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Original Research Article

Long-term leucine supplementation increases body weight in goats by controlling appetite and muscle protein synthesis under protein-restricted conditions

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ABSTRACT

An inadequate amino acid (AA) supply in animals under protein-restricted conditions can slow skeletal muscle growth. Protein translation can be activated by short-term leucine (Leu) stimulation; however, whether muscle mass increases under long-term Leu supplementation and how the gut and muscle respond to Leu supplementation are largely unknown. In this study, we investigated if muscle mass increases with long-term Leu supplementation under protein-restricted conditions. We identified changes in the link between the gut and muscles under different amino acid supply conditions, using goats as the study object. A total of 27 Xiangdong black male goats with average initial body weight (BW) of 10.88 ± 1.22 kg were randomly divided into three dietary treatments: a normal protein diet (NP, 14.24% crude protein [CP]); a low protein diet (LP, 8.27% CP with supplemental 1.66% rumen-protected lysine [RPLys] and 0.09% rumen-protected methionine [RPMet]); and LP diet with rumen-protected Leu (RPLeu) (LP + RPLeu, 8.75% CP with supplemental 1.66% RPLys, 0.09% RPMet and 1.46% RPLeu). The animal trial lasted for 110 d, consisting of 20 d of adaptation and a 90 d of experimental period. The results showed that long-term protein restriction increased gut tryptophan hydroxylase 1 (TPH1) activity ($P < 0.001$), tryptophan (Trp) catabolism ($P < 0.001$), and 5-hydroxytryptamine (5-HT) synthesis ($P < 0.001$), which all subsequently reduced goat appetite. Long-term Leu supplementation inhibited 5-HT synthesis ($P < 0.001$), decreased Trp catabolism in the gut, and increased appetite in goats. Long-term protein restriction enhanced jejunal and ileal branched-chain amino acid transferase (BCAT) ($P < 0.001$) and branched-chain α-Keto acid dehydrogenase (BCKD) ($P = 0.048$) activities, which increased branched-chain amino acid (BCAA) catabolism. Immunofluorescence results showed that protein restriction decreased the intestinal mucosal expression of solute carrier family 1 member 5 (*SLC1A5*) ($P = 0.032$) and solute carrier family 7 member 5 (*SLC7A5*) ($P < 0.001$), reduced BCAA transport from the mucosa to the blood, lowered BCAA levels in the blood ($P < 0.001$). Western blot results showed that protein restriction inhibited mammalian target of rapamycin (mTOR) pathway activation in goat muscles. Leu supplementation increased BCAA translocation from the intestine to the blood and promoted activation of the muscle mTOR pathway and protein synthesis. In conclusion, our results suggest that Leu

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supplementation in low-protein diets improves appetite and alleviates the inhibition of muscle protein synthesis in goats.

1. Introduction

In mammals, skeletal muscle growth is the fastest during adolescence (Davis and Fiorotto, 2009). However, adequate dietary protein intake is needed to support rapid muscle growth (Landi et al., 2016). The body responds to nutrient signal inputs, and changes in amino acids (AAs) and insulin concentrations can alter the rate of protein synthesis in the muscles (Wilson et al., 2009). The body reduces protein synthesis to adapt to an inadequate supply of dietary protein and AAs.

In low-income countries, a slow rate of muscle growth has been observed in adolescents due to insufficient protein intake resulting from a lack of animal-based products (Ochola and Masibo, 2014). In humans, an insufficient dietary protein intake is harmful to health (Wu, 2016). Overfishing and reductions in land and water resource availability for growing crops have led to higher prices for protein components such as fishmeal, oil, and soybean meal (DiGiacomo and Leury, 2019). Identifying new solutions to meet the high demand for feed protein in animal production is crucial for sustainable farming practice. In animal production, reducing the dietary protein intake of livestock (e.g., pig, chicken, cattle, sheep, and goat) has become popular to save feed cost. However, a lack of protein can limit animal growth. Adding AAs to a protein-restricted diet may be a suitable strategy for combatting this issue as AAs can enhance protein synthesis in the body by promoting translation initiation (Kimball and Jefferson, 2006; Torrazza et al., 2010). Over the last 35 years, research has demonstrated that branched-chain amino acids (BCAAs) enhance (Wolfe, 2017a) and control protein synthesis via mechanistic target of rapamycin complex 1 (mTORC1) (Soultoukis and Partridge, 2016). Based on the results in animals (e.g., mice, rat, and pig) and cell experiments (Rieu et al., 2007; Columbus et al., 2015; Dijk et al., 2018), leucine (Leu) is the most potent mTORC1 activator among BCAAs and effectively promotes protein synthesis (Areta et al., 2014; Duan et al., 2016). However, the effect of Leu supplementation over a short period is insufficient to conclude that Leu could promote muscle growth. Muscle growth requires efficient protein synthesis over the long term (Wolfe, 2017b). If the stimulation of protein synthesis cannot be sustained, it is of little physiological significance (Wolfe, 2017a). Hence, it is necessary to test whether long-term Leu supplementation affects muscle protein anabolism and growth.

BCAAs are also capable of modulating appetite due to changes in the relative amounts of dietary BCAAs to other AAs, especially tryptophan and threonine. Increasing the ratio of BCAAs to these AAs leads to overfeeding and is associated with central serotonin depletion (Solon-Biet et al., 2019). Increase in appetite and food intake are indicators of a sufficient nutritional supply in the body, in addition to increased protein anabolism (Rennie et al., 2004). Therefore, we explored how Leu supplementation regulates appetite and muscle protein anabolism in this study.

Protein anabolism in muscle is influenced by AAs level in the blood. AA transporters present in the intestinal epithelium absorb and transport AAs and are important determinants and regulators of AA fluxes between mammalian tissues (Brosnan, 2003). In the fed state, the main flow of AAs occurs from the gut to other tissues, whereas AAs flow in the postabsorptive state occurs in the muscles, liver, and kidneys (Karinch et al., 2007; He et al., 2010). Measuring the effect of Leu supplementation on muscle protein anabolism alone does not fully reveal the mechanism by which Leu affects the muscle. Therefore, the link between the gut and muscle (intestinal absorption and transport and AAs metabolism in the muscle) must be considered and elucidated during Leu supplementation. Hence, we sought to establish a link between intestinal AAs transport and muscle protein anabolism after Leu supplementation.

In this study, we selected goats as experimental animals to determine whether 1) Leu supplementation under protein-restricted conditions influences muscle protein anabolism and appetite, and 2) how long-term (90 d) supplementation of Leu affects this mechanism. Goats were introduced into the diet at 2 months of age, which is roughly equivalent to the human teenage years. To clarify this phenomenon, we focused on AAs transport, and AAs metabolism and protein synthesis in the goat intestine and muscles, respectively.

2. Materials and methods

2.1. Animal ethics statement

The experimental protocol was approved by the Animal Protection Committee and all operations were performed by following the Animal Protection Guidelines of the Institute of Subtropical Agriculture, Chinese Academy of Sciences. All animals were kept in accordance with the Council Regulation (EC) No. 1/2005 on the protection of animals, Directives 64/432/EEC and 93/119/EC, and Regulation (EC) No. 1255/97.

2.2. Animal experiment

At 60 days of age, 27 Xiangdong black male goats with an average initial body weight (BW) of 10.88 ± 1.22 kg were randomly allocated to three dietary treatments: a normal protein diet (NP, 14.24% crude protein [CP]); a low protein diet (LP, 8.27% CP with supplemental 1.66% rumen-protected lysine [RPLys] and 0.09% rumen-protected methionine [RPMet]); and LP diet with rumen-protected Leu (RPLeu) (LP + RPLeu, 8.75% CP with supplemental 1.66% RPLys, 0.09 % RPMet and 1.46% RPLeu). Diets are described in Table 1. The effect of Lys and Met on the results was minimized by adding RPLys and RPMet to make the Lys and Met levels of the LP and LP + RPLeu groups consistent with those of the NP group. The diets were formulated according to NRC (2007). The RPLeu, RPLys, and RPMet used in this experiment were supplied by King Techina (Hangzhou, Zhejiang, China). Leu content in the RPLeu was 70.7%, with a ruminal passing rate of 85.4%. Met content in the RPMet was 75.0% (rumen passing rate: 85.0%) and Lys content in the RPLys was 65% (rumen passing rate: 61.7%). RPLeu, RPLys, and RPMet were coated with the microencapsulated slow-release composite materials. The animal trial lasted for 110 d, consisting of 20 d of adaptation and a 90 d of experimental period. All goats had access to complete pellet feed (including concentrate and roughage) and ad libitum water. Goats were fed twice daily (08:00 and 16:00) during the experimental period. The feed and residue contents were recorded daily. Goats were weighed monthly to record changes in

Table 1
Composition and nutrient levels of diets (dry matter basis, %).

Item	Treatments[1]		
	NP	LP	LP + RPLeu
Ingredients			
Corn	35.38	49.73	48.21
Soybean meal	22.37	4.85	4.89
Straw	30.00	30.00	30.00
Fat powder	8.78	10.03	10.05
Rumen protected methionine		0.09	0.09
Rumen protected lysine		1.66	1.66
Rumen protected leucine			1.46
Calcium carbonate	0.65	0.48	0.48
Calcium hydrogen phosphate	1.33	1.66	1.66
Salt	0.50	0.50	0.50
Premix[2]	1.00	1.00	1.00
Nutrient levels			
ME, mKal/kg[3]	3.07	3.12	3.07
CP	14.24	8.27	8.75
Ca	0.82	0.82	0.82
TP	0.55	0.55	0.55
NDF	29.49	28.48	28.34
ADF	17.08	16.07	16.01
Methionine	0.10	0.10	0.10
Leucine	1.21	0.64	1.21
Lysine	0.82	0.82	0.82

ME = metalizable energy; CP = crude protein; TP = total phosphorus; NDF = neutral detergent fiber; ADF = acid detergent fibre.
[1] NP = normal protein diet (14.24% crude protein [CP]); LP = low protein diet (8.27% CP with supplemental 1.66% rumen-protected lysine [RPLys] and 0.09 % rumen-protected methionine [RPMet]); LP + RPLeu = LP with rumen-protected Leu (RPLeu) (8.75% CP with supplemental 1.66% RPLys, 0.09% RPMet and 1.46% RPLeu).
[2] The premix provided the following per kilogram of diets: Fe 1.5 g, Cu 0.5 g, Co 0.0055 g, I 0.0075 g, Mn 3 g, Zn 2.5 g, Se 0.0025 g, VA 419,491.5254 IU, VE 12,711.86441 IU.
[3] Metalizable energy was calculated according to NRC (2007).

body weight. The average daily feed intake (ADFI) was measured weekly by subtracting feed left and spillages from the feed offered. The calculation formula of average daily Leu intake is as follows:

Average daily Leu intake = ADFI × dietary Leu content.

Carcass traits included dressing percentage (DP), the calculation formula is as follows:

DP (%) = (slaughter weight/live weight) × 100.

Carcass weight (CW) is the weight after slaughter, after removing the head, hoof, tail, and viscera, and retaining the oil and kidney.

2.3. Sample collection

Approximately 500 g of feed samples were collected and dried in an oven at 65 °C for 48 h. Feed samples were stored at −20 °C until further analysis. On the morning of d 90 before feeding, blood samples were collected from the goats via jugular vein into a 10 mL vacuum tube containing Na-heparin. After centrifugation at 3000 × *g* for 20 min at 4 °C, plasma samples were split and frozen at −80 °C until analysis. On d 90 of the trial, goats were taken to the farm slaughterhouse, anaesthetized with sodium pentobarbital, and euthanized by jugular venous bleeding. Immediately after slaughter, the jejunal and ileal mucosa were rinsed with a cold phosphate-buffered saline (PBS) solution. Epithelial samples were collected ventrally from the mid-jejunum (5 cm) and mid-ileum (5 cm) and fixed in 10% formalin (v/v). For immunofluorescence investigation, the jejunum and ileum tissues were paraffin-embedded and kept at 4 °C. Jejunum, ileal mucosa, and vastus lateralis samples for real-time quantitative PCR (RT-qPCR), AAs content, enzyme activity and Western blotting detection were stored at −80 °C.

2.4. Feed sample analysis

The feed samples were dried at 65 °C in a forced air oven (101–3 A B, Tianjin Taisite Instrument Co., Ltd., Tianjin, China) to a constant weight. Samples were analyzed for dry matter (DM; method 930.15), CP (method 2001.11), neutral detergent fiber (NDF; method 2002.04), and acid detergent fiber (ADF; method 973.18) according to the Association of Official Agricultural Chemists (AOAC) procedures (2005). Calcium (Ca) and total phosphorus (TP) were analyzed using inductively coupled plasma spectroscopy (AOAC, 2005; method 985.01). The AAs content of the feed samples was measured using an ion-exchange amino acid analyzer (Hitachi L-8900; Tokyo, Japan) by the AOAC 2005 (method 982.30). Metabolisable energy (ME) value in the feed was calculated according to the NRC (2007).

2.5. Blood AAs, enzyme activity, and hormone assays

Free AAs profile of plasma samples was determined using the method described by Lv et al. (2022a). Injected 600 μL of plasma into a 1.5-mL centrifuge tube, then an equal volume of 8% sulfosalicylic acid was added, mixed thoroughly, and stored at 4 °C overnight. The centrifuge tube was removed and centrifuged at 3000 × *g* for 10 min at 4 °C, and the supernatant was filtered through a 0.22-μm membrane and transferred to a sample bottle, and then the free AAs content was determined by L-8900 analyzer (Hitachi, Tokyo, Japan). The enzymatic activities of branched chain amino acid transaminase (BCAT), branched chain keto acid dehydrogenase (BCKDH), and acetyl-CoA dehydrogenase (ACAD) were analyzed as reported by Lv et al. (2022a). According to the manufacturer's instructions, the tryptophan hydroxylase 1 (TPH1) enzyme-linked immunosorbent assay (ELISA) kit (#CSB-E13984r, CUSABIO, Wuhan, China) was used to detect TPH in plasma. The goat 5-hydroxytryptamine (5-HT) ELISA kit (goat# ml061860, Mlbio, Shanghai, China) was used to assess 5-HT in plasma taken from goats over a 90-day period according to the manufacturer's instructions.

2.6. AAs content in jejunum, ileum mucosa and vastus lateralis

Free AAs profiles (except Trp) were determined in the jejunal and ileal mucosa, plasma, and vastus lateralis using the method described by Li et al. (2015). About 100 mg samples were dissolved in water with methanol (1:1) at 48 °C for 30 min and centrifuged at 10,000 × *g* for 10 min, then the supernatant was filtered through glass wool and stored at −80 °C until analyses. After centrifugation to separate soluble from insoluble material, 40 mL of the supernatant were labelled with iTRAQ reagents (AA 45/32 kit; Applied Biosystems) as recommended by the manufacturer, and analysed on an Applied Biosystems 3200 QTRAP LC/MS/MS system equipped with an RP-C18 column (length 150 mm, diameter 4.6 mm and particle size 5 mm). The Trp content was determined according to GB/T 15400-2018. The content of each type of AAs was determined using the dry weight and protein content.

2.7. RT-qPCR

Following the methods used in a previous study (Lv et al., 2022a), mRNA expression of the AAs transporter vectors was assayed using RT-qPCR. Briefly, total RNA was extracted from collected epithelial samples using RNAiso Plus (TaKaRa, Dalian, China; Code No. 9108/9109) following the manufacturer's instructions. The genomic DNA was eliminated by digestion with DNase I (Thermo Scientific, Waltham, MA, USA). Total RNA quantification, purity, and integrity were evaluated by the NanoDrop

Table 2
Effects of supplementing Leu with low protein diet on the growth performance of goats.

Item	Treatments[1]			SEM	*P*-value
	NP	LP	LP + RPLeu		
0 d BW, kg	10.68	10.68	10.90	0.240	0.914
30 d BW, kg	16.35[a]	13.50[b]	14.49[ab]	0.442	0.021
60 d BW, kg	19.32[a]	15.02[b]	16.67[b]	0.491	<0.001
90 d BW, kg	22.38[a]	16.96[b]	18.48[ab]	0.553	<0.001
CW, kg	9.56[a]	7.39[c]	8.02[ab]	0.231	0.007
DP, %	42.72	43.57	43.39	0.402	0.699
0 to 90 d ADG, g/d	126.30[a]	69.63[c]	84.82[b]	4.984	<0.001
0 to 90 d ADFI, g/d	881.63[a]	516.86[c]	688.54[b]	31.640	<0.001
0 to 90 d AD Leu intake, g/d	10.67[a]	3.31[c]	8.33[b]	0.622	<0.001

BW = body weight; CW = carcass weight; DP = dressing percentage; ADG = average daily gain; ADFI = average daily feed intake; AD Leu intake = average daily leucine intake.
[a–c] Different letters in the same row mean significant difference between the treatments ($P < 0.05$). $n = 9$.
[1] NP = normal protein diet (14.24% crude protein [CP]); LP = low protein diet (8.27% CP with supplemental 1.66% rumen-protected lysine [RPLys] and 0.09% rumen-protected methionine [RPMet]); LP + RPLeu = LP with rumen-protected Leu (RPLeu) (8.75% CP with supplemental 1.66% RPLys, 0.09% RPMet and 1.46% RPLeu).

1000 and Bioanalyzer. RNA integrity numbers (RINs) of the samples were between 7.8 and 9.6 (Table S1). Afterward, 1-μg total RNA was reverse transcribed to cDNA in a 20-μL system using the Evo M-MLV RT Kit (AG11706, Changsha, China) following the manufacturer's instructions. The synthesized cDNA was saved at −20 °C until used for RT-qPCR analysis. The target gene primer sequences are presented in Table S2. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels of mRNA expression (Livak and Schmittgen, 2001).

Table 3
Effects of supplementing Leu with a low protein diet on the plasma amino acids (AAs) profile of goats (45 d) (μg/mL).

Item	Treatments[1]			SEM	*P*-value
	NP	LP	LP + RPLeu		
Asp	5.61	5.45	5.40	0.114	0.094
Thr	23.66	23.37	23.84	0.691	0.501
Ser	8.07	8.11	8.14	0.129	0.101
Glu	41.01	41.46	42.23	1.302	0.130
Gly	75.44	77.12	76.09	2.591	0.203
Ala	21.10	20.57	21.50	0.811	0.115
Cys	3.87	3.83	3.91	0.094	0.161
Val	36.55[a]	29.03[c]	32.66[b]	1.034	0.043
Met	6.98	7.05	7.13	0.235	0.117
Ile	13.65[a]	10.27[c]	11.30[b]	0.310	<0.001
Leu	16.31[a]	12.04[c]	15.60[ab]	0.512	<0.001
Tyr	25.31	22.54	24.68	1.013	0.204
Phe	10.68	10.66	10.29	0.261	0.511
Lys	28.61	26.04	27.50	1.604	0.305
His	7.15	6.98	7.55	0.191	0.312
Arg	33.75	30.61	32.64	1.805	0.763
Pro	12.69	11.98	12.77	0.550	0.631
Trp	2.15[b]	3.94[a]	2.63[b]	0.068	<0.001
BCAA[2]	66.51[a]	51.34[c]	59.56[b]	3.513	<0.001
EAA[3]	138.59[a]	122.40[c]	130.95[b]	4.316	<0.001
NEAA[4]	234.00[a]	228.65[b]	234.91[a]	6.117	<0.001
TAA[5]	372.59[a]	351.05[b]	365.86[a]	5.016	<0.001

[a–c] Different letters in the same row mean significant difference between the treatments ($P < 0.05$). $n = 9$.
[1] NP = normal protein diet (14.24% crude protein [CP]); LP = low protein diet (8.27% CP with supplemental 1.66% rumen-protected lysine [RPLys] and 0.09% rumen-protected methionine [RPMet]); LP + RPLeu = LP with rumen-protected Leu (RPLeu) (8.75% CP with supplemental 1.66% RPLys, 0.09% RPMet and 1.46% RPLeu).
[2] Branched chain amino acids (BCAA) = Leu + Ile + Val.
[3] Essential amino acid (EAA) = Lys + Trp + Thr + Leu + Ile + Val + Met + Phe.
[4] No-essential amino acids (NEAA) = Asp + Ser + Glu + Gly + Ala + Cys + Tyr + His + Arg + Pro.
[5] Total amino acids (TAA) = EAA + NEAA.

Table 4
Effects of supplementing Leu with a low protein diet on the plasma amino acids (AAs) profile of goats (90 d) (μg/mL).

Item	Treatments[1]			SEM	*P*-value
	NP	LP	LP + RPLeu		
Asp	5.84	5.01	5.32	0.262	0.470
Thr	24.73	23.24	24.48	0.811	0.310
Ser	8.64	8.87	8.52	0.452	0.114
Glu	49.42	48.27	49.67	1.285	0.221
Gly	80.07	79.85	78.84	3.193	0.341
Ala	25.44[a]	19.85[b]	24.91[ab]	0.930	0.004
Cys	3.88	3.93	4.04	0.064	0.250
Val	38.27[a]	24.85[c]	30.47[b]	1.121	<0.001
Met	7.63[a]	6.10[b]	6.99[a]	0.171	0.015
Ile	14.55[a]	8.44[b]	10.62[b]	0.520	<0.001
Leu	19.46[a]	11.78[c]	17.11[ab]	0.661	<0.001
Tyr	24.27	19.36	23.67	0.942	0.191
Phe	11.15	10.24	10.99	0.332	0.724
Lys	29.97	27.94	28.39	1.270	0.413
His	9.52[a]	6.40[c]	8.29[b]	0.334	0.005
Arg	31.94	32.53	32.57	1.313	0.326
Pro	13.39	11.59	13.59	0.471	0.444
Trp	2.78[b]	4.62[a]	2.39[b]	0.180	<0.001
BCAA[2]	126.30[a]	69.63[c]	84.82[b]	4.982	<0.001
EAA[3]	148.54[a]	117.21[c]	131.44[b]	3.161	<0.001
NEAA[4]	252.41[a]	235.66[b]	249.42[ab]	3.572	0.009
TAA[5]	400.95[a]	352.87[b]	380.86[ab]	7.990	0.049

[a–c] Different letters in the same row mean significant difference between the treatments ($P < 0.05$). $n = 9$.
[1] NP = normal protein diet (14.24% crude protein [CP]); LP = low protein diet (8.27% CP with supplemental 1.66% rumen-protected lysine [RPLys] and 0.09% rumen-protected methionine [RPMet]); LP + RPLeu = LP with rumen-protected Leu (RPLeu) (8.75% CP with supplemental 1.66% RPLys, 0.09% RPMet and 1.46% RPLeu).
[2] Branched chain amino acids (BCAA) = Leu + Ile + Val.
[3] Essential amino acid (EAA) = Lys + Trp + Thr + Leu + Ile + Val + Met + Phe.
[4] No-essential amino acids (NEAA) = Asp + Ser + Glu + Gly + Ala + Cys + Tyr + His + Arg + Pro.
[5] Total amino acids (TAA) = EAA + NEAA.

2.8. Western blot analysis

Western blot analysis was used to determine relative protein abundance, as detailed in a previous study (Lv et al., 2022b). To extract the total protein, samples were lysed in radio immunoprecipitation assay lysis buffer (RIPA) (Beyotime, Shanghai, China) for 30 min at 4 °C. The total protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL, USA)

Table 5
Effects of supplementing Leu with low protein diet on plasma branched chain amino acids and Trp metabolism enzymes activity in goats (45 d).

Item	Treatments[1]			SEM	*P*-value
	NP	LP	LP + RPLeu		
BCAT, U/L	57.73[b]	59.49[b]	67.62[a]	1.511	0.011
BCKD, U/L	77.38	75.43	73.77	2.412	0.841
ACAD, U/L	551.14[b]	583.39[b]	674.21[a]	18.701	0.014
TPH, U/L	67.25[b]	81.92[a]	68.18[b]	1.814	0.001
5-HT, ng/mL	10.67[a]	3.31[c]	8.33[b]	0.624	<0.001

BCAT = branched-chain amino acid transferase; BCKD = branched-chain-keto acid dehydrogenase; ACAD = acetyl-CoA dAehydrogenase; TPH = tryptophan hydroxylase; 5-HT = 5-hydroxytryptamine.
[a–c] Different letters in the same row mean significant difference between the treatments ($P < 0.05$). n = 9.
[1] NP = normal protein diet (14.24% crude protein [CP]); LP = low protein diet (8.27% CP with supplemental 1.66% rumen-protected lysine [RPLys] and 0.09% rumen-protected methionine [RPMet]); LP + RPLeu = LP with rumen-protected Leu (RPLeu) (8.75% CP with supplemental 1.66% RPLys, 0.09% RPMet and 1.46% RPLeu).

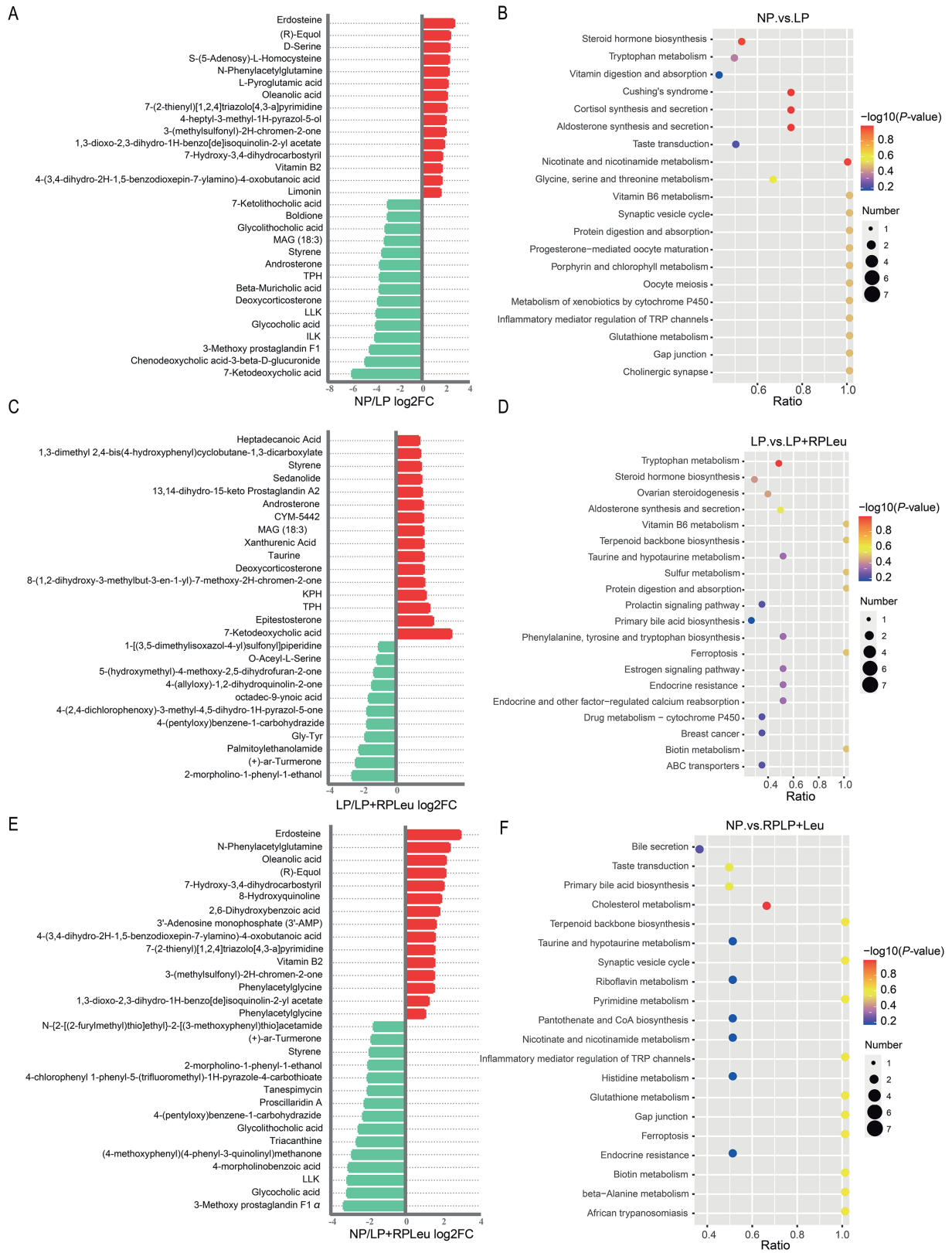


Fig. 1. Plasma metabolome reveals chronic protein restriction affects Trp metabolism. (A) Identified differential metabolites in positive (+) ion modes (NP vs. LP groups). (B) KEGG enrichment map of different metabolites based on LC-MS/MS in positive ion mode (NP vs. LP groups). (C) Identified differential metabolites in positive (+) ion modes (LP vs. LP + RPLeu groups). (D) KEGG enrichment map of different metabolites based on LC-MS/MS in positive ion mode (LP vs. LP + RPLeu groups). (E) Identified differential metabolites in positive (+) ion modes (NP vs. LP + RPLeu groups). (F) KEGG enrichment map of different metabolites based on LC-MS/MS in positive ion mode (NP vs. LP + RPLeu groups). Red indicates significantly up-regulated metabolite levels, while purple indicates significantly down-regulated metabolite levels. NP = normal protein diet (14.24% crude protein [CP]); LP = low protein diet (8.27% CP with supplemental 1.66% rumen-protected lysine [RPLys] and 0.09% rumen-protected methionine [RPMet]); LP + RPLeu = LP with rumen-protected

with a Nano-Drop ND 2000c Spectrophotometer. Twenty micrograms of proteins were denatured after boiling with 5 × protein loading buffer for 10 min, separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (80 V constant voltage for 90 min at 20 °C), and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Eschborn, Germany) by wet Trans-Blotsystem (Bio-Rad). After blocking with bovine serum albumin (BSA) for 1.5 h at room temperature, membranes were incubated overnight with the primary antibodies at 4 °C, followed by incubating with the corresponding secondary antibody (Proteintech, SA00001–1, SA00001–2). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalize the data (Lindner et al., 2013). Image Processing Software (Image-Proline Plus 6.0, Rockville, MD, USA) was used to quantify the output signals. Table S3 lists the antibodies used in this study.

2.9. Immunofluorescence and immunohistochemical analysis

Immunofluorescence analysis was used to detect AAs transporter protein abundance, as described in our previous study (Lv et al., 2022b). Immunohistochemistry was performed as previously described by Tian et al. (2020). Three slides were taken for each sample, with an average of eight images per slide. Image processing and analysis were conducted on ImageProline Plus 5.1 (Media Cybernetics), and exposure time and gain between all images on each slide were kept constant. Information on the antibodies used in our study is presented in Table S4.

2.10. Detection of metabolite composition of plasma by non-targeted metabolome

Metabolite extraction and liquid chromatography-mass spectrometry (LC-MS) analyses were performed according to the methods described by Song et al. (2022). Peak detection and alignment of raw data were performed using Progenesis QI 2.3 (Nonlinear Dynamics, Waters, USA). The samples with <80% metabolic profiles were excluded. Each metabolic profile was normalized to the corresponding sum. Metabolic profiles with a relative standard deviation (RSD) of >30% were excluded. After normalization and estimation procedures, the log10-transformed data were statistically analyzed to determine significant differences in metabolite levels between groups. The mass spectra of metabolic profiles were determined by searching biochemical databases such as the Human Metabolome Database (HMDB) and Kyoto Encyclopedia of Genomes (KEGG). Screening principles for differential metabolites between treatments were: variable importance in the projection (VIP) value > 1.0 for the partial least squares discriminant analysis (PLS-DA) model, *P*-value <0.05 for Student's *t*-test, fold change (FC) $\geq$ 2 or FC $\leq$ 0.5. Data are available at 10.6084/m9.figshare.25561899.

2.11. Cell culture

Skeletal muscle cells were obtained from isolated lateral goat vastus muscle tissue following procedures described by Wang et al. (2020). Cells were cultured in DMEM/F12 (Gibco; 12634010) under 37 °C, 5% CO_2. Muscle cell differentiation was induced in Dulbecco's modified eagle medium (DMEM) (Gibco, Waltham, MA, USA) supplemented with 2% horse serum (Gibco; 16050122). The medium treatments were DMEM/F12 (without fetal bovine serum [FBS]), control; DMEM/F12 (without FBS and Leu), -Leu; and Leu represented-Leu-treated cells, +Leu. Cell starvation: cells were washed three times in Hank's balanced salt solution (Invitrogen) and subsequently cultured in DMEM/F12 (Gibco, without FBS) at 37 °C for 12 h. After starvation, cell counting kit-8 (CCK-8), 5-Ethynyl-2′-deoxyuridine (EdU), SUface SEnsing of Translation (SUnSET), and co-localization assays were performed after 24 h incubation with the treated medium.

2.12. Cell counting assay and EdU staining

Cell proliferation was assayed using CCK8 (APExBIO, Houston, TX, USA) and EdU (C0071S; Beyotime). The proportion of EdU-positive cells was determined using a fluorescence microscope (Zeiss). Three slides were taken for each sample, with an average of eight images per slide. Image processing and analysis were conducted on ImageProline Plus 5.1 (Media Cybernetics), and exposure time and gain between all images on each slide were kept constant.

2.13. SUnSET analysis for protein synthesis

Cellular protein synthesis was analyzed following the SUnSET assay as described by Ravi et al. (2020). Cytofluorescence detection was performed with a Zeiss LSM880 confocal microscope (Carl-Zeiss-Straße 22, 73447 Oberkochen) equipped with argon and He–Ne laser sources. Three slides were taken for each sample, with an average of eight images per slide. Image processing and analysis were conducted on ImageProline Plus 5.1 (Media Cybernetics), and exposure time and gain between all images on each slide were kept constant.

2.14. Mammalian target of rapamycin (mTOR) distribution to lysosomal membranes

Use immunofluorescence to detect mTOR distribution to lysosomal membranes, following the immunofluorescence detection procedure described in section 2.1.3. Primary antibody: mouse mTOR (1:50, Proteintech, 66888-1-Ig); rabbit lysosomal associated membrane protein 2 (LAMP2) (1:50, Proteintech, 10397-1-AP). Secondary antibodies: donkey anti-mouse IgG (H + L) (1:200, Proteintech, SA00013–7); donkey anti-rabbit IgG (H + L) (1:200, Proteintech, SA00013–6) were used. Imaging was performed using a Zeiss LSM880 confocal microscope (Carl-Zeiss-Straße 22, 73447 Oberkochen). On average, 8 images were captured per section, and each image contained about 40 cells such that around 320 cells per time point (per slide) were used for analysis. Colocalization analysis of mTOR and LAMP2 was performed using the colocalization finder tool in the ImageJ plug-in.

2.15. Statistical analysis

All data were analyzed using SAS 9.2 (SAS Inc, Cary, NC, USA). The normality of data was verified and outliers were identified using the UNIVARIATE procedure (SAS Inst. Inc., Cary, NC). Data were analyzed using the PROC MIXED of SAS (SAS Institute Inc., Cary, NC).

The model used for data analysis was as follows:

$$Y_{ij} = \mu + T_i + e_{ij},$$

Leu (RPLeu) (8.75% CP with supplemental 1.66% RPLys, 0.09% RPMet and 1.46% RPLeu). The screening criteria for differential metabolites were: $P < 0.05$, variable importance in the projection (VIP) > 1, fold change (FC) > 2 or FC < 0.5. MAG (18:3) = monoglyceride (18:3); TPH = tryptophan hydroxylase; LLK = leucylleucyllysine; ILK = integrin-linked kinase; KPH = kalopanaxsaponin H.

where Y_{ij} is the dependent variable, μ is the overall mean, T_i ($I = 1$ to 3) is the fixed effect of treatment, e_{ij} is the random residual error, and animals were considered random effects.

A P-value < 0.05 was considered statistically significant. All statistical analyses and visualizations were performed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Chronic protein restriction reduced body weight, but Leu supplementation improved weight gain

The BW (30, 60, and 90 d), ADFI, ADG, and AD Leu intake differed significantly among the treatment groups ($P < 0.05$) (Table 2). The ADG and ADFI were higher for goats in the NP group than for those in the LP and LP + RPLeu groups ($P < 0.05$). Among the three treatment groups, the BW, ADFI, and ADG of the goats in the LP group were the lowest; however, the ADFI and ADG of the goats in the LP + RPLeu diet group were significantly higher than those in the LP group ($P < 0.05$) (Table 2).

3.2. Plasma AAs metabolism is changed in goats exposed to different dietary protein treatments

The plasma AAs profile (45 and 90 d) indicated that the levels of Val, Ile, and Leu in the plasma of the LP group were significantly lower than those in the plasma of the NP and LP + RPLeu groups ($P < 0.05$), whereas Trp was significantly higher ($P < 0.05$) (Table 3 and Table 4). Plasma BCAT ($P = 0.011$) and ACAD ($P = 0.014$) activities in the LP + RPLeu group were significantly higher than those in the NP and LP groups. BCKD activity did not differ significantly among the three treatment groups ($P = 0.841$) (Table 5). Considering the changes in feed intake and plasma Trp content and that Trp catabolism can produce 5-HT, 5-HT is an important regulator of appetite; therefore, we detected the 5-HT and TPH contents in plasma. Plasma 5-HT levels were significantly higher in the LP group than those in the NP and LP + RPLeu groups ($P < 0.001$) (Table 5). Plasma TPH enzyme activity was significantly higher in the LP group than in the NP and LP + RPLeu groups ($P = 0.001$) (Table 5).

3.3. Plasma metabolome reveals chronic protein restriction affects Trp metabolism

Principal components analysis (PCA) was applied to investigate the clustering trends of the metabolome among the NP, LP, and LP + RPLeu groups and to exclude possible outliers. A separation trend between the NP, LP, and LP + RPLeu groups was observed in the PCA score plot (Fig. S1), thus indicating that the plasma metabolome differed according to dietary protein treatment type. VIP plots in PLS-DA analysis of plasma metabolites detected by NP group and LP group (Fig. S2), LP group and LP + RPLeu group (Fig. S3), NP group and LP + RPLeu group (Fig. S4) in positive ion mode.

Eighty-five differential metabolites were identified in the NP versus LP group, of which 35 were upregulated and 50 were downregulated. We selected the 15 differential metabolites with the largest upward and downward adjustments for presentation (Fig. 1A). The metabolomic results showed that TPH content was significantly higher in the LP group than in the NP group ($P < 0.05$) and that TPH was able to metabolize Trp to 5-HT. We found that the contents of 7-ketolithocholic acid and 7-ketodeoxycholic acid were significantly lower in the NP group than in the LP group ($P < 0.05$).

Table 6
Effects of low protein diet supplemented with Leu on branched chain amino acids content (g/100 g) in goat ileal mucosa.

Item	Treatments[1]			SEM	*P*-value
	NP	LP	LP + RPLeu		
Leu	2.94[b]	3.03[a]	2.95[b]	0.051	0.011
Ile	2.41[b]	2.80[a]	2.28[c]	0.049	<0.001
Val	4.47[b]	5.17[a]	4.35[b]	0.101	<0.001
BCAA	9.82[b]	11.00[a]	9.58[b]	0.147	<0.001

BCAA = branched chain amino acids.
[a–c] Different letters in the same row mean significant difference between the treatments ($P < 0.05$). $n = 9$.
[1] NP = normal protein diet (14.24% crude protein [CP]); LP = low protein diet (8.27% CP with supplemental 1.66% rumen-protected lysine [RPLys] and 0.09% rumen-protected methionine [RPMet]); LP + RPLeu = LP with rumen-protected Leu (RPLeu) (8.75% CP with supplemental 1.66% RPLys, 0.09% RPMet and 1.46% RPLeu).

Table 7
Effects of low protein diet supplemented with Leu on branched chain amino acids metabolic enzyme activity (mU/mg·prot) in goat ileal mucosa.

Item	Treatments[1]			SEM	*P*-value
	NP	LP	LP + RPLeu		
BCAT	9.59[b]	14.11[a]	10.81[b]	0.479	<0.001
ACAD	11.92	13.36	12.28	0.561	0.761
BCKD	63.25[c]	102.00[a]	76.36[b]	3.531	0.048

BCAT = branched-chain amino acid transferase; ACAD = acetyl-CoA dehydrogenase; BCKD = branched-chain α-Keto acid dehydrogenase.
[a–c] Different letters in the same row mean significant difference between the treatments ($P < 0.05$). $n = 9$.
[1] NP = normal protein diet (14.24% crude protein [CP]); LP = low protein diet (8.27% CP with supplemental 1.66% rumen-protected lysine [RPLys] and 0.09% rumen-protected methionine [RPMet]); LP + RPLeu = LP with rumen-protected Leu (RPLeu) (8.75% CP with supplemental 1.66% RPLys, 0.09% RPMet and 1.46% RPLeu).

Table 8
Effects of low protein diet supplemented with Leu on the mRNA expression of branched chain amino acids metabolic enzymes and amino acid transporters in goat ileal mucosa.

Item	Treatments[1]			SEM	*P*-value
	NP	LP	LP + RPLeu		
BCKDHA	1.02[b]	1.65[a]	0.79[c]	0.079	0.031
BCKDHB	1.01	1.07	1.04	0.029	0.139
ACADS	1.01	0.96	1.03	0.035	0.438
ACADSB	1.00	1.11	1.04	0.030	0.610
BCAT2	1.00[b]	1.68[a]	0.80[c]	0.078	<0.001
SLC7A7	1.01	1.08	1.07	0.025	0.410
SLC38A1	1.00	1.04	0.97	0.023	0.211
SLC7A6	1.00	0.97	1.00	0.019	0.164
SLC1A1	1.00	1.05	1.04	0.018	0.625
SLC1A5	1.00[b]	0.76[c]	1.58[a]	0.078	<0.001
SLC7A5	1.00[b]	0.71[c]	1.64[a]	0.085	<0.001
SLC3A2	1.00	0.99	1.02	0.020	0.091
SLC6A19	1.02[b]	1.73[a]	1.03[b]	0.076	<0.001

BCKDHA = branched chain keto acid dehydrogenase E1 subunit alpha; *BCKDHB* = branched chain keto acid dehydrogenase E1 subunit beta; *ACADS* = acyl-CoA dehydrogenase short chain; *ACADSB* = acyl-CoA dehydrogenase short/branched chain; *BCAT2* = branched chain amino acid transaminase 2; *SLC7A7* = solute carrier family 7 member 7; *SLC38A1* = solute carrier family 38 member 1; *SLC7A6* = solute carrier family 7 member 6; *SLC1A1* = solute carrier family 1 member 1; *SLC1A5* = solute carrier family 1 member 5; *SLC7A5* = solute carrier family 7 member 5; *SLC3A2* = solute carrier family 3 member 2; *SLC6A19* = solute carrier family 6 member 19.
[a–c] Different letters in the same row mean significant difference between the treatments ($P < 0.05$). $n = 9$.
[1] NP = normal protein diet (14.24% crude protein [CP]); LP = low protein diet (8.27% CP with supplemental 1.66% rumen-protected lysine [RPLys] and 0.09% rumen-protected methionine [RPMet]); LP + RPLeu = LP with rumen-protected Leu (RPLeu) (8.75% CP with supplemental 1.66% RPLys, 0.09% RPMet and 1.46% RPLeu).

Thirty-four differential metabolites were identified in the LP versus LP + RPLeu groups, of which 23 were upregulated and 11 were downregulated. We selected the 15 differential metabolites with the greatest upward adjustments for presentation (Fig. 1C). The metabolomics results showed that TPH content was significantly higher in the LP group than in the LP + RPLeu group ($P < 0.05$). We found that the content of 7-ketodeoxycholic acid was significantly higher in the LP group than in the LP + RPLeu group ($P < 0.05$). Xanthurenic acid content was higher in the LP group than in the LP + RPLeu group ($P < 0.05$).

Eighty-three differential metabolites were identified in the NP vs. LP + RPLeu groups, of which 27 were upregulated and 29 were downregulated. We selected the 15 differential metabolites with the largest upward and downward adjustments for presentation

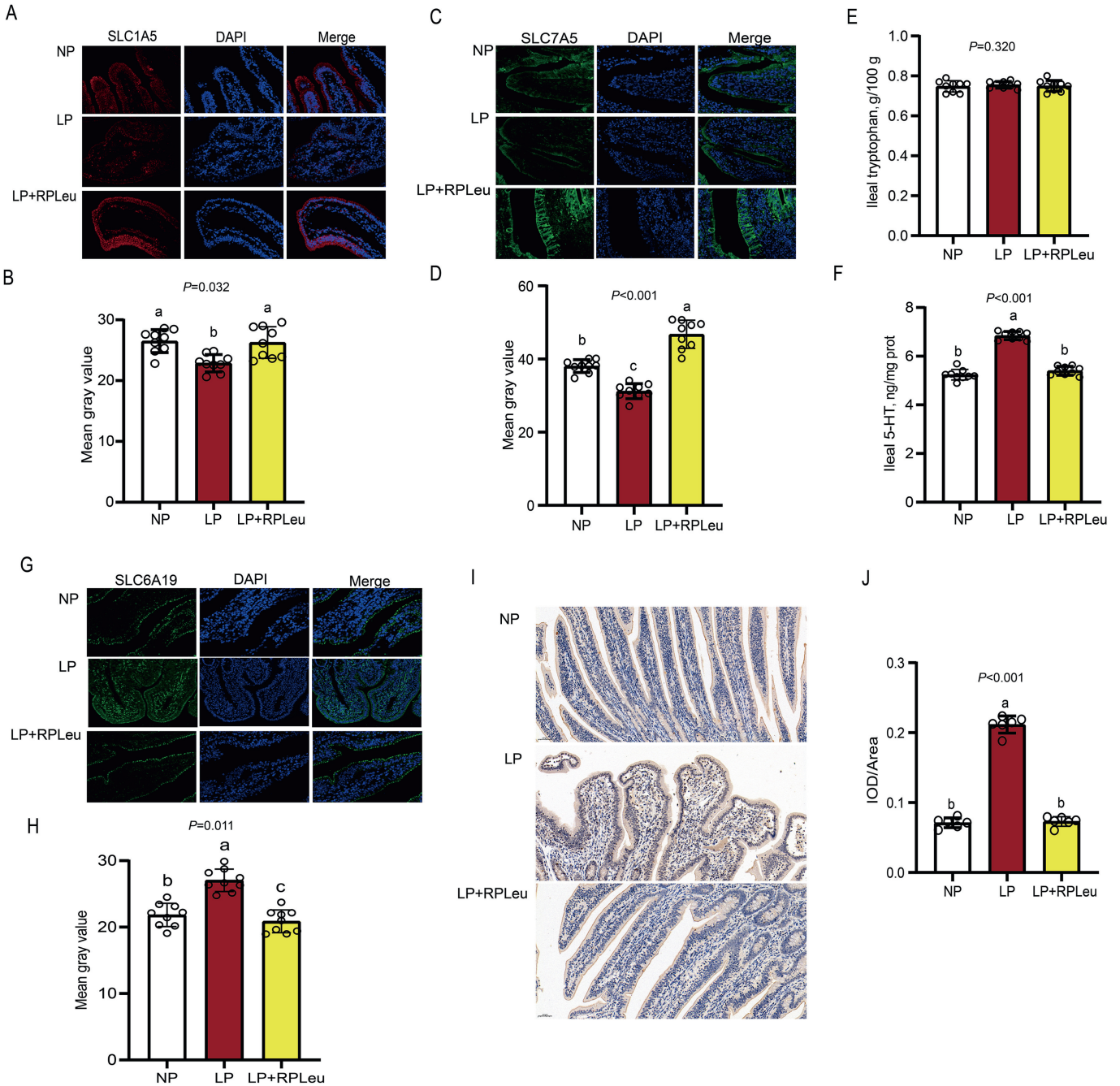


Fig. 2. Leu supplementation alters ileal branched-chain amino acid and Trp metabolism under chronic protein restriction conditions. (A-D) The protein expression levels of solute carrier family 1 member 5 (SLC1A5) (A, B) and solute carrier family 7 member 5 (SLC7A5) (C, D) were detected by immunofluorescence (400×). (E, F) Tryptophan (Trp) and 5-hydroxytryptamine (5-HT) content in ileal mucosa. (G, H) The protein expression levels of solute carrier family 6 member 19 (SLC6A19) were detected by immunofluorescence (400×). (I, J) The protein expression level of tryptophan hydroxylase 1 (TPH1) in ileal mucosa was detected by immunohistochemistry (100 ×). NP = normal protein diet (14.24% crude protein [CP]); LP = low protein diet (8.27% CP with supplemental 1.66% rumen-protected lysine [RPLys] and 0.09 % rumen-protected methionine [RPMet]); LP + RPLeu = LP with rumen-protected Leu (RPLeu) (8.75% CP with supplemental 1.66% RPLys, 0.09% RPMet and 1.46% RPLeu). Announcing different lowercase letters (a-c) in the bar chart indicate significant differences between groups. *P*-value < 0.05 was considered statistically significant. $n = 9$. DAPI = diamidino-phenyl-indole.

(Fig. 1E). We found that glycocholic acid and leucylleucyllysine (LLK) contents were significantly lower in the NP group than those in the LP + RPLeu group ($P < 0.05$).

3.4. Leu supplementation can alter intestinal BCAAs and Trp transport and metabolism under chronic protein restriction conditions

We observed that the contents of Leu ($P = 0.011$), Ile ($P < 0.001$), and Val ($P < 0.001$) in the ileal mucosa of the LP group were significantly higher than those in the NP and LP + RPLeu groups (Table 6). The LP diet increased the activities of BCAT ($P < 0.001$) and BCKD ($P = 0.048$) in the ileal mucosa (Table 7). The RT-qPCR data showed that the relative mRNA expressions of branched chain keto acid dehydrogenase E1 subunit alpha (*BCKDHA)* ($P = 0.031$) and branched chain amino acid transaminase 2 (*BCAT2*) ($P < 0.001$) in the LP group were significantly higher than those in the NP and LP + RPLeu groups (Table 8). Considering the differences in the BCAA content in the ileal mucosa among the three groups, we measured the mRNA and protein expression of BCAA transporters. No significant differences in the relative mRNA expression of solute carrier family 7 member 7 (*SLC7A7)*, solute carrier family 38 member 1(*SLC38A1)*, solute carrier family 7 member 6 (*SLC7A6)*, or solute carrier family 1 member 1 (*SLC1A1)* were observed among the three groups (Table 8). Among the three treatment groups, we found that mRNA expression of *SLC1A5* and *SLC7A5* was lowest in the LP group and highest in the LP + RPLeu group ($P < 0.05$) (Table 8). However, solute carrier family 6 member 19 (*SLC6A19)* relative mRNA expression in the ileal mucosa of the LP group was significantly higher than that in the NP and LP + RPLeu groups ($P < 0.001$) (Table 8).

Immunofluorescence data showed that the protein expression of SLC1A5 ($P = 0.032$) and SLC7A5 ($P < 0.001$) in the ileal mucosa of the LP group was the lowest (Fig. 2A–D) among the three treatment groups, whereas the protein expression of SLC6A19 ($P = 0.011$) was the highest (Fig. 2G–H). Therefore, the ileal mucosal transport capacity of BCAAs is reduced under protein-limiting conditions, whereas Trp transport is increased under the same conditions. The 5-HT content in the ileal mucosa of the LP group was significantly higher than that in the NP and LP + RPLeu groups ($P < 0.001$) (Fig. 2F). TPH1 protein expression in the ileal mucosa of the LP group was significantly higher than that in the NP and LP + RPLeu groups ($P < 0.001$) (Fig. 2I–J). Under protein-restricted conditions, this result demonstrates that the ileal mucosa produces more 5-HT by upregulating TPH1 expression, whereas Leu supplementation reduces 5-HT production. Supplementation with Leu under protein-limiting conditions caused the transport and catabolism of BCAAs, and Trp tended to be similar to that of the NP group.

The Leu ($P = 0.031$) and Ile ($P = 0.001$) contents in the jejunal mucosa of the LP group were significantly higher than those in the NP and LP + RPLeu groups (Table S5). We found that BCAT activity in the jejunal mucosa of the LP group was significantly higher than that in the NP and LP + RPLeu groups ($P < 0.001$) (Table S6). No significant differences in the relative mRNA expression of *SLC7A7*, *SLC38A1*, *SLC7A6*, solute carrier family 3 member 2 (*SLC3A2),* or *SLC1A1* were observed among the three groups (Table S7). Among the three treatment groups, we found that mRNA expression of *SLC1A5* and *SLC7A5* was the lowest in the LP group ($P < 0.05$) (Table S7). The 5-HT content in the jejunal mucosa was significantly higher in the LP group than in the NP and LP + RPLeu groups ($P = 0.002$) (Fig. S5F). Immunohistological observations indicated that the upregulation of TPH1 protein expression in the jejunal mucosa of the LP group was significantly higher than that of the NP and LP + RPLeu groups ($P < 0.001$) (Figs. S5I–J).

3.5. Leue supplementation promotes activation of the mTOR signalling pathway under chronic protein restriction conditions

We analyzed the AA content of the vastus lateralis and discovered that the content of Val ($P = 0.041$) and BCAA ($P = 0.003$) in the LP + RPLeu group were significantly higher than those in the LP and NP groups (Table 9). However, there was no significant difference in the content of other AAs in the vastus lateralis among the three groups (Table S8) ($P > 0.05$). BCAT activity of the vastus lateralis in the LP group was significantly lower than that in the NP and LP + RPLeu groups ($P < 0.001$) (Table 10). The RT-qPCR data indicated that the downregulation of *BCAT2* relative mRNA expression was higher in the LP group than in the NP and LP + RPLeu groups ($P < 0.001$) (Table 11). This suggests that chronic protein restriction impairs BCAA catabolism in the muscles. As shown in Fig. 3A and B, the downregulation of SLC7A5 ($P = 0.003$) and SLC1A5 ($P = 0.011$) protein expression is significantly higher in the LP + RPLeu and LP groups, respectively, than those in the NP group. Protein expression of p-mTOR in the LP + RPLeu group was significantly higher than that in the LP group ($P < 0.001$). mTOR, ribosomal protein S6 kinase B1 (RPS6KB1), p-RPS6KB1, and p-eIF4EBP1 protein expression levels did not differ significantly among the three treatment groups ($P > 0.05$) (Fig. 3C and D).

3.6. Leu regulates protein synthesis in goat muscle cells by affecting the activation of mTORC1

We performed immunofluorescence analysis on isolated goat skeletal muscle cells and found that both Desimin and Myhc6 were positively expressed (Fig. 4A). To evaluate the regulatory effects of Leu on goat skeletal muscle mass, we examined its effect on the

Table 9
Effects of low protein diet supplemented with Leu on branched chain amino acids content (g/100 g) in goat vastus lateralis.

Item	Treatments[1]			SEM	*P*-value
	NP	LP	LP + RPLeu		
Leu	5.37	5.55	5.37	0.051	0.513
Ile	3.27	3.41	3.43	0.032	0.710
Val	3.83[b]	3.77[b]	4.17[a]	0.039	0.041
BCAA	12.47[b]	12.73[b]	13.17[a]	0.092	0.003

BCAA = branched chain amino acids.
[a,b] Different letters in the same row mean significant difference between the treatments ($P < 0.05$). $n = 9$.
[1] NP = normal protein diet (14.24% crude protein [CP]); LP = low protein diet (8.27% CP with supplemental 1.66% rumen-protected lysine [RPLys] and 0.09% rumen-protected methionine [RPMet]); LP + RPLeu = LP with rumen-protected Leu (RPLeu) (8.75% CP with supplemental 1.66% RPLys, 0.09% RPMet and 1.46% RPLeu).

Table 10
Effects of low protein diet supplemented with Leu on branched-chain amino acid metabolic enzyme activity (mU/mg·prot) in goat vastus lateralis.

Item	Treatments[1]			SEM	*P*-value
	NP	LP	LP + RPLeu		
BCAT	28.33[a]	19.60[b]	24.53[a]	0.937	<0.001
ACAD	29.30	27.45	30.45	0.948	0.100
BCKD	219.90	203.51	261.26	10.967	0.201

BCAT = branched-chain amino acid transferase; BCKD = branched-chain α-Keto acid dehydrogenase; ACAD = acetyl-CoA dehydrogenase.
[a,b] Different letters in the same row mean significant difference between the treatments ($P < 0.05$). $n = 9$.
[1] NP = normal protein diet (14.24% crude protein [CP]); LP = low protein diet (8.27% CP with supplemental 1.66% rumen-protected lysine [RPLys] and 0.09% rumen-protected methionine [RPMet]); LP + RPLeu = LP with rumen-protected Leu (RPLeu) (8.75% CP with supplemental 1.66% RPLys, 0.09% RPMet and 1.46% RPLeu).

Table 11
Effects of low protein diet supplemented with Leu on the mRNA expression of branched-chain amino acid metabolic enzymes in goat vastus lateralis.

Item	Treatments[1]			SEM	*P*-value
	NP	LP	LP + RPLeu		
BCKDHA	1.00	0.98	0.96	0.016	0.510
BCKDHB	0.99	1.01	1.01	0.024	0.401
ACADS	1.00	0.57	1.25	0.020	0.081
ACADSB	0.99	1.01	1.01	0.014	0.210
BCAT2	1.13[a]	0.78[b]	1.23[a]	0.142	<0.001

BCKDHA = branched chain keto acid dehydrogenase E1 subunit alpha; *BCKDHB* = branched chain keto acid dehydrogenase E1 subunit beta; *ACADS* = acyl-CoA dehydrogenase short chain; *ACADSB* = acyl-CoA dehydrogenase short/branched chain; *BCAT2* = branched chain amino acid transaminase 2.
[a,b] Different letters in the same row mean significant difference between the treatments ($P < 0.05$). $n = 9$.
[1] NP = normal protein diet (14.24% crude protein [CP]); LP = low protein diet (8.27% CP with supplemental 1.66% rumen-protected lysine [RPLys] and 0.09% rumen-protected methionine [RPMet]); LP + RPLeu = LP with rumen-protected Leu (RPLeu) (8.75% CP with supplemental 1.66% RPLys, 0.09% RPMet and 1.46% RPLeu).

proliferation of goat skeletal muscle cells. Both EdU and CCK8 analyses showed that Leu starvation resulted in slow muscle cell proliferation ($P < 0.001$) (Fig. 4B–D). When Leu-starved cells were recultured in the Leu-containing medium, the cell proliferation rate was accelerated ($P < 0.001$) (Fig. 4B–C). To explore the regulatory effects of Leu on protein synthesis in goat skeletal muscle cells, SUnSET software was used to analyze intracellular protein synthesis. Reduced puromycin signalling in Leu-starved goat skeletal muscle cells indicated reduced protein synthesis ($P < 0.001$) (Fig. 4E–F). Our results showed that Leu starvation decreased the degree of mTOR and LAMP2 colocalization ($P < 0.001$) (Fig. 4G–H). When Leu was added to Leu-starved cells, mTOR redistribution into lysosomes increased ($P < 0.001$) (Fig. 4G–H). As presented in Fig. 4I–J, downregulation of p-mTOR ($P = 0.012$), p-RPS6KB1 ($P < 0.001$), and SLC7A5 ($P < 0.001$) protein expression is significantly higher in the Leu group than that in the Con group. The protein expression of p-mTOR, p-RPS6KB1 and SLC7A5 in the +Leu group was significantly higher than that in the −Leu group ($P < 0.05$). mTOR, RPS6KB1, eIF4EBP1, p-eIF4EBP1, and SLC1A5

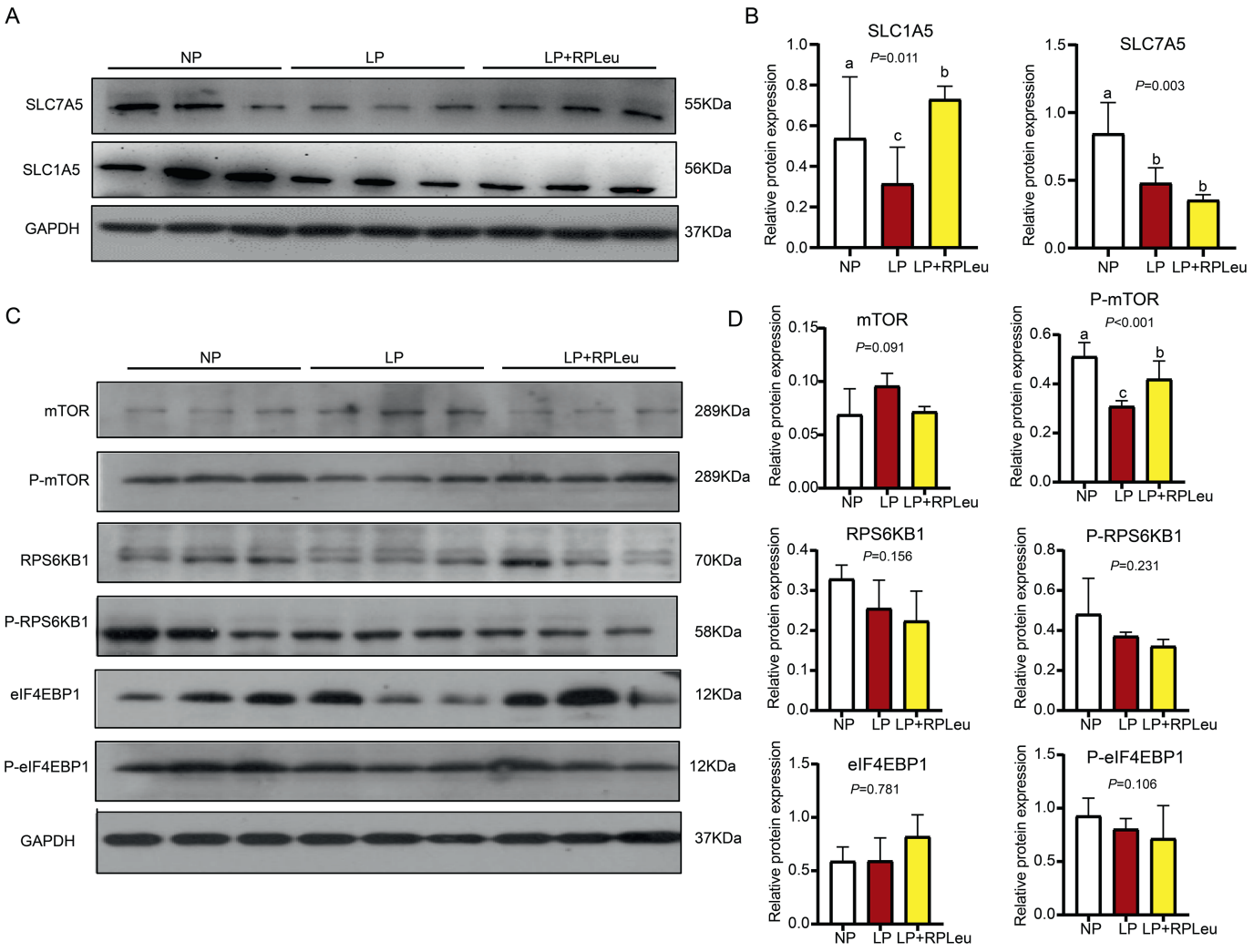
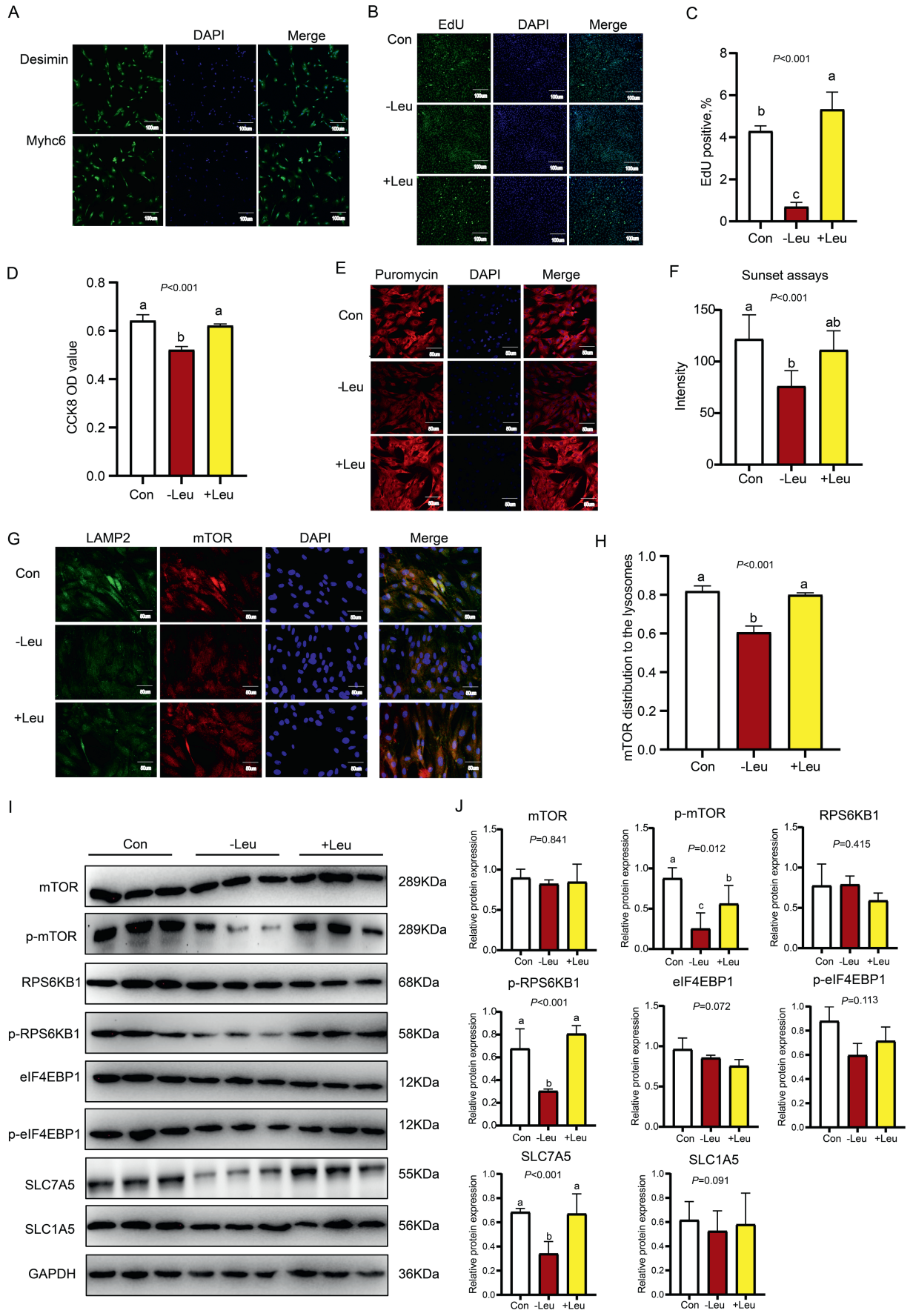


Fig. 3. Leu supplementation promotes activation of the mammalian target of rapamycin (mTOR) signalling pathway under chronic protein restriction conditions. (A, B) The protein expression levels of solute carrier family 7 member 5 (SLC7A5) and solute carrier family 1 member 5 (SLC1A5). (C, D) The protein expression levels of mTOR, p-mTOR, ribosomal protein s6 kinase (RPS6KB1), p-RPS6KB1, eukaryotic translation initiation factor 4E binding protein 1 (eIF4EBP1), and p-eIF4EBP1. NP = normal protein diet (14.24% crude protein [CP]); LP = low protein diet (8.27% CP with supplemental 1.66% rumen-protected lysine [RPLys] and 0.09% rumen-protected methionine [RPMet]); LP + RPLeu = LP with rumen-protected Leu (RPLeu) (8.75% CP with supplemental 1.66% RPLys, 0.09% RPMet and 1.46% RPLeu). Announcing different lowercase letters (a-c) in the bar chart indicate significant differences between groups. *P*-value < 0.05 was considered statistically significant. $n = 9$. GAPDH = glyceraldehyde-3-phosphate dehydrogenase.


A
DAPI
Merge
Desimin
Myhc6
B
EdU
DAPI
Merge
Con
-Leu
+Leu
C
P<0.001
EdU positive,%
Con -Leu +Leu
D
P<0.001
CCK8 OD value
Con -Leu +Leu
E
Puromycin
DAPI
Merge
Con
-Leu
+Leu
F
Sunset assays
P<0.001
Intensity
Con -Leu +Leu
G
LAMP2
mTOR
DAPI
Merge
Con
-Leu
+Leu
H
P<0.001
mTOR distribution to the lysosomes
Con -Leu +Leu
I
Con
-Leu
+Leu
mTOR 289KDa
p-mTOR 289KDa
RPS6KB1 68KDa
p-RPS6KB1 58KDa
eIF4EBP1 12KDa
p-eIF4EBP1 12KDa
SLC7A5 55KDa
SLC1A5 56KDa
GAPDH 36KDa
J
mTOR
P=0.841
p-mTOR
P=0.012
RPS6KB1
P=0.415
p-RPS6KB1
P<0.001
eIF4EBP1
P=0.072
p-eIF4EBP1
P=0.113
SLC7A5
P<0.001
SLC1A5
P=0.091
Relative protein expression
Con -Leu +Leu

protein expression levels did not differ significantly among the three groups ($P > 0.05$) (Fig. 4I–J).

4. Discussion

Previous research has focused on the connection of macronutrients (proteins, lipids, and carbohydrates) to humans and animals. Protein deficiency often results in limited growth and muscle development (Marin-Garcia and Llobat, 2021; Zhang et al., 2021). BCAAs (Leu, Ile, and Val) have received widespread attention as the most potent AAs stimulating protein synthesis (Columbus et al., 2015). In addition, BCAAs can affect appetite and thus physical health. For example, mice chronically exposed to a diet high in BCAAs experienced a large increase in appetite and weight gain (Solon-Biet et al., 2019). The purpose of our study was to reveal the effects of a low-protein diet on AAs and protein synthesis in goats and to further elucidate the regulatory effect of long-term (90 d) Leu supplementation on goats fed low-protein diets.

Our study showed that both ADFI and BW were lower in protein-restricted goats than those in control goats, and Leu supplementation improved appetite and BW in goats under chronic protein-restricted conditions. Previous studies have shown that dietary protein levels regulate feed intake because animals primarily eat to meet their protein requirements. Thus, feed and energy intake is expected to increase when dietary protein is reduced according to the protein leverage hypothesis (Simpson and Raubenheimer, 2011). Some researchers believe that a reduction in dietary protein is closely related to increased feed intake (Solon-Biet et al., 2014), which is inconsistent with our findings. The contrast in results may be because we restricted the protein intake by 40% in our study, and the regulatory effect of large protein restriction and mild protein restriction on the goat body differed. Similarly, studies in mice have shown that low-protein diets do not stimulate feed intake (Hu et al., 2018). Research has shown that appetite is not stimulated, but is suppressed in very low-protein diets (Wu et al., 2021).

Elevated levels of circulating BCAAs in humans are considered markers of obesity. Our results showed that heavier goats have higher concentrations of circulating BCAAs. However, elevated concentrations of circulating BCAAs are not markers of compromised health (Solon-Biet et al., 2019), and the goats remained healthy throughout the experimental period. Numerous studies have suggested that AAs metabolism is involved in appetite regulation (Hill and Blundell, 1988; Hall et al., 2003; Korompokis et al., 2016). Our findings suggest that protein restriction affects the levels of BCAAs and Trp in the plasma. Protein restriction decreased plasma BCAAs levels and increased Trp levels. Previous studies have observed that plasma levels of essential AAs (in particular, BCAAs) decrease with a low-protein diet (Solon-Biet et al., 2015; Fontana et al., 2016) and increase with a high-protein diet (Solon-Biet et al., 2019), consistent with our results. Dietary BCAAs can regulate appetite through interactions with other AAs, particularly Trp (Solon-Biet et al., 2019). 5-HT acts as a neurotransmitter by transmitting gut signals to the brain and regulating appetite (Wargent et al., 2020; Han et al., 2021). There are two main pathways for the production of 5-HT in the brain: the peripheral pathway (gut) and the central synthesis pathway. We found that protein restriction increased plasma 5-HT levels, whereas Leu supplementation decreased 5-HT levels. Previous studies have shown that mice subjected to protein or calorie restriction have elevated levels of 5-HT in the brain and plasma (Hernandez et al., 1989; Manjarrez et al., 2003). Research on mice showed that long-term exposure to foods high in BCAAs results in increased appetite (Solon-Biet et al., 2019), which is similar to our findings. Our metabolomics data showed that the LP group exhibited higher plasma TPH activity than that in the NP and LP + RPLeu groups. In addition, the metabolomic data showed that the plasma levels of 7-ketodeoxycholic acid were higher in the LP group than those in the NP and LP + RPLeu groups. 7-ketodeoxycholic acid is a type of bile acid, which can have appetite suppressant effects (Perino et al., 2021). This may explain why protein-restricted goats had lower feed intake, while Leu supplementation increased their appetite in our study.

Almost 90% of circulating 5-HT is generated by intestinal chromophores (ECs), which metabolize Trp to 5-HT via TPH1 (Yano et al., 2015). In this study, we observed that protein restriction resulted in elevated levels of 5-HT in peripheral plasma. In addition, 5-HT was produced in the gut and transported to the peripheral blood. Therefore, we examined 5-HT levels in the jejunal and ileal mucosa and found that 5-HT levels were elevated under protein-restricted conditions. 5-HT production in the gut increases and enters the peripheral plasma, which can suppress appetite (Crowell et al., 2004; McLean et al., 2007; Bellono et al., 2017). Since the BCAA and Trp levels in the peripheral plasma were altered, we aimed to elucidate the reasons for this phenomenon. The process of AA absorption from the gut into the peripheral blood is completed by various AA transporters (Poncet et al., 2013). Therefore, we measured the mRNA and protein expression levels of AA transporters (SLC7A7, SLC38A1, SLC7A6, SLC1A1, SLC1A5, SLC7A5, SLC3A2, and SLC6A19) in the jejunum and ileum. We found that under protein-restricted conditions, the mRNA expression of *SLC1A5* and *SLC7A5* in the jejunum and ileum of goats decreased significantly, Leu mainly enters cells through the L transport system, including SLC7A5, SLC3A2, SLC7A7, and solute carrier family 43 member 1 (SLC43A1) (Krokowski et al., 2013). Previous studies have indicated that Leu transport into cells requires reverse glutamine transport, and SLC1A5 first transports glutamine into cells, which is a prerequisite for Leu transport (Baird et al., 2009). Therefore, SLC1A5 may play a major role in Leu transport. The Leu transport capacity of the goat jejunal and ileal mucosa was reduced under protein-limiting conditions and increased after long-term Leu supplementation. In this study, we observed that ileal *SLC6A19* mRNA and protein expression levels were significantly higher in protein-restricted goats than those in the other two treatment groups. SLC6A19 is the primary transporter of neutral AAs in intestinal epithelial cells and is the main mediator of neutral AA delivery to the systemic circulation (Broer et al., 2004; Broer, 2009). SLC6A19 is required for Trp transport from the gut into the peripheral blood (Broer et al., 2011; Javed and Broer, 2019). Our results revealed that protein restriction enhanced ileal SLC6A19 expression and increased Trp transport into blood. To further

Fig. 4. Leu can regulate protein synthesis in goat muscle cells by affecting the activation of mechanistic target of rapamycin complex 1 (mTORC1). (A) The immunofluorescent staining of Desimin and Myhc6 in cells. (B) Proliferation cells labelled with 5-ethynyl-2′-deoxyuridine (EdU), where green shows EdU-positive cells and blue shows cell nuclei. (C) The percentage of EdU-positive cells. (D) CCK-8 assay. (E) Representative images of immunofluorescence SUface SEnsing of Translation (SUnSET) analysis of protein synthesis in goat muscle cells. (F) Immunofluorescence intensity analysis. (G, H) Representative pictures (G) and quantification (H) of colocalization between mammalian target of rapamycin (mTOR) (red) and lysosomal associated membrane protein 2 (LAMP2) (green) in goat muscle cells. (I, J) The protein expression levels of mTOR, p-mTOR, ribosomal protein s6 kinase (RPS6KB1), p-RPS6KB1, eukaryotic translation initiation factor 4E binding protein 1 (eIF4EBP1), p-eIF4EBP1, solute carrier family 7 member 5 (SLC7A5) and solute carrier family 1 member 5 (SLC1A5). Con = the medium treatments were DMEM/F12 (without fetal bovine serum [FBS]); -Leu = DMEM/F12 (without FBS and Leu); +Leu = Leu represented -Leu-treated cells. Announcing different lowercase letters (a-c) in the bar chart indicate significant differences between groups. *P*-value < 0.05 was considered statistically significant. DAPI = diamidino-phenyl-indole; LAMP2 = lysosomal associated membrane protein 2; OD = optical density.

explore the effects of protein restriction and Leu supplementation on intestinal AAs metabolism, we measured the mRNA expression and activity of enzymes related to BCAAs catabolism in the jejunal and ileal mucosa. BCAAs are catabolized and utilized in the intestinal mucosa (Wu, 1998). BCAT2 encodes mitochondrial BCAT (BCAT2), which is required for BCAA catabolic processes (Bledsoe et al., 1997). In addition, BCAA catabolism requires the BCKDH complex and acyl-CoA dehydrogenase (ACAD) (Alfardan et al., 2010; Wanders et al., 2012). Protein restriction increases jejunal mucosal BCAT activity and enhances *BCAT2* mRNA expression. Under protein-restricted conditions, BCAT and BCKD activities in the ileal mucosa increased and *BCKDHA* and *BCAT2* mRNA expression increased significantly. This result suggests that a portion of the BCAA in the ileum and jejunum of goats may be degraded to maintain body requirements under the restriction of protein intake, which may be a stress mechanism to compensate for the dysregulated intestinal energy supply due to protein deficiency and inadequate AAs supply. As the Trp content in the jejunal and ileal mucosa is altered, Trp metabolism may have been altered. Typically, >90% of 5-HT is produced by TPH1 enzyme in enterochromaffin cells (ECs). Therefore, we detected TPH1 protein expression in the jejunum and ileum using immunohistochemistry. We found that under protein-restriction conditions, TPH1 protein expression in the jejunum and ileum was enhanced, and Leu supplementation decreased TPH1 expression. Increased TPH1 expression in the intestines of goats in the protein restriction group resulted in increased 5-HT production, which resulted in higher levels of 5-HT to enter the peripheral blood and suppress goat appetite. Previous studies have shown that different protein diets can alter gut and serum 5-HT levels (Chi et al., 2017; Sandhu et al., 2017; Xie et al., 2019). To the best of our knowledge, our results are the first to reveal changes in AAs transport and metabolism in the gut under conditions of protein restriction and long-term Leu supplementation.

AAs concentration in the skeletal muscle reflects the nutritional and health status of the animal (He et al., 2012; Sales et al., 2013). Our study found that protein restriction and Leu supplementation did not affect Leu or total AAs content in the lateral thigh muscle. However, Leu supplementation increased Val content in the lateral thigh muscles. This may be due to increased BCAAs catabolism in the muscles of the NP and LP + RPLeu goats, as indicated by our BCAAs metabolic enzyme mRNA expression data. AA transporters regulate AAs concentration in cells. AA transporters are widely present in the membranes of many types of cells (Broer, 2008; Miyazaki and Esser, 2009). High AA receptor expression is associated with mTORC1 signalling pathway overactivation, which is a major contributor to the anabolic response following AAs supplementation (Tennant et al., 2009). We observed that protein restriction weakened SLC7A5 and SLC1A5 protein expression in the lateral thigh muscle. After Leu supplementation, SLC7A5 protein expression was enhanced. SLC7A5 transports BCAA; therefore, muscle tissue can obtain more BCAA from the blood to maintain continuous activation of the mTOR pathway. SLC1A5 and SLC7A5 increase intracellular leucine concentrations and stimulate mTORC1 activation (Evans et al., 2008). Previous studies have demonstrated that SLC7A5 protein expression increases 1 h after young people are injected with AAs (Drummond et al., 2010). A high dose of Leu can promote *SLC7A5* expression in skeletal muscles (Davuluri et al., 2016). In addition, we detected the mRNA expression and activity of BCAA catabolism-related enzymes in the lateral thigh muscle. We found that protein restriction lowered *BCAT* expression in the lateral thigh muscles and reduced the BCAA catabolism. Previous studies found that mice were thinner with high protein degradation in the skeletal muscle as a result of BCAT knockout (She et al., 2010; Lynch et al., 2015). In addition, Leu-ammonia in α-ketoisocaproic acid (KIC) is necessary to inhibit protein degradation in skeletal muscles (Tischler et al., 1982). After exercise, mTORC1 in the muscle was activated and the ratio of KIC to Leu was higher than that in the resting state (Knapik et al., 1991). To better clarify the mechanism by which Leu supplementation promotes muscle growth in goats, isolated goat muscle cells were treated. We found that a lack of Leu caused a slow proliferation of goat muscle cells and reduced protein synthesis. When Leu was added back, the proliferation rate of the muscle cells and protein synthesis increased. Similarly, we found that Leu deletion lowered SLC7A5 protein expression in muscle cells but had no effect on SLC1A5 expression. In contrast to what we reported, a lack of BCAAs caused elevated muscle *LAT1* expression in piglets (Li et al., 2016; Liu et al., 2016). We hypothesized that the reduced expression of SLC7A5 protein in goat muscle cells deficient in Leu may be due to the inability of muscle cells to fully metabolize and utilize all intracellular Leu after protein synthesis is inhibited. This may be an adaptive mechanism in muscle cells (in response to inhibition of protein synthesis under Leu deficiency). The metabolism of BCAAs in ruminants progressively decreases as the organism develops, a phenomenon not observed in humans, mice, or pigs (Faure et al., 2001), suggesting that the ruminant-specific physiological metabolism of BCAAs may influence the response of muscle amino acid transporters to amino acids. Functional coupling exists between SLC1A5 and SLC7A5 (Nicklin et al., 2009). ASCT2 has been shown to regulate increases in the intracellular glutamine concentration. LAT1 uses glutamine as an efflux substrate to promote extracellular leucine uptake, thus activating mTORC1 (Hayashi et al., 2012). However, functional coupling between SLC7A5 and SLC1A5 is not necessary in all cell types (Cormerais et al., 2018). Studies in both goat and skeletal muscle cell models have suggested that Leu regulates mTOR signalling pathway activation by affecting SLC7A5 expression. This suggests that SLC7A5 is a key target of Leu in the regulation of protein synthesis in goat muscle. Although Leu supplementation upregulated SLC1A5 expression in the animal model, similar results were not observed in the skeletal muscle cell model. Further research is needed to identify the mechanisms by which SLC1A5 and SLC7A5 respond to Leu in goat muscle cells.

5. Conclusion

These findings support the conclusion that long-term (three-month) Leu supplementation alleviates growth restriction due to protein deficiency in goats, which is related to goat muscle mTOR pathway activation by leucine through SLC7A5 to promote protein synthesis. Moreover, long-term Leu supplementation reduced intestinal tryptophan transport and metabolism, decreased 5-HT synthesis, and improved goat appetite.

Credit Author Statement

Xiaokang Lv: Writing – review & editing, Writing – original draft, Investigation, Data curation. **Aoyu Jiang:** Formal analysis, Data curation. **Jinling Hua:** Data curation. **Zixin Liu:** Data curation. **Qiongxian Yan:** Validation. **Shaoxun Tang:** Validation, Data curation. **Jinhe Kang:** Validation, Data curation. **Zhiliang Tan:** Conceptualization. **Jian Wu:** Data curation, Formal analysis. **Chuanshe Zhou:** Supervision, Conceptualization.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal

interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2024.09.005.

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