RESEARCH ARTICLE

Branched-chain amino acids modulate the expression of hepatic fatty acid metabolism-related genes in female broiler chickens

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Scope: The effects and roles of branched-chain amino acids (BCAAs) in hepatic fat metabolism are still unknown.

Methods and results: Here, we used broiler chickens, in which lipogenesis occurs essentially in the liver as in human, to investigate the effects of three levels of BCAAs (control "C," low "L" and exogenous supplemented diet "L+S") on growth, carcass traits, immunity, and hepatic fat metabolism. Despite the same productive performance, immunity, and plasma BCAA levels between all groups, low BCAA levels significantly downregulated the hepatic expression of lipogenic genes particularly acetyl-CoA carboxylase alpha (ACCα) and stearoyl-coA desaturase 1 ($p = 0.0036$ and $p = 0.0008$, respectively) and upregulated the hepatic expression of mitochondrial β-oxidation- (uncoupling protein and NRF-1, $p < 0.05$) and dynamic-related genes (DNM1, $p < 0.05$). Concomitant with these changes, low BCAA levels increased the phosphorylation of AMP-activated protein kinase (AMPK)α^{Thr172}, ACCα^{Ser79}, and forkhead box protein O1 (FoxO1^{Ser256}) and decreased the phosphorylation of mTOR^{Ser2481} and P70 S6 kinase ^{Thr389}. The mRNA abundance of the transcription factors SREBP1/2, peroxisome proliferator activated receptor alpha/beta, and FoxO1 were also increased in the liver of L group compared to the control.

Conclusion: Together our data indicate that low BCAA levels inhibit fatty acid synthesis and enhanced fatty acid β-oxidation in the liver of female broiler chickens and these effects were probably mediated through AMPK-mTOR-FoxO1 pathway.

Keywords:
AMPK / BCAAs / FoxO1 / Lipogenesis / mTOR / β-Oxidation

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1 Introduction

The essential amino acids, branched-chain amino acids (BCAAs) are not synthesized endogenously and need to be supplemented to the diet. They exert several critical roles in metabolism homeostasis and cell functions including immunity [1–3], survival and growth [4–6], energy homeostasis [7], and protein and lipid metabolism regulation [8–12]. Thus, their dosage needs to be closely managed because insufficient or excessive level of BCAAs in the diet can be detrimental to cell metabolism and cell growth.

BCAAs are not only substrates for various metabolic pathways, but can also serve as signaling molecules controlling signal transduction pathways and gene transcription [13–16]. They are internalized in conjunction with the system L amino acid transporter and recruit/activate the mechanistic target of rapamycin complex through Rag GTPase and Ras homolog enriched in brain (Rheb) and in turn phosphorylate several targets, including ribosomal S6 kinase (P70S6K), eukaryotic

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Abbreviations: **ACC**, acetyl-CoA carboxylase; **AMPK**, AMP-activated protein kinase; **BCAA**, branched-chain amino acid; **FCR**, feed conversion ratio; **FoxO1**, forkhead box protein 1; **LXR**, liver X receptor; **ME**, malic enzyme; **SCD-1**, stearoyl-coA desaturase 1; **Ski**, nuclear sarcoma viral oncogene homolog; **TF**, transcription factor; **UCP**, uncoupling protein

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initiation factor 4E-binding protein 1(4E-BP1), Unc-51-like kinase 1 (ULK1), forkhead box protein O (FoxO), and the transcription factor EB (TFEB) to control the regulation of protein synthesis/degradation, autophagy, and several other cell functions [17–21].

Although the effects of BCAAs are well established in muscle, i.e. stimulation of protein synthesis [22–24] and reduction of proteasome-mediated proteolysis [25, 26], their effects on fat metabolism has yet to be elucidated.

As in human, chicken liver, not adipose tissue, is the primary site for lipid synthesis [27–29]; however, in rodents lipogenesis occurs in both adipose tissue and liver [30]. In addition, chickens are characteristically hyperglycemic compared with mammals, with their blood glucose levels averaging three times that found in humans (300 versus 100 mg/dL) [31]. Genetic selection for rapid growth rate driven by economic demands has resulted in hyperphagic, heavy, and obese broiler chickens. They voraciously consume approximately 4.1 kg of feed to achieve a 40-fold increase in body weight arising from breast (pectoralis) muscle and abdominal fat during a period of 42 days [32, 33]. Chickens are insulin resistant [34, 35] and lacking glucose transporter glucose transporter 4 [36]. They require insulin doses greater than four times that required in mammals to achieve hypoglycemia [37]. These peculiarities make chicken an interesting model for understanding the role of BCAAs in the regulation of hepatic fat metabolism. Since BCAAs are metabolic signature in obesity and insulin resistance, insights into their underlying molecular mechanisms in fat metabolism are of uppermost interest not only in animal biology for health and feed efficiency improvement, but also in molecular nutrition and medicine for potential nutritional supplement optimization and therapeutic perspectives.

Therefore, the present experimental study aimed to assess the effects of different levels of BCAAs on Cobb 500 female broiler growth, carcass traits, immunity, and hepatic fat metabolism.

2 Materials and methods

2.1 Animal, diet, and experimental design

Cobb 500 female chickens ($n = 832$) where obtained from a local commercial hatchery and were housed in a conventional environmentally controlled poultry shed. The lighting schedule was 23-h-light/1-h-dark cycle for the first 7 d of life, 18-h-light/6-h-dark cycle thereafter (dark period from 0000 to 0400). Environmental temperature was progressively reduced from 32°C to 23°C. Clean water and food were freely available during the whole experiment period. All the chickens received the same starter feed (2900 kcal/kg ME; crude protein 21%).

During the grower rearing phase (14–28 d of age), chickens were divided in three body weight-matched groups. The first group was fed with low BCAA-enriched diet (L group) containing 72% Val/Lys-63% Ile/Lys (ten replicate pens, 32 birds/pen). The second group (L + S group) was subjected to 72% Val/Lys-63% Ile/Lys and then Val and Ile were added to reach 77% Val/Lys and 69% Ile/Lys (ten replicate pens). The control group (C) was fed with a control diet containing 77% Val/Lys-69% Ile/Lys that conforms to levels in practice (six replicate pens). The diet composition is presented in Supporting Information Table 1. Birds were weighed on 14 and 28 d and feed consumption was recorded. At the end of the experiment, chickens were cervically dislocated after blood sampling ($n = 10$). Breast, tender, wings, thigh bones, thigh skin, thigh meat, and drumsticks weights were recorded ($n = 60$). Liver tissues ($n = 6$–10) were removed, quickly snap frozen in liquid nitrogen and stored at –80°C until analysis.

The present study was conducted in accordance with the recommendations in the guide for the care and use of laboratory animals of the National Institutes of Health and the protocol was approved by the University of Arkansas Institutional Animal Care and Use Committee.

2.2 Blood samples

Plasma amino acid levels were measured by HPLC as previously described [38, 39]. Hematologic parameters were measured using CELL-DYN 3500 system as previously described [40]. Plasma triglyceride and cholesterol levels were measured using commercial kit (CHIRON Diagnostics, Cergy Pontoisr, France) according to manufacturer's recommendations. Circulating glucose concentrations were determined using commercial kit from CIBA Corning (OH, USA). Plasma uric acid levels were measured using reagents from Pointe Scientific (MI, USA) according to manufacturer's recommendations.

2.3 Reverse transcription and real-time quantitative PCR

Total RNA was extracted from liver tissues using Trizol reagent (Life Technologies, Grand Island, NY) according to manufacturer's recommendations, DNAse treated and reverse transcribed (Quanta Biosciences, Gaithersburg, MD). The RT products (cDNAs) were amplified by real-time quantitative PCR (Applied Biosystems 7500 Real-Time PCR system) with Power SYBR green Master Mix. Oligonucleotide primers specific for chicken fatty acid synthase, acetyl-CoA carboxylase alpha (ACCα), malic enzyme (ME), ATP citrate lyase, stearoyl-CoA desaturase-1 (SCD-1), sterol regulatory element-binding protein-1 and 2 (SREBP-1 and 2), peroxisome proliferator-activated receptor alpha and gamma (PPARα and PPARγ), nuclear respiratory factor-1 (NRF-1), forkhead box protein O1 (FoxO1), avian uncoupling protein (av-UCP), the nuclear sarcoma viral oncogene homolog (Ski), dynamin 1 (DNM1), Mitofusin 1 and 2 (MFN1 and 2), optic atrophy 1 (OPA1), and ribosomal 18S as a housekeeping gene were used (Supporting Information Table 2).

The qPCR cycling conditions were 50°C for 2 min, 95°C for 10 min followed by 40 cycles of a two-step amplification program (95°C for 15 s and 58°C for 1 min). At the end of the amplification, melting curve analysis was applied using the dissociation protocol from the Sequence Detection system to exclude contamination with unspecific PCR products. The PCR products were also confirmed by agarose gel and showed only one specific band of the predicted size. For negative controls, no RT products were used as templates in the qPCR and verified by the absence of gel-detected bands. Relative expressions of target genes were determined by the $2^{-\Delta\Delta Ct}$ method [41].

2.4 Western blot analysis

Liver tissues were homogenized in lysis buffer (10 mM Tris base, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% Triton X-100, 0.5% NP-40, protease, and phosphatase inhibitor cocktail). Protein concentrations were determined using Synergy HT multimode microplate reader (BioTek, Winooski, VT) and a Bradford assay kit (Bio-Rad, Hercules, CA) with BSA as a standard. Proteins (70 μg) were run on 4–12% Novex Bis–Tris gels (Life Technologies, Grand Island, NY) or 10% tris-glycine gel (Bio-Rad, Hercules, CA). The transferred membranes were blocked for 1 h at room temperature, and incubated with primary antibodies (diluted 1:500–1:1000) at 4°C overnight. The polyclonal antibodies used were as follows: anti-phospho AMP-activated protein kinase (AMPK)α^{Thr172}, anti-phospho mTOR^{Ser2448}, anti-phospho FoxO1^{Thr24}/FoxO3a^{Thr32}, anti-phospho FoxO1^{Ser256}, anti-phospho FoxO1^{Thr24}/FoxO3a^{Thr32}/FoxO4^{Thr28}, anti-phospho ACCα^{Ser79}, anti-phospho P70 S6 Kinase^{Thr389}, anti-AMPKα, anti-mTOR, anti-FoxO1, anti-beclin 1, anti-Atg3, anti-β actin, and anti-vinculin. All the primary antibodies were purchased from Cell Signaling Technology (Danvers, MA) except anti-beclin1, anti-atg3, anti-phospho P70 S6 kinase^{Thr389} from Thermo Scientific Pierce Antibodies (Rockford, IL), and anti-vinculin from Sigma-Aldrich (St. Louis, MO). The secondary antibodies were used (1:5000) for 1 h at room temperature. The signal was visualized by enhanced chemiluminescence (ECL plus) (GE Healthcare, Buckinghamshire, UK) and captured by FluorChem M MultiFluor System (Proteinsimple, Santa Clara, CA). Image Acquisition and Analysis were performed by AlphaView software (Version 3.4.0, 1993–2011, Proteinsimple, Santa Clara, CA).

2.5 Statistical analyses

Data were analyzed by one-factor ANOVA. Significant differences among individual group means were determined with Student–Newman–Keuls's multiple range test using the Graph Pad Prism version 6.00 for Windows, Graph Pad Software, La Jolla California USA. Significance was set at $p < 0.05$. Data are expressed as the mean ± SEM.

3 Results

3.1 Effects of BCAAs availability on body weight, feed conversion ratio, and carcass traits

All groups of chicken exhibited similar body weights and feed conversion ratios (FCRs) (Supporting Information Table 3). Next, we evaluated the weight of several organs including the liver, the paw and the thigh bone, the abdominal fat, the minor, the major, the carcass, the wing, the breast, and the thigh meat and no significant differences were observed between the groups (Supporting Information Table 3).

3.2 BCAAs did not alter the immune response and plasma amino acid levels

There was no difference in circulating BCAA levels and percentage of immune cells (neutrophils, lymphocytes, monocytes, eosinophils, and basophils) among treatments (Supporting Information Table 3).

3.3 BCAAs downregulated the hepatic expression of fatty acid synthesis-related genes

The mRNA abundance of all lipogenic genes studied here tended to decrease in the liver of L group compared to that of the control (C) group with a dramatic downregulation of ACCα (–34%) and SCD-1 (–50%) gene expression ($p = 0.0036$ and $p = 0.0008$, respectively) (Fig. 1B and E). The addition of synthesized BCAAs in the diet (L + S group) increased the mRNA levels of SCD-1 close to that of the control (C group), but did not affect the expression of ACCα gene (Fig. 1B and E).

Since the transcription of these genes is under the control of several transcription factors (TFs), we next determined the expression of the most common TFs; SREBP-1, SREBP-2, PPARα, and PPARγ genes. Surprisingly, the hepatic expression of these transcription factors, except PPARα, was significantly upregulated in the L group compared to the control group ($p < 0.05$) (Fig. 2). The addition of BCAAs in the diet was able to bring PPARγ and SREBP-2 mRNA abundance, but not the other mRNAs, to the same level observed in the control group (Fig. 2B and D).

3.4 BCAAs affected AMPK-mTOR-FoxO1 pathway

Since the TOR pathway-mediated BCAA effects have been well documented in the muscle we sought to evaluate this pathway in the liver. Low BCAA levels (L group) significantly induced the phosphorylation of AMPKα at Thr^{172} site ($p = 0.038$) and decreased mTOR phosphorylation at Ser^{2448} ($p = 0.0012$) and p 70 S6 kinase at Thr^{389} ($p = 0.008$) compared

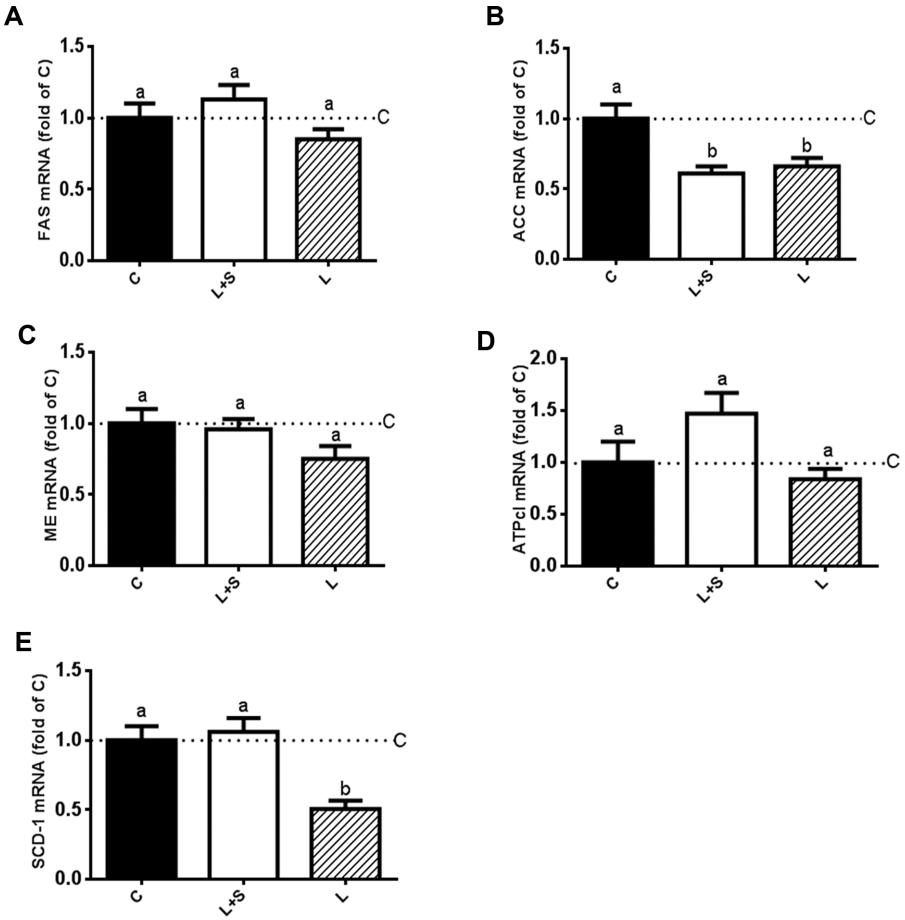

Figure 1. Hepatic expression of lipogenic genes in female broiler chickens fed different BCAA levels. Total RNA was isolated from the liver of female broiler chickens fed different BCAA ratios: the control diet "C" containing Ile and Val ratios to Lys of 69 and 77%, respectively, the low diet "L" containing 63% Ile/Lys and 72% Val/Lys, and supplemented group "L + S" where exogenous Ile and Val were added to L diet to reach the same BCAA ratio as the control. The RT-qPCR was performed as described in materials and methods [fatty acid synthase (A), ACCα (B), ME (C) and ATP citrate lyase (D) and SCD-1 (E)]. Relative expression was determined by $2^{-\Delta\Delta CT}$ method and data are presented as means ± SEM of six chickens per group. Means without a common letter differ, $p < 0.05$.

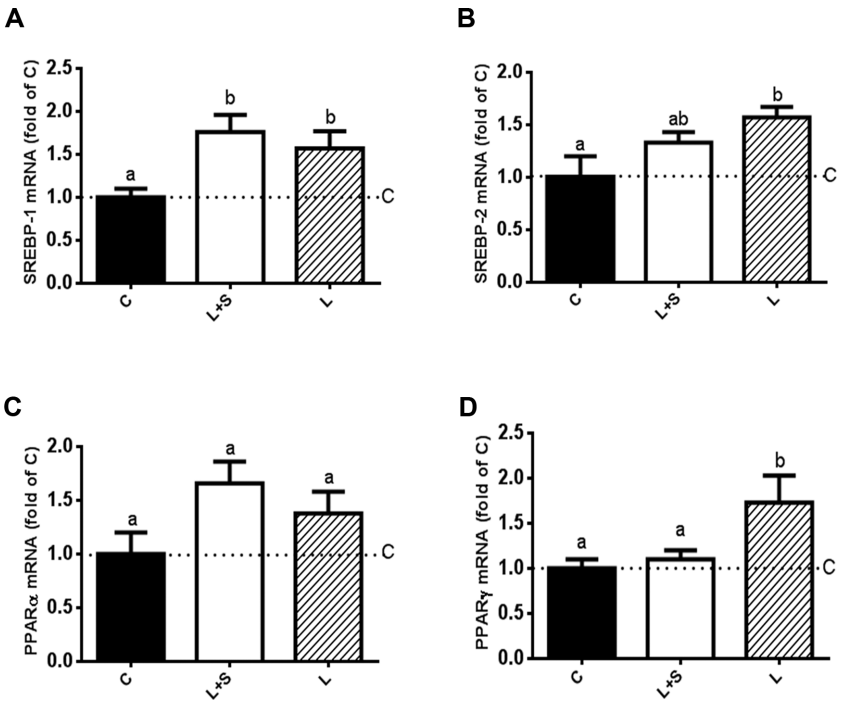

Figure 2. Hepatic expression of transcription factors in female broiler chickens fed different BCAA levels. The relative expression of SREBP-1 (A), SREBP-2 (B), PPARα (C), and PPARγ (D) was determined, using $2^{-\Delta\Delta CT}$ method, by RT-qPCR in three groups of chicken fed different BCAA-enriched diets: the control diet "C" containing Ile and Val ratios to Lys of 69 and 77%, respectively, the low diet "L" containing 63% Ile/Lys and 72% Val/Lys, and supplemented group "L + S" where exogenous Ile and Val were added to L diet to reach the same BCAA ratio as the control. Data are presented as means ± SEM of six chickens per group. Means without a common letter differ, $p < 0.05$.

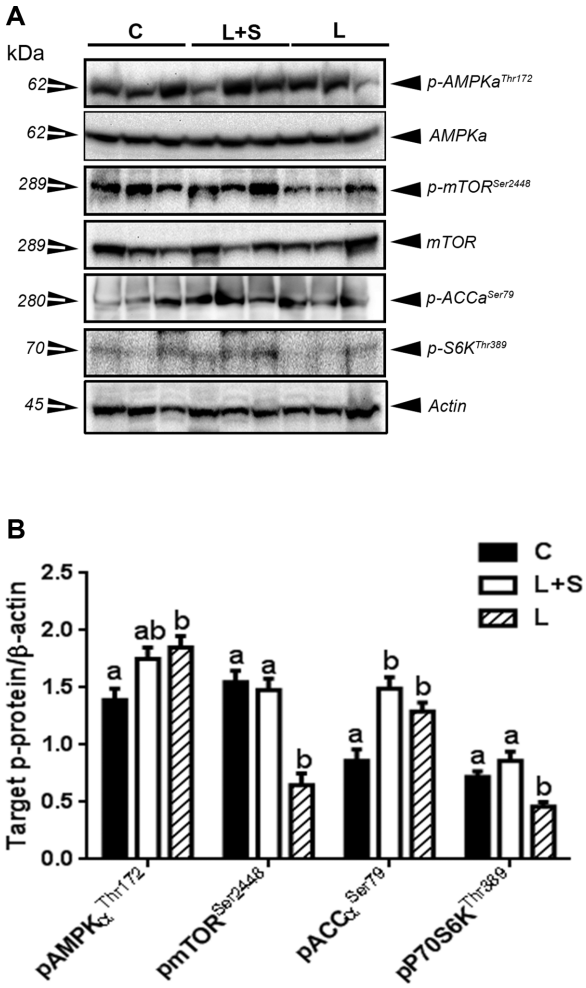

Figure 3. Western blot analysis of AMPK, mTOR, ACCα, and P70 S6 kinase phosphorylation in female broiler chicken liver fed different BCAA-enriched diets. (A) Comparison of control (C) fed with 69% Ile/Lys-77% Val/Lys-enriched diet with L group fed with 63% Ile/Lys-72% Val/Lys-enriched diet and L + S group where exogenous BCAA were added to the L group to reach the same ratio as the control group. (B) Graphs represent the ratio between the phosphorylated protein and the housekeeping protein β-actin. Data are represented as arbitrary unit and results are mean ± SEM of six chickens per group. Means without a common letter differ, $p < 0.05$.

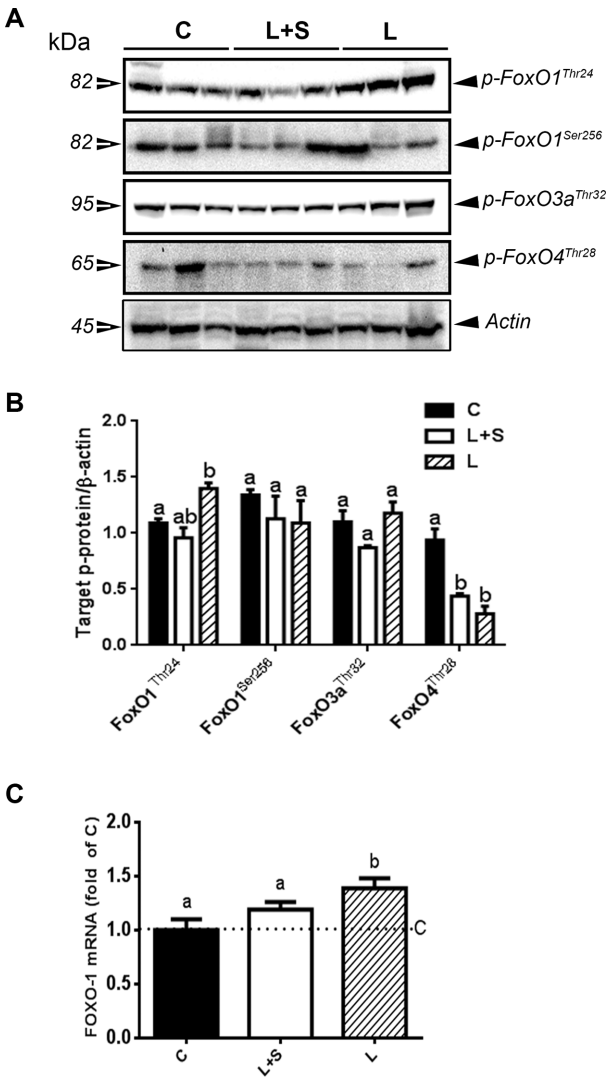

Figure 4. Effects of BCAAs on hepatic FoxO mRNA levels and phosphorylation in female broiler chickens. Representative immunoblots (A) and densitometric analysis (B) of phospho FoxO1, phospho FoxO3a, and phospho FoxO4 in the liver of broiler chickens fed different ratios of BCAA: C (69% Ile/Lys-77% Val/Lys), L (63% Ile/Lys and 72% Val/Lys) and L + S (where exogenous Ile and Val were added to L diet to reach the same BCAA ratio as the control). Data are represented as arbitrary unit. (C) Relative expression of FoxO1 determined, using $2^{-\Delta\Delta CT}$ method, by RT-qPCR. Results are mean ± SEM of six chickens per group. Means without a common letter differ, $p < 0.05$.

to the control (Fig. 3A and B). The (L + S) group, where synthesized BCAAs were added, exhibited the same hepatic level of phospho mTOR, phospho P70 S6 kinase, and phospho AMPK as the control group (Fig. 3A and B). Low BCAA levels significantly induced both the mRNA expression and the phosphorylation of FoxO1 at Thr^{24} but not at Ser^{256} compared to the control group ($p = 0.015$ and 0.0072, respectively, Fig. 4A, B, and C). The phosphorylated FoxO3a^{Thr32} levels remain the same between the three groups, however the phosphorylated FoxO4^{Thr28} levels were significantly decreased in L and (L + S) groups compared to the control ($p < 0.05$, Fig. 4A and B).

3.5 BCAAs did not affect autophagy-related proteins in chicken liver

The expression of beclin-1 and Atg-3, key proteins in autophagic/lysosomal pathway, did not differ between all studied groups (beclin1, 1 ± 0.01 versus 0.96 ± 0.08 and 1.01 ± 0.1, Atg-3, 1 ± 0.03 versus 0.92 ± 0.2 and 1.05 ± 0.1 for C, S + L and L respectively, mean ± SEM, $n = 6$).

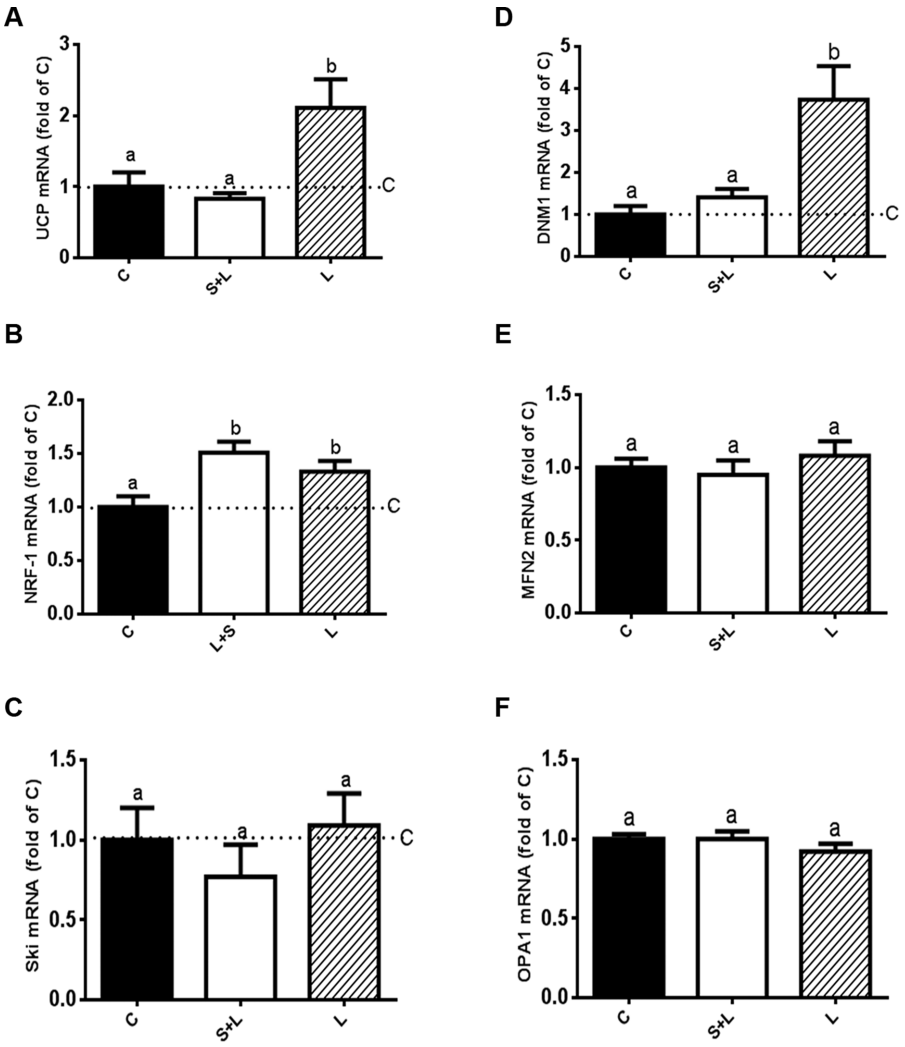

Figure 5. Effects of BCAAs on hepatic expression of fatty acid β-oxidation-related genes in female broiler chickens. The relative expression of UCP (A), NRF-1 (B), and Ski (C) was determined, using $2^{-\Delta\Delta CT}$ method, by RT-qPCR in three groups of chickens fed different ratios of BCAAs: control (C) fed with 69% Ile/Lys-77% Val/Lys-enriched diet, L group fed with 63% Ile/Lys-72% Val/Lys-enriched diet and L + S group where exogenous BCAAs were added to the L group to reach the same ratio as the control group. Data are presented as means ± SEM of six chickens per group. Means without a common letter differ, $p < 0.05$.

3.6 BCAAs altered the hepatic expression of mitochondrial β-oxidation- and biogenesis-related genes

Low BCAA levels significantly induced the hepatic expression of av-UCP ($p = 0.007$, Fig. 5A), and the nuclear respiratory factor 1 (NRF-1) ($p = 0.008$, Fig. 5B) but did not affect the Ski mRNA abundance (Fig. 5C). Interestingly, low BCAA levels significantly upregulated the hepatic expression of the mitochondrial fission-related gene, DNM1 ($p < 0.05$, Fig. 5D), but not that of the mitochondrial fusion-related genes, MFN2 and OPA1 (Fig. 5E and F).

3.7 BCAAs altered plasma metabolite levels

Low BCAA levels significantly reduced plasma triglyceride levels compared to the control (C) and L+S groups ($p < 0.05$, Fig. 6) without affecting circulating cholesterol, glucose, or uric acid levels (Fig. 6).

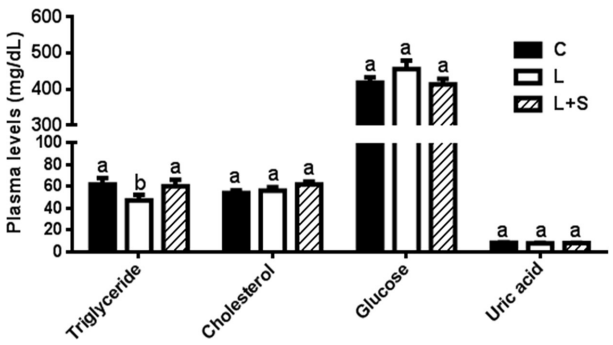

Figure 6. Effect of BCAA on plasma metabolite levels in female broiler chickens. Values are means ± SEM of six chickens per group. Means without a common letter differ, $p < 0.05$.

4 Discussion

Obesity is a devastating multifactorial disease with continuous increasing prevalence in many countries around the world [42] and is linked to diabetes and cardiovascular

diseases. Broiler chickens are hyperphagic and prone to obesity and therefore may represent a model of choice for mechanistic understanding of metabolic disorders. Dysregulation in BCAA metabolism has recently been linked to development of metabolic disorder [43] however the underlying molecular mechanisms are not completely defined.

Here, we used female broiler chickens as model of choice to study the effect of BCAA on fat metabolism because chicken liver is responsible for more than 90% of de novo fatty acid synthesis [27, 28] and it is therefore, as in human, the main site for lipogenesis. We found that variation of dietary BCAAs did not affect the chicken live performances (BW, FCR, and carcass traits) corroborating previous findings in chickens [44] and in rats [45]. In contrast, other studies showed a different patterns of responses (improvement or depression of body weight gain and FCR) [46–48]. The discrepancies between these findings might be related to several factors including gender, chicken strain and age, environmental and/or experimental conditions (diet composition, stress, density, feeding system, and BCAA ratio). For instance, the ideal BCAA ratio during the different phases of broiler rearing is still being debated and BCAA deficiency and/or excess has been reported to affect animal performances [44, 49]. From an overall live performance viewpoint, the BCAA levels used in our experimental conditions seemed to be above their minimum requirements and satisfied the female broiler chicken needs.

Next, we determined the effects of BCAA levels on hepatic lipogenic gene expression. Although, the variation of BCAA levels in the diet appeared to not influence the female broiler chicken performances, the circulating levels of BCAA and the immune response, the low level of BCAA downregulated the hepatic expression of ACCα and SCD-1 genes. ACCα is a complex multifunctional enzyme system that catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, the rate-limiting step for both synthesis and elongation of fatty acid synthesis [50, 51]. SCD-1, however, catalyzes the biosynthesis of mono unsaturated fatty acid from dietary or de novo synthesized saturated fatty acid precursors [52]. In agreement with previous findings in isolated rat hepatocytes [53, 54], together our data indicate that low BCAA levels inhibit fatty acid synthesis and disrupt the lipid composition of the cell. The decreased level of plasma triglyceride supports our hypothesis and indicates that triglycerides might be hydrolyzed to glycerol and free fatty acids, indicators of fat mobilization.

The expression of lipogenic genes including ACCα and SCD-1 is under the control of many key transcription factors [55]. Because of their high expression levels in the liver, we choose to determine the mRNA abundance of the most common TFs; sterol regulatory element-binding protein ½ (basic helix-loop-helix leucine zipper (bHLH-LZ) TF involved in the control of the biosynthesis of fatty acids and cholesterol, respectively [56]) and peroxisome proliferator activated receptor alpha/beta (ligand-activated TF involved in regulating lipid metabolism, adipogenesis, and glucose homeostasis, [57]). Unexpectedly the regulation of these TFs seemed to be opposite to that of lipogenic genes in our in vivo experimental conditions. However, we know that in vivo the networks between TFs and their target gene responses are extremely complex and distinctly regulated in a cell type- and context-dependent manner. TFs tightly interact with transcriptional cofactors to modulate chromatin compaction and the accessibility of coding regions to the transcriptional machinery [58]. Thus, the same TF can have opposite effects, depending on the nature of the coregulator (coactivator or corepressor) to which they are bound [59]. In addition TF can form heterodimer with other nuclear receptors, for example SREBP-1 with liver X receptor (LXR) [60] and PPARγ with retinoid-X receptor [57]. Our study was limited by our inability to determine the nuclear levels of these TF proteins (some antibodies did not work in our experimental protocols) and their interaction with cofactors and orphan nuclear receptors that is difficult to perform in vivo. Further in vitro experiments analyzing the interaction of these TF with coregulators in isolated chicken hepatocytes treated with different levels of BCAA will provide insight into these mechanisms.

Since the TF profile is not conclusive, and since the control of lipogenic gene (ACCα) is the product of integrated changes in substrate supply, allosteric ligands, the phosphorylation of multiple serine residues and interaction with other proteins, we sought to determine the involvement of AMPK-mTOR pathway that has been shown to mediate the effect of BCAA in muscle tissue [13]. ACCα is a substrate for AMPK and its activity is negatively regulated by phosphorylation [61]. The increase of ACCα phosphorylation at Ser^{79} and its inactivation in L group (received low BCAA-enriched diet), might be mediated through the activation of AMPK, supporting the notion of fatty acid synthesis inhibition. AMPK is a key energy sensor that regulates cellular metabolism to maintain energy homeostasis and has been shown to control ACC activity [61]. Consistent with previous data in mammals [62], the activation of AMPK might inhibit the activity of mTOR, another energy/nutrient-sensitive kinase that in turn inhibits the phosphorylation of P70S6K in the liver of L group indicating an inhibition of protein synthesis. The exact mechanism by which AMPK altered mTOR activity is not clear here and further investigations are warranted. It is possible that AMPK modulates hamartin/tuberin (also known as tuberous sclerosis 1/2, respectively) through GAP/Rheb [62] or directly phosphorylates raptor leading to the allosteric inhibition of mTOR [63]. There is emerging evidence to support that FoxO, the winged helix/forkhead class of transcription factor that acts downstream of the mTOR pathway and regulates a plethora of biological processes including lipid, energy metabolism, and glucose production [64]. In agreement with previous finding in mouse liver [65] the upregulation of hepatic FoxO1 at mRNA levels and its phosphorylation at Thr^{24} (and not Ser^{256}) indicated that low BCAA levels inhibit fatty acid synthesis through AMPK-mTOR-FoxO1 pathway. FoxO1 might directly repress the transcription of lipogenic genes that contain putative FoxO1 (FKHRs) binding sites, which are similar to insulin response element (IREs) in their

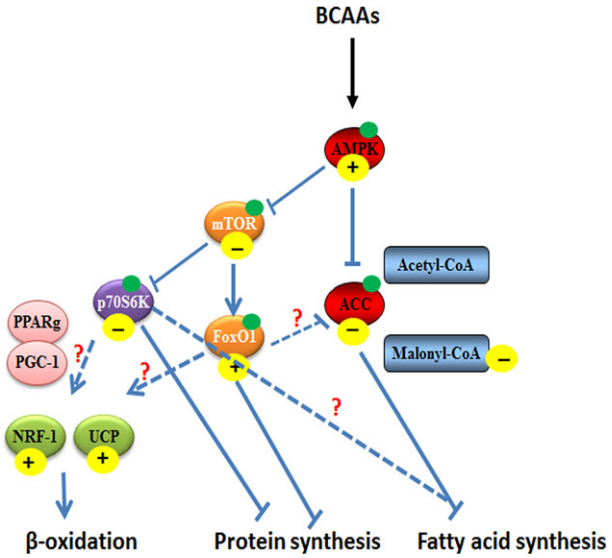

Figure 7. Schematic representation of hypothesized mechanism of BCAA effects on hepatic fatty acid synthesis and β-oxidation in female broiler chickens (from mRNA and protein expression analysis). Low levels of BCAA induced the phosphorylation of AMPK which in turn inactivated ACCα by increasing its phosphorylation and thereby inhibited fatty acid synthesis. Activated AMPK inhibited also the activation of mTOR by reducing its phosphorylation that leads to the inhibition of P70 S6 kinase and inhibition of protein synthesis. Inactivation of mTOR increased the phosphorylation of FoxO1 that has been proposed to inhibit fatty acid synthesis probably through ACCα. FoxO1 might induce β-oxidation via UCP and NRF-1 and its effect might be mediated through PPARγ and PGC-1. Phosphorylated site, + induction, –inhibition.

promoter sequences [66]. Interaction of FoxO1 with other transcription factors such as SREBP-1c/LXR/PPARγ and/or PGC-1α might account for the observed downregulation of lipogenic gene expression [65, 67, 68]. More detailed studies will be needed to dissect the mechanism(s) mediating the effect of FoxO1 on hepatic lipogenic genes. It is also probable that mTOR control hepatic fatty acid synthesis through an additional pathway involving P70S6K [69, 70].

Since both energy/nutrient sensors AMPK and mTOR were altered indicating an increased energy demand, we hypothesize that low level of BCAA might affect autophagy, mitochondrial biogenesis, and fatty acid β-oxidation. The upregulation of the hepatic expression of av-UCP (the mitochondrial anion carrier protein) and nuclear respiratory factor 1 (principal modulator of mitochondrial protein expression and mitochondrial biogenesis [71]) supports our hypothesis. It has been shown that overexpression of NRF-1 results in inhibition of ACC gene promoter activity in the mammalian heart and enhances mitochondrial fatty acid β-oxidation rate [72]. In addition, Su and coworkers reported that FoxO1 activation result in increased fatty acid β-oxidation through translocation of CD36 [73]. PPARγ has been shown to control the transcription of fatty acid β-oxidation related protein including UCP [74] and AMPK may increase the activity of PPARγ and PGC-1 by activating sirtuin 1 (SIRT1) [75, 76]. Furthermore, mitochondria are dynamic organelles that constantly fuse and divide to maintain mitochondrial morphology, metabolism, and function [77]. Although their roles are still unknown in avian species, the upregulation of hepatic mitofusin MFN1 and dynamin DNM1 in our experimental conditions indicated that low BCAA levels modulate mitochondrial biogenesis and fatty acid oxidation [78]. The constant levels of Atg-3 and beclin1, key autophagy-related proteins, between all groups indicated that low level of BCAA did not affect autophagy.

In conclusion, the present study provided the first evidence, to our knowledge, that low BCAA levels alter hepatic lipogenic gene expression in female broiler chickens resulting in a shift of fatty acid synthesis toward fatty acid β-oxidation and this is likely mediated through AMPK-mTOR-FoxO1 pathway (Fig. 7). Unraveling the molecular mechanisms of BCAA action of fat metabolism may have broader implications not only in animal biology for health and feed efficiency improvement, but also in molecular nutrition and medicine for potential nutritional supplement optimization and therapeutic perspectives.

S.D. and M.K. designed research. J.B. and E.G. performed research. S.D. analyzed data and wrote the paper.

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