shown in Figures 4A and 4B, respectively. Dietary betaine significantly ($P < 0.05$) affect GR binding to GRE at promoter regions of *SREBP1* and *ApoB* gene (Figure 4C). Other genes were not affected by betaine.

Effect of Betaine on the Expression of ApoV1, GR, and Methionine Cycle-Related Genes In Vitro

Embryonic hepatocytes were treated with β-estradiol (E2) to initiate the synthesis of ApoV1 because its expression is dependent on estrogen. Betaine together with E2 have a maximal effect on ApoV1 expression than E2 alone (Figure 5A). Betaine supplementation greatly ($P < 0.05$) enhanced GR expression at the mRNA and protein levels, whereas E2 treatment had no effect (Figures 5B and 5C, respectively). Hepatocytes treated with betaine, but not E, had significantly ($P < 0.05$) higher mRNA and protein level of genes involved in liver methionine cycle named *BHMT*, *GNMT,* and *DNMT1* (Figures 5D and 5E). The expression of *MAT2b* was not changed by either betaine or E2.

DISCUSSION

In the current study, betaine supplementation enhanced egg laying rate accompanied with increased plasma and liver TG concentration and induced hepatic mRNA expression of the major lipogenic genes, *SREBP1*, *ACC*, *FAS*, and *SCD,* as well as *MTTP*, *APOB*, and *APOV1* which are involved in TG synthesis and export. In vitro experiments revealed that betaine treated cells had higher expression of *GR, APOV1,* and the methyl-transfer enzymes *BHMT*, *GNMT*, and *DNMT1.*

In laying hens, the physiological mechanism of egg formation requires the synthesis of yolk precursors, VTG, and VLDL-yolk in the liver (Vézina et al., 2003). Previously, we reported activation of hepatic *VTGII* expression plays a role in improving egg production in betaine-fed laying hens (Omer et al., 2018). Similar to

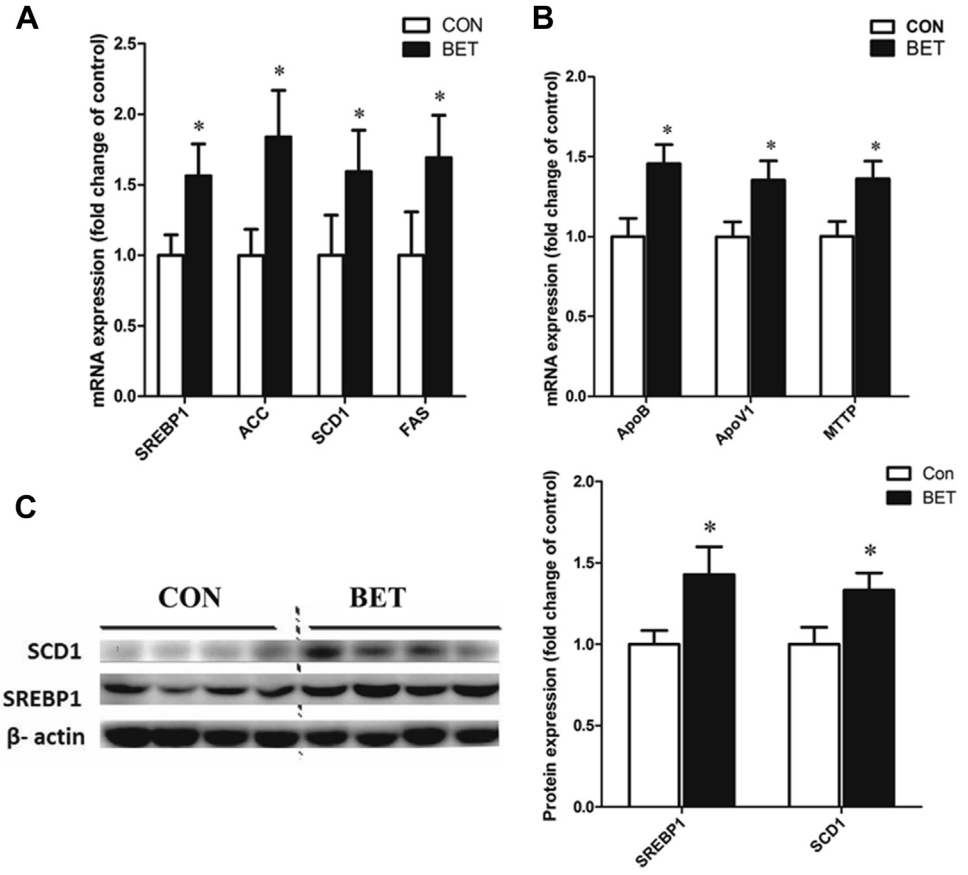

Figure 2. Effect of betaine supplemented diet on the expression of main genes involved in hepatic lipogenesis and TG and VLDL formation and export in hens. (A) mRNA expression of *SREBP1, ACC, SCD1,* and *FAS.* (B) mRNA expression of *ApoB, ApoV1,* and *MTTP.* (C) Protein expression of SREBP1 and SCD1. Values are means ± SEM, $^*P < 0.05$, compared with control (n = 8). Abbreviations: ACC, acetyl-CoA carboxylase; ApoB, apolipoprotein B; ApoV1, apolipoprotein-V1; FAS, fatty acid synthase; MTTP, microsomal triglyceride transfer protein; SCD1, stearoyl-CoA desaturase 1; SREBP1, sterol regulatory element-binding protein-1.

other studies, our study also revealed an increase liver synthesis of FA and TG during egg laying in birds to maintain the demand for yolk formation (Furuse et al., 1991; Nys and Guyot, 2011).

However, part of our results is contradicted with other studies in mice and rats, which concluded inhibiting lipogenesis as a result of betaine administration. In apolipoprotein E–deficient mice, Wang et al. reported that betaine reduced hepatic TG and alter the expression of hepatic lipid metabolism-related genes and especially reduced the expression of FAS (Wang et al., 2013). Another study revealed that betaine significantly attenuated the high-sucrose diet–induced hepatic steatosis which was associated with reduced hepatic lipogenic enzyme activities and gene expression in mice (Song et al., 2017). In rats, *Ahn* et al. reported that inhibition of lipid synthesis was one of the factors responsible for the antisteatotic activity of betaine (Ahn et al., 2015). It is noticed that most of these studies were conducted in male rather than female animals. Another observation is the use of induced hepatic steatosis model and trying to alleviate the change with betaine.

Yet, our data indicating betaine enhanced hepatic lipid export and increased serum TG were in line with some studies in human and rats. For instance, Olthof et al. reported increasing blood TG concentrations in healthy human subject when betaine supplemented orally for 6 wks (Olthof et al., 2005). Sparks et al. reported increased hepatic ApoB mRNA level accompanied by higher serum concentration of TG and ApoB in rats fed betaine-supplemented diet (Sparks et al., 2006). Xu et al. and Ahn et al. stated that betaine improved liver lipid accumulation via increasing hepatic lipid exportation and FA oxidation in rats given high-fat diet (Ahn et al., 2015; Xu et al., 2015). Nevertheless, sex differences, physiological status, diet composition, experimental condition, organ specificity, as well as species differences should be considered when dealing with the effect of betaine on lipids metabolism.

We observed increased oil droplets in the liver section of betaine fed hens. Again this result disagrees with other findings assumed hypolipidimic effect of betaine. But it is in agreement with Hayes et al. (Hayes et al., 2003) who observed increased fat droplets in rat hepatocytes following betaine intake. He suggested that betaine might stimulate both synthesis and secretion of VLDL. However, owing to a limitation in the protein supply, there was a brake on apolipoprotein formation. The consequence leads to an imbalance between synthesis and export which lead to increasing fat droplets in the

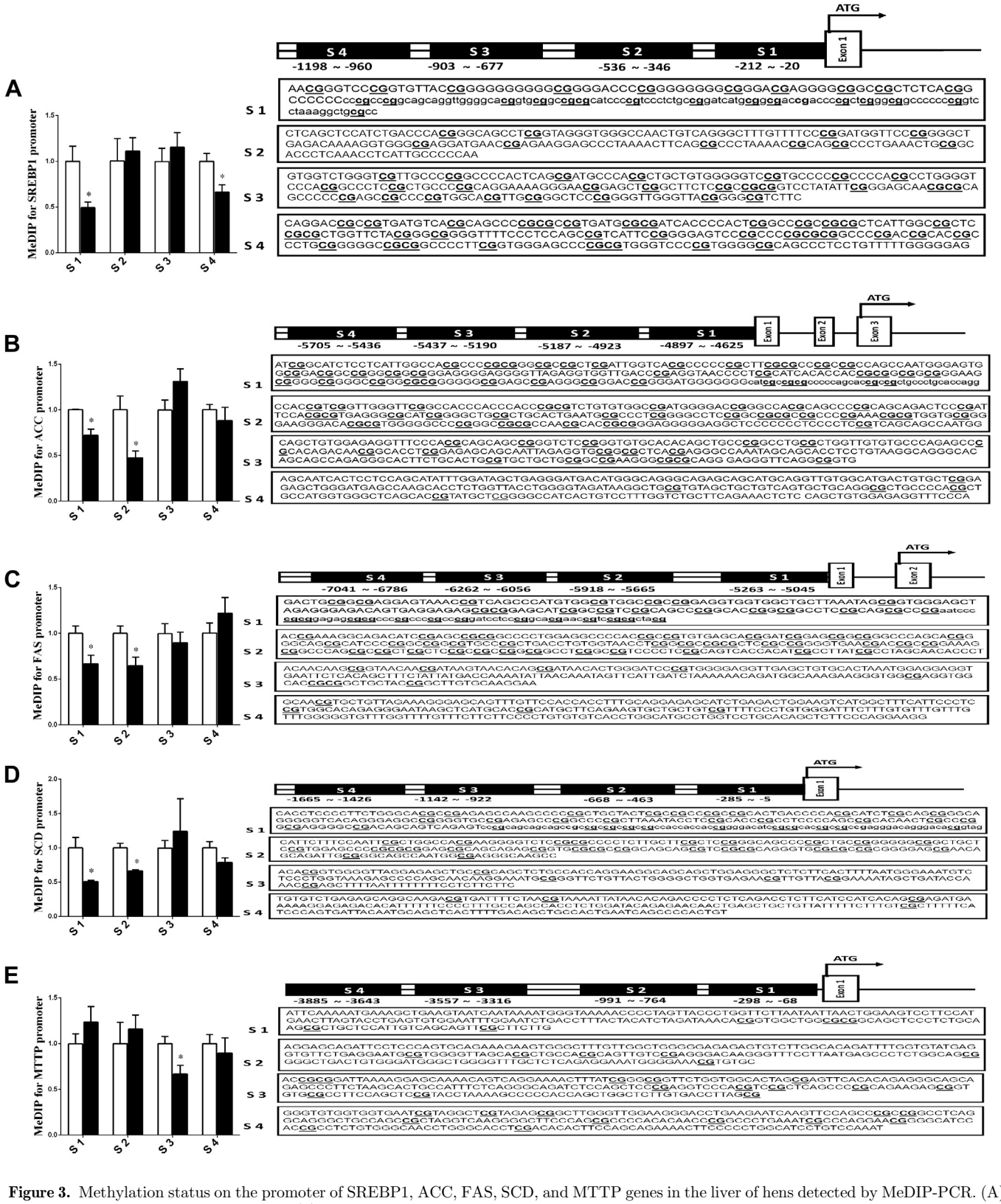

Figure 3. Methylation status on the promoter of SREBP1, ACC, FAS, SCD, and MTTP genes in the liver of hens detected by MeDIP-PCR. (A) Left: DNA methylation status on 4 segments (S) of *SREBP1* promoter; right: schematic diagram showing the amplified segments and their sequences. (B) Left: DNA methylation status on 4 segments of *ACC* promoter; right: schematic diagram showing the amplified segments and their sequences. (C) Left: DNA methylation status on 4 segments of *FAS* promoter; right: schematic diagram showing the amplified segments and their sequences. (D) Left: DNA methylation status on 4 segments of *SCD* promoter; right: schematic diagram showing the amplified segments and their sequences. (E) Left: DNA methylation status on 4 segments of MTTP promoter; right: schematic diagram showing the amplified segments and their sequences. Values are means ± SEM, $^*P < 0.05$, compared with control (n = 3). Abbreviations: ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; MeDIP, methylated DNA immunoprecipitation; MTTP, microsomal triglyceride transfer protein; SCD, stearoyl-CoA desaturase; SREBP1, sterol regulatory element-binding protein-1.

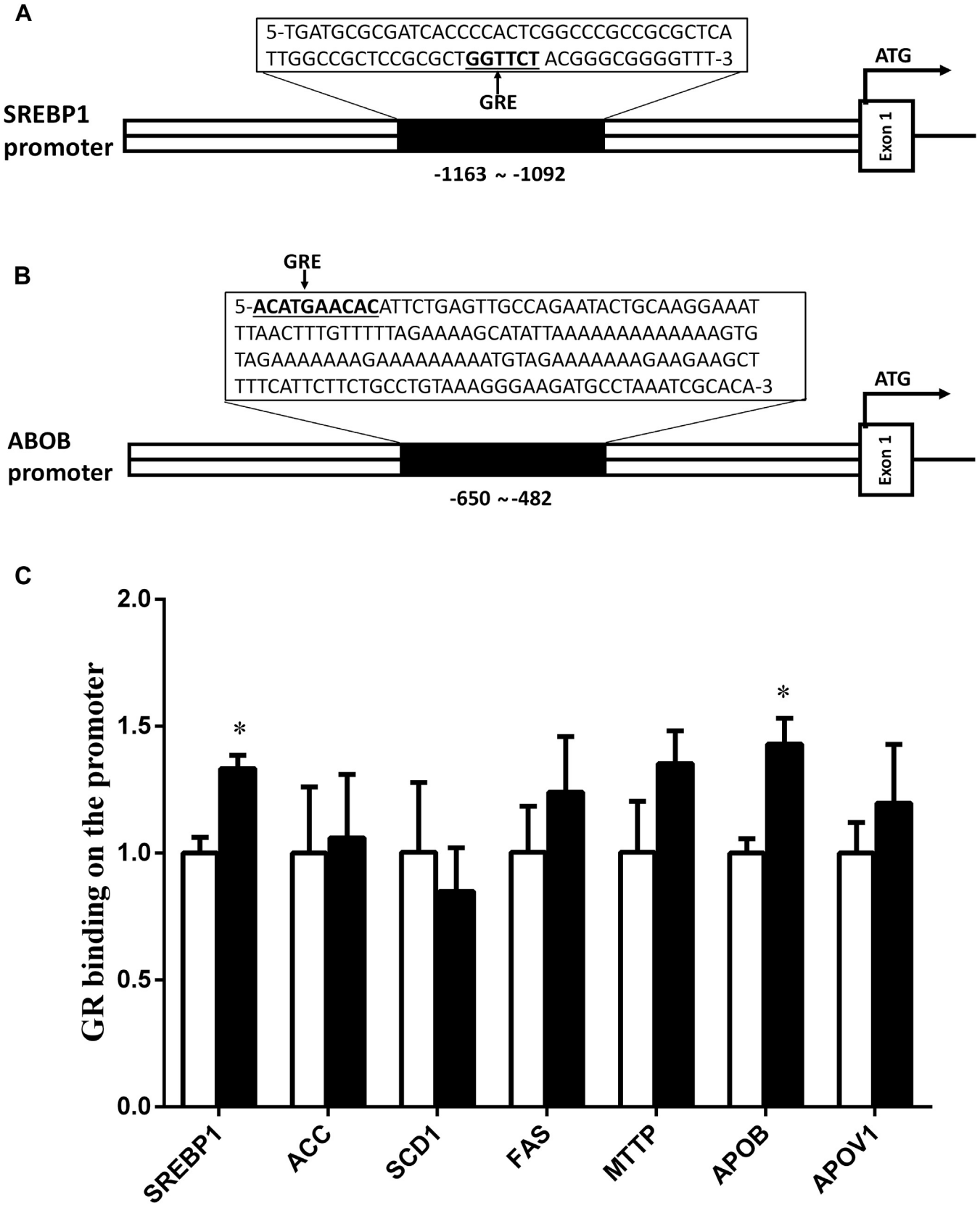

Figure 4. GR binding to the promoter of affected genes. (A) Schematic diagram showing the promoter sequences of chicken *SREBP1* gene. (B) Schematic diagram showing the promoter sequences of chicken *ApoB* gene. (C) ChIP assays of GR binding on the promoter of *SREBP1, ACC, SCD1, FAS, MTTP, ApoB*, and *ApoV1*. Values are means ± SEM, $*P < 0.05$, compared with control (n = 3). Abbreviations: ACC, acetyl-CoA carboxylase; APOB1, apolipoprotein B1; APOV1, apolipoprotein-V1; FAS, fatty acid synthase; GR, glucocorticoid receptor; MTTP, microsomal triglyceride transfer protein; SCD1, stearoyl-CoA desaturase 1; SREBP1, sterol regulatory element-binding protein-1.

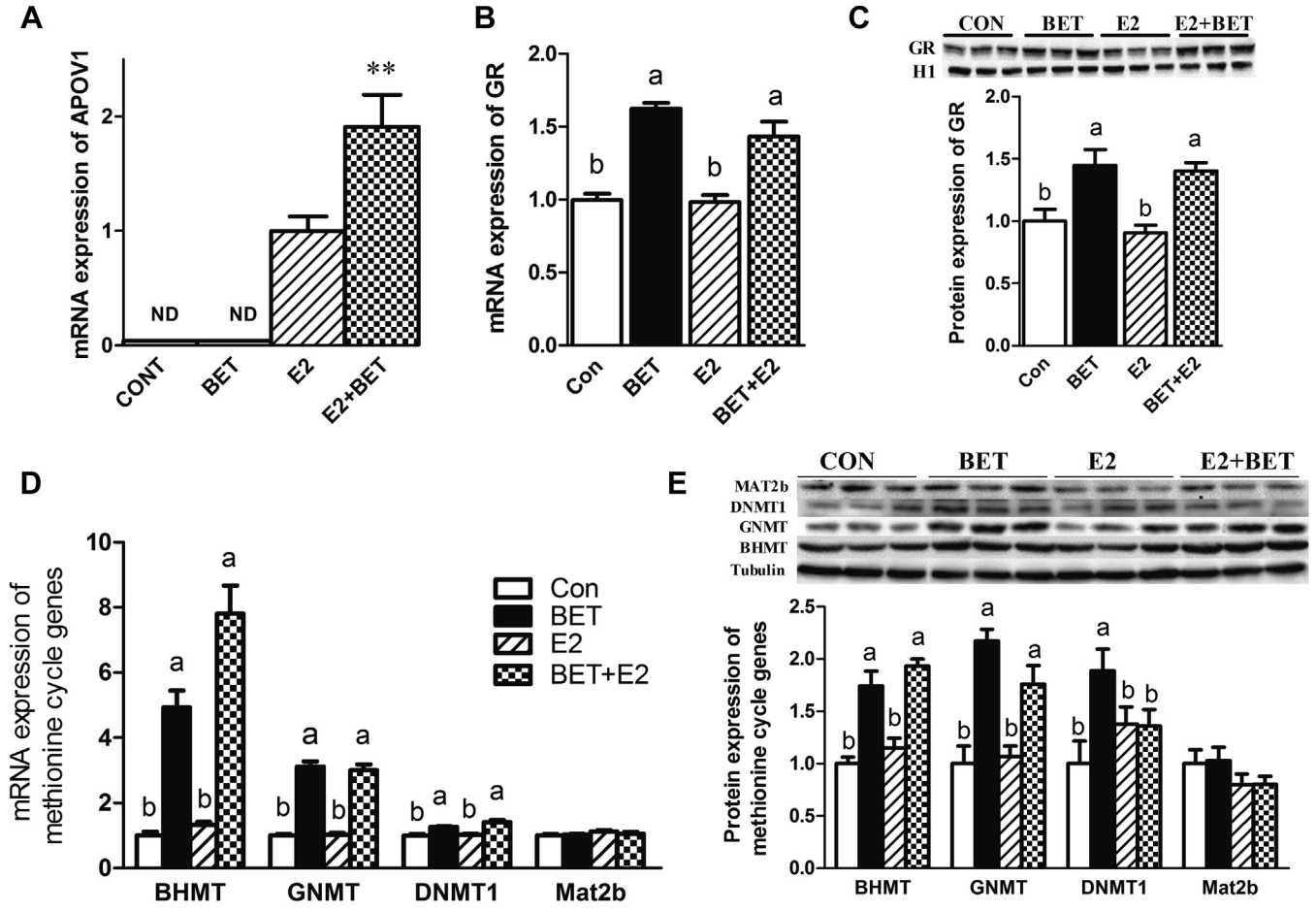

Figure 5. Effect of betaine on the expression of ApoV1, GR, and methionine cycle related genes in vitro. (A) mRNA expression of *ApoV1*, (B) mRNA expression of *GR*. (C) Protein expression of GR. (D) mRNA expression of *BHMT*, *GNMT*, *DNMT1*, *MAT2b*. (E) Protein expression of BHMT, GNMT, DNMT1, MAT2b. Values are means ± SEM, different letters above the bars indicate significantly different mean value at $P < 0.05$ compared with control, $^{*}P < 0.05$, $^{**}P < 0.01$, (n = 3). ND: *Ct* was not detectable. Abbreviations: APOV1, apolipoprotein-V1; BHMT, betaine homocysteine methyltransferase; DNMT1, DNA (cytosine-5-)-methyltransferase 1; GNMT, glycine-N-methyltransferase; GR, glucocorticoid receptor; MAT2b, methionine adenosyltransferase 2 subunit beta; MTTP, microsomal triglyceride transfer protein.

liver. It is worth to do a further in-depth investigation to test the above hypothesis with different conditions, such as higher dietary protein level.

In the present study, MeDIP results showed that betaine supplementation lowered the methylation status of the promoter region of target genes. It is not new phenomenon that betaine as a methyl donor, induced promoter hypomethylation. Previously, we showed that betaine reduced the methylation status of the GR gene promoter (Omer et al., 2018). Moreover, Wang et al. reported that addition of betaine decreased methylation level of hepatic PPARα promoter in mice and suggested that betaine selectively decrease the methylation status of certain genes involved in lipid metabolism (Wang et al., 2013). CpG hypomethylation of gene promoter may promote the binding activity of transcription factor to its specific response element (Moore ey al., 2013). In fact, hypomethylation on one of the analyzed segment of SREBP1 gene promoter was matched by a predicted GRE and might contribute to the increased GR binding, which was detected by ChIP method in the liver of betaine-fed hens. Glucocorticoid receptor is known regulator of several genes encoding enzymes in FA and TG synthesis (Wang et al., 2012). Furthermore, apart from GR effect, SREBP1 alone is well-known master regulator of lipids homeostasis (Horton et al., 2002; Eberlé et al., 2004).

This study demonstrated that dietary betaine supplementation induced hepatic lipogenesis as well as TG formation and VLDL export in laying hens. These changes were associated with alteration in the expression, promoter methylation, and GR binding to some genes involved. Our findings suggested a distinct mechanism for improved eggs production by betaine supplementation in hens. However, further studies are necessary to determine the limitation regarding timing and dose, in addition to impact of treatment on animal health and welfare.

ACKNOWLEDGMENTS

Funding: This work was supported by the National Natural Science Foundation of China (31672512), the Fundamental Research Funds for the Central Universities (KYZ201212), the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), and Jiangsu Collaborative Innovation

Centre of Meat Production and Processing, Quality and Safety Control.

Ethics Statement: The Animal Ethics Committee in Nanjing Agricultural University approved the experimental protocol, with the project number 31672512. The sampling procedures complied with the "Guidelines on Ethical Treatment of Experimental Animals" (2006) No. 398 set by the Ministry of Science and Technology, China.

Conflict of interest statement: The authors did not provide a conflict of interest statement.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at http://doi.org/10.1016/j.psj.2020.01.017.

REFERENCES

Ahn, C. W., Y. J. Choi, S. H. Hong, D. S. Jun, J. D. Na, Y. J. Choi, and Y. C. Kim. 2015. Involvement of multiple pathways in the protection of liver against high-fat diet-induced steatosis by betaine. J. Funct. Foods. 17:66–72.

Anderson, O. S., K. E. Sant, and D. C. Dolinoy. 2012. Nutrition and epigenetics: an interplay of dietary methyl donors, one-carbon metabolism and DNA methylation. J. Nutr. Biochem. 23:853–859.

Cai, D., Y. Jia, H. Song, S. Sui, J. Lu, Z. Jiang, and R. Zhao. 2014a. Betaine supplementation in maternal diet modulates the epigenetic regulation of hepatic gluconeogenic genes in neonatal piglets. PLoS One. 9:e105504.

Cai, D., Y. Jia, J. Lu, M. Yuan, S. Sui, H. Song, and R. Zhao. 2014b. Maternal dietary betaine supplementation modifies hepatic expression of cholesterol metabolic genes via epigenetic mechanisms in newborn piglets. Br. J. Nutr. 112:1459–1468.

Chan, L., W. A. Bradley, and A. R. Means. 1980. Amino acid sequence of the signal peptide of apoVLDL-II, a major apoprotein in avian very low density lipoproteins. J. Biol. Chem. 255:10060–10063.

Cherian, G. 2015. Nutrition and metabolism in poultry: role of lipids in early diet. J. Anim. Sci. Biotechnol. 6:28.

Choi, S. H., and H. N. Ginsberg. 2011. Increased very low density lipoprotein (VLDL) secretion, hepatic steatosis, and insulin resistance. Trends Endocrinol. Metab. 22:353–363.

Craig, S. A. 2004. Betaine in human nutrition. Am. J. Clin. Nutr. 80:539–549.

Day, C. R., and S. A. Kempson. 2016. Betaine chemistry, roles, and potential use in liver disease. Biochim. Biophys. Acta. 1860:1098–1106.

Eberlé, D., B. Hegarty, P. Bossard, P. Ferré, and F. Foufelle. 2004. SREBP transcription factors: master regulators of lipid homeostasis. Biochimie. 86:839–848.

Farré, D., R. Roset, M. Huerta, J. E. Adsuara, L. Roselló, M. M. Albà, and X. Messeguer. 2003. Identification of patterns in biological sequences at the ALGGEN server: PROMO and MALGEN. Nucleic Acids Res. 31:3651–3653.

Furuse, M., A. Murai, K. Kita, K. Asakura, and J. Okumura. 1991. Lipogenesis depending on sexual maturity in female Japanese quail (Coturnix coturnix Japonica). Comp. Biochem. Physiol. B. 100:343–345.

Gudev, D., S. Popova-Ralcheva, I. Yanchev, P. Moneva, E. Petkov, and M. Ignatova. 2011. Effect of betaine on egg performance and some blood constituents in laying hens reared indoor under natural summer temperatures and varying levels of air ammonia. Bulg. J. Agric. Sci. 17:859–866.

Hayes, K. C., A. Pronczuk, M. W. Cook, and M. C. Robbins. 2003. Betaine in sub-acute and sub-chronic rat studies. Food Chem. Toxicol. 41:1685–1700.

Horton, J. D., J. L. Goldstein, and M. S. Brown. 2002. SREBPs: activators of the complete program of cholesterol and synthesis in the liver. J. Clin. Invest. 109:1125–1131.

Hu, Y., Q. Sun, X. Li, M. Wang, D. Cai, X. Li, and R. Zhao. 2015. In Ovo injection of betaine affects hepatic cholesterol metabolism through epigenetic gene regulation in newly hatched chicks. PLoS One. 10:e0122643.

Kathirvel, E., K. Morgan, G. Nandgiri, B. C. Sandoval, M. A. Caudill, T. Bottiglieri, S. W. French, and T. R. Morgan. 2010. Betaine improves nonalcoholic fatty liver and associated hepatic insulin resistance: a potential mechanism for hepatoprotection by betaine. Am. J. Physiol. Gastrointest. Liver Physiol. 299:G1068–G1077.

Kirchgessner, T. G., C. Heinzmann, K. L. Svenson, D. A. Gordon, M. Nicosia, H. G. Lebherz, A. J. Lusis, and D. L. Williams. 1987. Regulation of chicken apolipoprotein B: cloning, tissue distribution, and estrogen induction of mRNA. Gene. 59:241–251.

Li, H., Z. Li, and X. Liu. 2017. An overall view of the regulation of hepatic lipid metabolism in chicken revealed by new-generation sequencing. In Poultry Science. M. Manafi ed. IntechOpen, London, UK.

Liu, Z., Q. Li, R. Liu, G. Zhao, Y. Zhang, M. Zheng, H. Cui, P. Li, X. Cui, and J. Liu. 2016. Expression and methylation of microsomal triglyceride transfer protein and acetyl-CoA carboxylase are associated with fatty liver syndrome in chicken. Poult. Sci. 95:1387–1395.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 25:402–408.

Messeguer, X., R. Escudero, D. Farré, O. Núñez, J. Martínez, and M. M. Albà. 2002. PROMO: detection of known transcription regulatory elements using species-tailored searches. Bioinformatics 18:333–334.

Moore, L. D., T. Le, and G. Fan. 2013. DNA Methylation and its basic function. Neuropsychopharmacology. 38:23.

Nys, Y., and N. Guyot. 2011. Egg formation and chemistry. Pages 83–132. in Improving the Safety and Quality of Eggs and Egg Products. Y. Nys, M. Bain and F. Van Immerseel eds. Woodhead Publishing Limited, Cambridge, UK.

Olthof, M. R., T. van Vliet, P. Verhoef, P. L. Zock, and M. B. Katan. 2005. Effect of homocysteine-lowering nutrients on blood lipids: results from four randomised, placebo-controlled studies in healthy humans. Plos Med. 2:e135.

Omer, N. A., Y. Hu, Y. Hu, A. A. Idriss, H. Abobaker, Z. Hou, H. Dong, and R. Zhao. 2018. Dietary betaine activates hepatic VTGII expression in laying hens associated with hypomethylation of GR gene promoter and enhanced GR expression. J. Anim. Sci. Biotechnol. 9:2.

Razin, A. 1998. CpG methylation, chromatin structure and gene silencing-a three-way connection. EMBO J. 17:4905–4908.

Riegler, B., C. Besenboeck, R. Bauer, J. Nimpf, and W. J. Nimpf. 2011. Enzymes involved in hepatic acylglycerol metabolism in the chicken. Biochem. Biophys. Res. Commun. 406:257–261.

Song, Z., I. Deaciuc, Z. Zhou, M. Song, T. Chen, D. Hill, and C. J. McClain. 2007. Involvement of AMP-activated protein kinase in beneficial effects of betaine on high-sucrose diet-induced hepatic steatosis. Am. J. Physiol. Gastrointest. Liver Physiol. 293:G894–G902.

Song, Z., I. Deaciuc, Z. Zhou, M. Song, T. Chen, D. Hill, and C. J. McClain. 2017. Involvement of AMP-activated protein kinase in beneficial effects of betaine on high-sucrose diet-induced hepatic steatosis. Am. J. Physiol. Gastrointest. Liver Physiol. 293:G894–G902.

Sparks, J. D., H. L. Collins, D. V. Chirieac, J. Cianci, J. Jokinen, M. P. Sowden, C. A. Galloway, and C. E. Sparks. 2006. Hepatic very-low-density lipoprotein and apolipoprotein B production are increased following in vivo induction of betaine-homocysteine S-methyltransferase. Biochem. J. 395:363–371.

Speake, B. K., A. M. Murray, and R. C. Noble. 1998. Transport and transformations of yolk lipids during development of the avian embryo. Prog. Lipid Res. 37:1–32.

Vézina, F., K. G. Salvante, and T. D. Williams. 2003. The metabolic cost of avian egg formation: possible impact of yolk precursor production? J. Exp. Biol. 206:4443–4451.

Walzem, R. L., R. J. Hansen, D. L. Williams, and R. L. Hamilton. 1999. Estrogen induction of VLDLy assembly in egg-laying hens. J. Nutr. 129:467S–472S.

Wang, J., N. E. Gray, T. Kuo, and C. A. Harris. 2012. Regulation of triglyceride metabolism by glucocorticoid receptor. Cell Biosci. 2:19.

Wang, L., L. Chen, Y. Tan, J. Wei, Y. Chang, T. Jin, and H. Zhu. 2013. Betaine supplement alleviates hepatic triglyceride accumulation of apolipoprotein E deficient mice via reducing methylation of peroxisomal proliferator-activated receptor alpha promoter. Lipids Health Dis. 12:34.

Wang, L. J., H. W. Zhang, J. Y. Zhou, Y. Liu, Y. Yang, X. L. Chen, C. H. Zhu, R. D. Zheng, W. H. Ling, and H. L. Zhu. 2014. Betaine attenuates hepatic steatosis by reducing methylation of the MTTP promoter and elevating genomic methylation in mice fed a high-fat diet. J. Nutr. Biochem. 25:329–336.

Wetterau, J. R., M. C. Lin, and H. Jamil. 1997. Microsomal triglyceride transfer protein. Biochim. Biophys. Acta. 1345:136–150.

Wu, J., W. Fu, Y. Huang, Y. Ni, and R. Zhao. 2013. Kisspeptin-10 enhanced egg production in quails associated with the increase of triglyceride synthesis in liver. Asian-australas J. Anim. Sci. 26:1080–1088.

Xing, J., L. Kang, and Y. Jiang. 2011. Effect of dietary betaine supplementation on lipogenesis gene expression and CpG methylation of lipoprotein lipase gene in broilers. Mol. Biol. Rep. 38:1975–1981.

Xing, J., and Y. Jiang. 2012. Effect of dietary betaine supplementation on mRNA level of lipogenesis genes and on promoter cpg methylation of fatty acid synthase (fas) gene in laying hens. Afr. J. Biotechnol. 11:6633–6640.

Xu, L., D. Huang, Q. Hu, J. Wu, Y. Wang, and J. Feng. 2015. Betaine alleviates hepatic lipid accumulation via enhancing hepatic lipid export and fatty acid oxidation in rats fed with a high-fat diet. Br. J. Nutr. 113:1835–1843.

Yu, C. Y., O. Mayba, J. V. Lee, J. Tran, C. Harris, T. P. Speed, and J. Wang. 2010. Genome-wide analysis of glucocorticoid receptor binding regions in adipocytes reveal gene network involved in triglyceride homeostasis. PLoS One. 5:e15188.

Zou, X. T., and J. J. Lu. 2002. Effect of betaine on the regulation of the lipid metabolism in laying hen. Agric. Sci. China. 1:1043–1049.