Animal Nutrition 19 (2024) 117–130

Contents lists available at ScienceDirect

Animal Nutrition

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

Original Research Article

Dietary protein re-alimentation following restriction improves protein deposition via changing amino acid metabolism and transcriptional profiling of muscle tissue in growing beef bulls

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ARTICLE INFO

Article history:
Received 20 October 2023
Received in revised form
25 April 2024
Accepted 26 April 2024
Available online 23 July 2024

Keywords:
Amino acid
Beef cattle
Compensatory growth
Metabolism
Protein

ABSTRACT

This study aimed to develop a compensatory growth model using growing beef cattle by changing dietary protein and to investigate the underlying mechanisms of compensatory protein deposition in muscle tissue. Twelve Charolais bulls were randomly assigned to one of two groups with two periods: 1) a control group (CON) fed a 13% crude protein (CP) diet for 6 weeks; 2) a treatment group (REC) fed a 7% CP diet for 4 weeks (restriction period) and fed a 13% CP diet in the following 2 weeks (re-alimentation period). Growth performance, digestibility, nitrogen balance, targeted metabolomics of amino acids (AA) in plasma, and transcriptional profiling in muscle tissue were analyzed. Protein restriction decreased average daily gain (ADG; $P < 0.05$), while protein re-alimentation increased ADG relative to the CON ($P < 0.05$). Compared to the CON, REC reduced retained N ($P < 0.05$), and protein re-alimentation increased retained N and N utilization efficiency ($P < 0.05$), due to reduced urinary urea, hippuric acid, and creatinine excretions ($P < 0.05$). Ruminal NH_3-N in the REC was lower than that in the CON in the protein re-alimentation period ($P < 0.05$). However, there was no difference in microbial protein and plasma urea nitrogen concentrations. Dietary protein restriction decreased plasma valine and aspartic acid concentrations relative to the CON ($P < 0.05$), and increased proline and 3-methyl-L-histidine concentrations ($P < 0.05$). After dietary protein re-alimentation, REC increased plasma citrulline concentrations ($P < 0.05$). The transcriptional profiling revealed that REC upregulated the AA transporter *SLC3A1*, and protein re-alimentation downregulated *SLC7A8* in the muscle cell membrane. Within the muscle cell, upregulated cytosolic arginine sensor for mTORC1 subunit 2 (*CASTOR2*) inhibited protein synthesis by inhibiting the mammalian target of rapamycin complex 1 phosphorylation in the protein restriction period, while DNA-damage-inducible transcript 4 (*DDIT4*) activated the mTOR signaling pathway and promoted protein synthesis in the protein re-alimentation period. In summary, the targeted metabolomics and transcriptomics analyses demonstrated that protein re-alimentation following restriction can promote protein synthesis and reduce muscle breakdown by regulating AA metabolism and functional transcripts related to AA transporters and the mTOR signaling pathway.

1. Introduction

Livestock production accounts for 77% of global agricultural land and contributes to 18% and 37% of global food energy and protein supplies, respectively (Ritchie and Roser, 2020). This sector plays a critical role in the agri-food system (Spiegal et al., 2022). Beef cattle can effectively convert low-quality forage and grain byproducts that are inedible to humans into nutritious human edible products. In addition to high-quality protein, beef contains essential nutrients, including iron, zinc, selenium, potassium, vitamin B, and fatty

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Peer review under the responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

https://doi.org/10.1016/j.aninu.2024.04.028

acids (Cashman and Hayes, 2017). Because of its nutritional value and palatability, beef has become the third most consumed meat product in the world (OECD, 2018). Rapid global population growth and increased pressure on arable land for livestock production require improvements in productivity, efficiency, and other aspects of the sustainability of the beef production system (Capper and Bauman, 2013; Greenwood, 2021).

Compensatory growth (CG) is a physiological phenomenon in which growth rate is accelerated in cattle upon re-alimentation following a prior period of dietary restriction (Hornick et al., 2000). For example, Keogh et al. (2015) reported that growth rate increased to 2.5 kg/d in the CG stage, compared with a control growth rate of 1.4 kg/d in beef cattle. Meanwhile, the ratio of feed consumption to body weight gain was decreased from 9.98 to 4.87, suggesting that CG significantly improved the feed utilization efficiency (Keogh et al., 2015). Skeletal muscle accounts for 50% of the animal's body weight and 25% of the basal metabolic expenditure (Costa et al., 2015; Henriksson, 1990). Hornick et al. (1998) reported that the protein content in the skeletal muscle in the CG group was greater than that in control group (89% versus 85.6%), indicating that the digested and absorbed nutrients might have been more efficiently used for protein synthesis and deposition in muscle tissue during the CG of beef cattle (Hornick et al., 2000). Keogh et al. (2016b) demonstrated that the expression of nutrient transporters and ribosome-related functional genes was upregulated to increase protein synthesis and muscle growth during the CG phase.

Most CG studies in beef cattle have been designed to feed ad libitum after feed restriction or supplementary feeding after starvation (Connor et al., 2010; Lehnert et al., 2006; Mullins et al., 2020). Given such designs, it is not possible to separate the effects of individual nutrients (protein, energy, vitamins, and mineral elements, etc.). It is important to understand which nutrients impact CG the most, and what nutrient levels are needed to achieve production targets during and after periods of nutrient restriction. In an intensive feeding system, beef cattle are usually fed ad libitum, so dietary energy intake might not be the primary factor to restrict growth rates. Conversely, previous studies demonstrated that dietary crude protein (CP) can increase ADG and protein deposition in beef cattle (Amaral et al., 2018; Moriel et al., 2015). Therefore, dietary protein may be the primary growth limiter in the growing period of beef cattle.

Although the phenomenon of CG has been widely utilized in beef production systems, the underlying biological mechanisms of protein restriction and re-alimentation on compensatory protein deposition are ambiguous and need to be elucidated fully. The objectives of this study were to develop a beef cattle model of CG by manipulating dietary protein supply and to investigate the underlying mechanisms of protein compensatory deposition in bulls.

2. Materials and methods

2.1. Animal ethics statement

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee at China Agricultural University (AW91104202-1-1).

2.2. Animals, experimental design, and diets

Twelve Charolais bulls with similar body weight (BW) and age (282.7 ± 27.3 kg and 6 to 8 months) were selected as experimental animals. The animals were blocked by initial BW and randomly assigned to one of two treatment groups with two periods, including a dietary protein restriction period and a subsequent re-alimentation period (Fig. 1). For the control group (CON), all the animals were offered a 13% CP diet for the entire 6 weeks (restriction period and re-alimentation period). For the treatment group (REC), the animals received a 7% CP diet for 4 weeks (restriction period). Following the restriction period, a 13% CP diet was offered to the REC animals for 2 weeks (re-alimentation period). The animals that received the low protein diet were expected to achieve a target mean daily growth rate of 0.4 kg/d, whereas the animals that received the high protein diet were expected to gain 1.0 kg/d (NASEM, 2016). A 5-d sample collection period followed

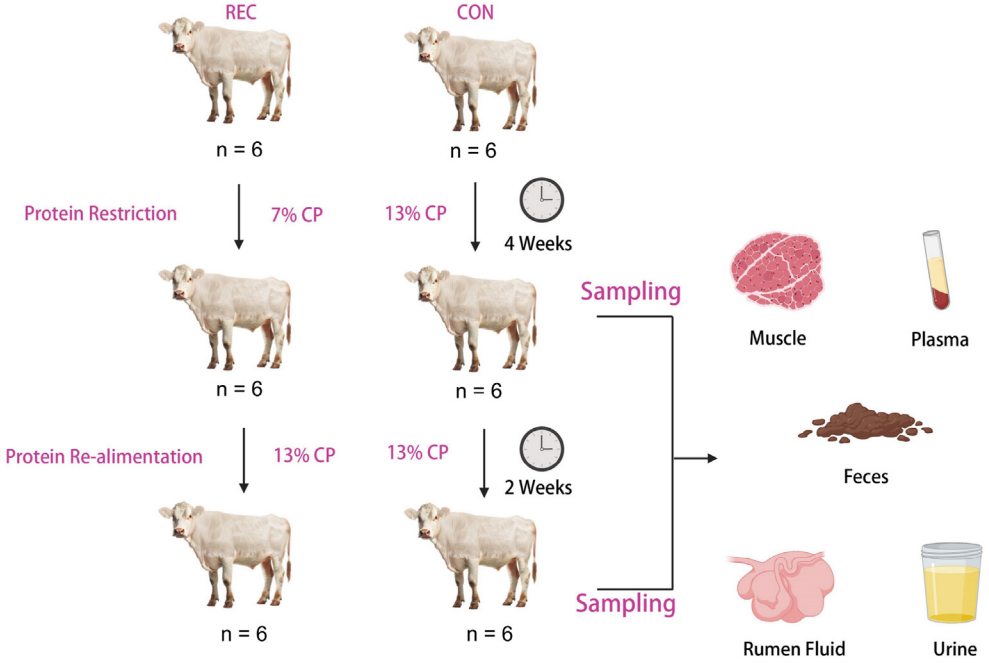

Fig. 1. Experimental design and timeline for protein restriction, re-alimentation, and sampling. CON: a 13% crude protein (CP) diet was fed both in the restriction and re-alimentation period; REC: a 7% CP diet was fed for 4 weeks in the restriction period and a 13% CP diet was fed subsequently for 2 weeks in the re-alimentation period.

the restriction and re-alimentation period. The animals were fed the same diet as the previous stage in the sampling period. The collected samples included feces, urine, muscle, plasma, and rumen fluid. Diet composition and nutritional contents are listed in Table 1.

All the animals were tethered and housed individually in metabolic stalls. The stalls were disinfected before the animal's arrival and were cleaned regularly throughout the experiment. The animals were adapted to the environment and experimental diets over a 7-d pre-feeding period at the beginning of the experiment. The cattle were fed ad libitum and had free access to water. Diets were offered twice daily at 07:00 and 16:00. The diet supply for each cattle was adjusted to ensure an average of 5% refusals. The diet supplies and refusals were monitored and recorded daily to calculate daily feed intake.

2.3. Sample collection

2.3.1. Body weight, average daily gain, and diet samples

Animal body weights (BW) were recorded before morning feedings on two consecutive days at the beginning of the experiment (d 0), at the end of the restriction period (d 28), and the end of the re-alimentation period (d 48), respectively. Average daily gain (ADG) for each period was calculated from the initial and final BW based on the average of the two consecutive BW records. Diet ingredient samples were collected weekly before feeding, and diet refusals were weighed and sampled daily. Diet ingredients and refusals were composited within period and treatment and stored at −20 °C for future analysis.

Table 1
Diet ingredients and nutrient composition, % DM.

Item	CON	REC
Ingredients		
Corn straw	52.88	63.82
Corn	28.09	29.15
DDGS	0	5.95
Soybean meal	13.63	0
Wheat bran	4.39	0
Premix[1]	0.47	0.42
Salt	0.44	0.39
$CaHPO_4$	0	0.27
Limestone	0.27	0
Nutrient composition[2]		
OM	95.50	95.82
CP	12.83	7.04
EE	1.05	1.84
NDF	57.06	66.33
ADF	31.78	37.02
ME, Mcal/kg	2.34	2.24
NE_m, Mcal/kg	1.47	1.38
NE_g, Mcal/kg	0.88	0.79
AA composition, % total AA		
Alanine	6.45	4.13
Arginine	9.68	8.61
Asparagine	11.83	28.10
Aspartic acid	2.80	1.18
Glutamine	1.64	3.01
Glutamic acid	10.17	12.63
Glycine	1.13	1.82
Histidine	23.64	6.91
Isoleucine	4.37	2.35
Lysine	2.81	4.99
Methionine	2.39	0.17
Phenylalanine	4.30	1.87
Proline	5.06	9.39
Serine	3.81	7.85
Threonine	5.62	3.49
Tryptophan	1.18	0.58
Tyrosine	1.07	1.09
Valine	2.04	1.82

DM = dry matter; DDGS = distillers dried grains with solubles; OM = organic matter; CP = crude protein; EE = ether extracts; NDF = neutral detergent fiber; ADF = acid detergent fiber; ME = metabolizable energy; NE_m = net energy for maintenance; NE_g = net energy for gain.

[1] Vitamin and minerals provided per kilogram of premix: vitamin A ≥ 22,000 IU; vitamin D_3 ≥ 6300 IU; vitamin E ≥ 700 IU; nicotinic acid ≥ 370 mg; Cu 350 mg; Fe 1300 mg; Zn 1300 mg; Mg 3000 mg; Mn 900 mg; I 15 mg; Se 7.5 mg; Co 5 mg.

[2] ME, NE_m, and NE_g were calculated based on feed library values in Beef NASEM (2016), others are analyzed values.

2.3.2. Ruminal fluid, blood, feces, and urine samples

On the first day of each sampling period, an aliquot of 200 mL rumen fluid was collected before the morning feeding via the esophagus using a stomach tube. The first tube of rumen fluid was discarded to avoid saliva contamination. Ruminal pH was immediately measured using a digital pH meter (PHS-3C; Yueping Scientific Instrument, Shanghai). The ruminal fluid samples were transferred to centrifuge tubes after being strained through four layers of cheesecloth and stored at −20 °C. A 10-mL blood sample was collected from the jugular vein of each animal into vacutainer tubes containing sodium heparin (Greiner Bio-one, Shanghai). The blood samples were centrifuged at 3000 × *g* at 4 °C for 15 min. The resulting plasma was collected and stored at −20 °C until later analysis.

Total feces and urine were collected over a 5-d sampling collection period as previously stated. Feces excreted from each animal on rubber mats were immediately collected and stored individually in a plastic bucket with a cover. Daily excretion of feces from each bull was recorded, homogenized, and composited within each period, and stored at −20 °C until later analysis. Urine was collected using a urine funnel. The urine funnel was connected to a polyethylene hose into a sealed plastic bucket which was surrounded with ice packs to keep the temperature close to 0 °C. Excreted urine was weighed every 4 h, and 90 mL urine samples were collected after through mixing. A 10-mL sulfuric acid (H_2SO_4) solution (10%, vol/vol) was added to the urine sample to preserve urinary N. Urine samples were composited based on daily excretion within each bull and period and stored at −20 °C until further analysis.

2.3.3. Muscle biopsy

Muscle biopsies were conducted at 09:00 on the last day of each sample collection period. Briefly, a 5 × 5 cm biopsy area between 12th and 13th transverse of the *longissimus thoracis et lumborum* muscle was shaved and wiped three times with betadine and 75% alcohol in a circular motion starting with an inside-out approach for disinfection. Using an 18-gauge needle, 7 mL of 5% lidocaine hydrochloride was injected under the skin and intramuscular at the biopsy area. After 5 min of anesthesia, the skin was incised using a scalpel blade, and the muscle samples were collected using a sterile puncture instrument inserted to a depth of about 4 to 5 cm. The collected muscle samples were rinsed with saline, frozen in liquid nitrogen, and transferred to the −80 °C refrigerator for further analysis. The incision site was sealed and a thin layer of erythromycin ointment was applied. The left *longissimus thoracis et lumborum* muscle was sampled in the restriction period, and the right *longissimus thoracis et lumborum* muscle was sampled in the re-alimentation period, allowing the muscle to properly regenerate and animals to heal.

2.4. Sample analysis

2.4.1. Nutrient composition analysis

The composited corn straw, concentrate mixture, diet refusals, and fecal samples were dried at 65 °C in a forced air oven for 48 h. The dried samples were ground through a Wiley mill (1 mm

screen). Feed, diet refusals, and feces were analyzed for dry matter (DM), ash, and ether extract (EE) according to AOAC (2005) method no. 930.15, 942.05, and 920.39, respectively. Organic matter (OM) content was calculated by subtracting the percentage of the ash from 100. Total N in feed, diet refusals, feces, and urine was analyzed using the Kjeldahl method according to AOAC (2005; method no. 994.12), and the N values were converted to CP by multiplying by 6.25. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed with additions of sodium sulfite and heat-stable α-amylase using a fiber analyzer (ANKOM Technology Corp, Fairport, NY) based on the method reported by Van Soest et al. (1991). Dietary AA profiles were analyzed according to China national standard (GB/T 18246-2019). Dietary metabolizable energy (ME), net energy for maintenance (NE_m), and net energy for gain (NE_g) were estimated using feed library values from the NASEM (2016).

2.4.2. Chemical composition of urine, ruminal ammonia N, and microbial crude protein

Urinary urea and creatinine were analyzed using commercial assay kits (Jiangsu Addison Biotechnology Co., Ltd., China) according to the diacetyl monoxime method (Friedman, 1953) and Jaffe's method (Yatzidis, 1974), respectively. Allantoin and uric acid in urine were analyzed using a spectrophotometer (UV-1801; Beijing Rayleigh Analytical Instrument, China) as reported by Chen and Gomes (1995). Hippuric acid in urine was analyzed according to China national standard (WS/T 52-1996) on a microplate reader (Synergy 4, BioTek, USA). Ruminal ammonia N (NH_3-N) was analyzed using the colorimetric method of Broderick and Kang (1980) and a spectrophotometer (UV-1801; Beijing Rayleigh Analytical Instrument, China). Ruminal microbial protein (MCP) concentrations were determined using the improved Lowry's assay (Makkar et al., 1982).

2.4.3. Plasma urea and targeted metabolomics of amino acids

The plasma urea N (PUN) concentrations were analyzed using a commercial assay kit (Jiangsu Addison Biotechnology Co., Ltd., China) on a microplate reader (Synergy 4, BioTek, USA) based on the urease method. Targeted metabolomics of amino acids (AA) in plasma was conducted using the ultra-high performance liquid chromatography–mass spectrometry (UHPLC–MS/MS) technique (Simura et al., 2018). Briefly, approximately 1 g of plasma samples were added into a 2-mL centrifuge tube and mixed with 1 mL of a methanol:acetonitrile:water solution (2:2:1, vol:vol:vol) that contained an isotope-labeled internal standard mixture (Merck Chemical Technology Co., Ltd, Shanghai, China). The resulting solution was homogenized at 45 Hz for 4 min and ultrasonicated for 5 min in an ice water bath. The homogenization and ultrasonic steps were repeated 3 times followed by storage at −20 °C for 1 h, and then were centrifuged at 15,000 × *g* and 4 °C for 15 min. The supernatant (200 μL) was collected and passed through a 0.22-μm membrane. The filtered sample (1 μL) was injected into a Waters ACQUITY I-Class (Waters Corporation, Milford, MA, USA) UHPLC system equipped with a Waters ACQUITY UPLC BEH Amide (150 × 2.1 mm, 1.7 μm) liquid chromatographic column. The mobile phases were 0.2% formic acid in water (A) and 0.2% formic acid in acetonitrile (B). The column temperature was 35 °C. The autosampler temperature was 10 °C. Mass spectrometric detection was carried out in a 6500 QTRAP triple quadrupole mass spectrometer (SCIES, USA) equipped with an IonDrive Turbo V electrospray ionization (ESI) interface. The following conditions were used for the analysis: curtain gas = 35 psi, ion spray voltage = +5500 V, −4500 V, temperature = 550 °C, ion source gas 1 = 50 psi, ion source gas 2 = 55 psi. The multiple reaction monitoring (MRM) parameters for each of the targeted components were optimized before the UHPLC–MS/MS analysis. The instrument was controlled, and data was collected using the Analyst 1.6.2 software (SCIEX, USA). Chromatograms were processed using the MultiQuant 2.1 software (SCIEX, USA).

2.4.4. RNA isolation and transcriptome sequencing

RNA was extracted from approximately 500 mg of muscle sample using the TRIZOL RNA extraction kit according to the manufacturer's instructions (Invitrogen Corp., Carlsbad, CA, USA). The concentration and purity of the extracted RNA were analyzed using the Nanodrop 2000 (Nanodrop Technologies, Wilmington, DE, USA), and RNA integrity (RIN) was evaluated using the RNA Nano 6000 kit (Agilent Technologies Inc., USA) in the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). RNA samples with a RIN greater than 8 were selected for subsequent sequencing analysis. One sample was discarded because of low RIN, leaving 23 RNA samples for further sequencing analysis. mRNA was isolated from total RNA using the Ribo-Zero rRNA Removal Kit (Epicentre, Madison, WI, USA). cDNA libraries were prepared using the Illumina TruSeq RNA sample prep kit following the manufacturer's instructions (Illumina, San Diego, CA, USA). Pair-end libraries (2 × 150 bp) were sequenced on the Illumina HiSeq 4000 platform (Illumina, San Diego, California, United States).

2.4.5. Quality control and sequence alignment

The quality statistics of raw sequence data obtained from the Illumina platform were primarily analyzed using FASTQC software (version 0.10.0). Reads with a low sequence quality (<30 at the 3′ end) were removed. The reads containing more than 10% of unknown nucleotides were removed, and sequences with lengths less than 50 bp after adapter removal and mass pruning were discarded. After verifying the FASTQC results of the trimmed reads, the clean reads were mapped to the bovine reference genome using TopHat (v2.0.9), and the number of reads mapped to gene features was counted using HTSeq (v0.5.4p5).

2.4.6. Identification of differentially expressed genes and functional annotation

Gene expression was normalized and quantified as transcripts per million (TPM). All the data analyses and visualization were performed using R Statistical Software (v4.1.2; R Core Team, 2021). The criteria for differentially expressed genes (DEGs) between treatments was set at $P < 0.05$ and $|\log_2\text{FoldChange}| > 1$. The DEGs were clustered by the pheatmap library. To detect the biological functions of the DEGs, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were conducted using the clusterProfiler library. The pathways meeting the conditions of $P < 0.05$ were defined as significant enrichment pathways. Specific genes related to protein metabolism and AA metabolism pathways (Table S1) were analyzed. For the GO enrichment analysis, genes were grouped into cellular composition (CC), molecular function (MF), and biological processes (BP), respectively.

2.5. Data calculations and statistical analysis

All data calculations and analyses were conducted in R Statistical Software (v4.1.2; R Core Team, 2021). Apparent total tract nutrient digestibility was calculated by subtracting fecal nutrient excretions from nutrient intake, then dividing by nutrient intake. Nitrogen retention was calculated by subtracting urinary and fecal

N from N intake. Nitrogen utilization efficiency (NUE) was calculated by dividing nitrogen retention by N intake and multiplying by 100. The microbial N supply was estimated according to Chen and Gomes (1995) based on the total urinary excretions of purine derivatives including uric acid and allantoin.

The data pertaining to growth performance, apparent nutrient digestibility, N balance, and targeted metabolomics of amino acids were analyzed using the mixed linear model:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha_i \times \beta_j + \delta_k + \varepsilon_{ijk}$$

where Y_{ijk} is the dependent variable; μ is the population mean; α_i is the fixed treatment effect (i = 1, 2); β_j is the fixed period effect (j = 1, 2); $\alpha_i \times \beta_j$ is the interaction between treatment and period; δ_k is the random effect of experimental block divided according to initial BW (k = 1, 2, 3, 4, 5, 6); ε_{ijk} is the residual error. The Tukey–Kramer test was conducted to make post-hoc pairwise multiple comparisons (Benjamini and Braun, 2002). Differences among treatments were considered significant when P-values were less than or equal to 0.05 and were considered not significant when P-values were greater than 0.05. All results are reported as least squares means.

The Wilcoxon non-parametric test was used to analyze the transcriptome profiles and differential gene expression. Enrichment of DEGs with KEGG and GO, and the calculation formula for enrichment significance was:

$$P\text{-value} = 1 - \sum_{j=0}^{x-1} \frac{\binom{M}{j}\binom{N-M}{n-j}}{\binom{N}{n}}$$

where N is the number of genes participating in GO/KEGG annotation among all genes; n is the number of DEGs in N; M is the number of genes annotated to a certain GO term or KEGG pathway among all genes; x is the number of DEGs annotated to a certain GO term or KEGG pathway.

The correlations between DEGs and plasma targeted AA metabolites were analyzed using the Spearman correlation method and visualized in a network graph. To decrease the correlation matrix size, correlation coefficients were filtered, and only coefficients with an absolute value greater than 0.3 and a P-value less than or equal to 0.05 were considered significant.

3. Results

3.1. Effects of dietary protein restriction and subsequent re-alimentation on dry matter intake and growth performance

Significant interaction effects were observed between treatment and period for DMI, BW, and ADG ($P < 0.001$; Table 2; Fig. 2). Compared with the CON, dietary CP restriction decreased DMI, BW, and ADG ($P < 0.05$). In the subsequent re-alimentation period, although there was no significant difference in DMI, ADG in the REC was greater than that in the CON ($P < 0.05$), but the BW in the REC was still lesser than the BW in the CON at the end of the re-alimentation period ($P < 0.05$).

3.2. Effects of dietary protein restriction and subsequent re-alimentation on apparent total tract nutrient digestibility

There was a significant interaction effect between treatment and period on apparent total tract digestibility of CP ($P < 0.01$) and EE ($P = 0.03$; Table 2). There was a significant period effect on nutrient digestibility for DM ($P < 0.001$), OM ($P < 0.001$), ADF ($P = 0.009$). No significant treatment effect was observed on the apparent total tract digestibility of DM, OM, NDF, and ADF within the restriction or re-alimentation period. Dietary CP restriction increased CP digestibility ($P < 0.05$) compared to the CON, but decreased EE digestibility ($P < 0.05$). Regardless of treatment group, the digestibility of DM and OM in the re-alimentation period was greater than that in the restriction period ($P < 0.05$).

3.3. Effects of dietary protein restriction and subsequent re-alimentation on ruminal pH, NH_3-N, microbial protein, and PUN concentrations

As displayed in Table 3, significant interaction effects were observed on concentrations of PUN, ruminal NH_3-N, and MCP ($P < 0.001$). Compared with the CON, dietary CP restriction decreased the concentrations of PUN, ruminal NH_3-N, and MCP ($P < 0.05$). In the re-alimentation period, no significant treatment effect was observed on the concentrations of MCP and PUN between the CON and the REC, while the REC had lesser ruminal NH_3-N concentration than the CON ($P < 0.05$). There was no significant difference in ruminal pH between the two groups during the whole experimental period.

Table 2
Effects of dietary crude protein restriction and subsequent re-alimentation on DMI, growth performance and apparent total tract digestibility of growing beef bulls.[1]

Item	Restriction		Re-alimentation		SEM	*P*-value		
	CON	REC	CON	REC		Treatment	Period	Treatment × Period
DMI, kg/d	6.65^{a}	4.87^{b}	7.22^{a}	6.55^{a}	0.184	<0.001	<0.001	<0.001
ADG, kg/d	1.12^{b}	0.39^{c}	1.38^{b}	2.03^{a}	0.132	<0.001	<0.001	<0.001
BW, kg	310^{b}	286^{c}	330^{a}	308^{b}	9.3	<0.001	<0.001	<0.001
Apparent total tract digestibility, %								
DM	51.4^{b}	50.3^{b}	62.0^{a}	64.8^{a}	2.02	0.430	<0.001	0.328
OM	58.3^{b}	57.2^{b}	67.1^{a}	69.6^{a}	2.52	0.415	<0.001	0.326
CP	62.9^{a}	46.9^{b}	68.7^{a}	66.3^{a}	2.36	<0.001	<0.001	<0.001
EE	62.3^{b}	80.1^{a}	61.3^{b}	60.2^{b}	4.89	0.009	0.002	0.033
NDF	51.0^{b}	57.0^{ab}	60.5^{ab}	63.3^{a}	2.44	0.234	0.067	0.513
ADF	46.9^{b}	50.1^{ab}	58.2^{ab}	61.3^{a}	3.03	0.727	0.009	0.977

DM = dry matter; BW = final body weight; ADG = average daily gain; OM = organic matter; CP = crude protein; EE = ether extracts; NDF = neutral detergent fiber; ADF = acid detergent fiber.
Within a row, means with different superscripts differ at $P \leq 0.05$.
[1] CON: a 13% crude protein (CP) diet was fed both in the restriction and re-alimentation period; REC: a 7% CP diet was fed for 4 weeks in the restriction period and a 13% CP diet was fed subsequently for 2 weeks in the re-alimentation period.

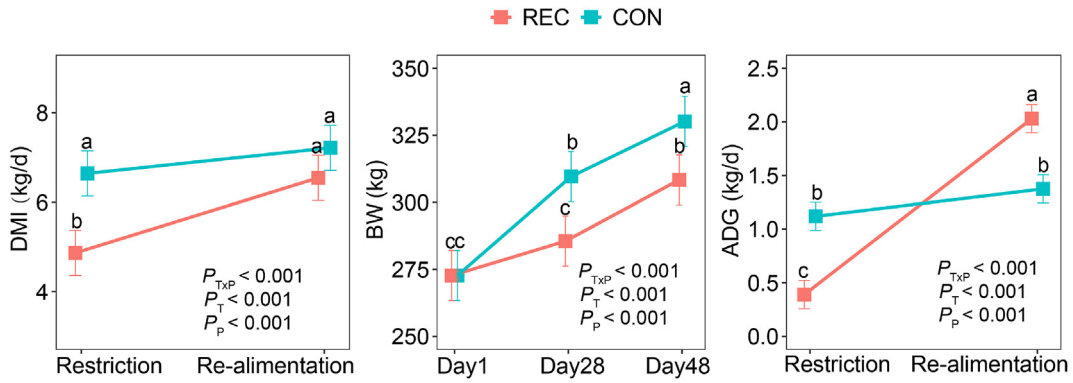

Fig. 2. Effects of dietary protein restriction and subsequent re-alimentation on dry matter intake (DMI), body weight (BW), and average daily gain (ADG) in growing beef bulls. Means with different superscripts differ at $P \leq 0.05$. T: treatment effect; P: period effect; T × P: interaction between treatment and period. CON: a 13% crude protein (CP) diet was fed both in the restriction and re-alimentation period; REC: a 7% CP diet was fed for 4 weeks in the restriction period and a 13% CP diet was fed subsequently for 2 weeks in the re-alimentation period.

Table 3
Effects of dietary protein restriction and subsequent re-alimentation on ruminal pH, NH_3-N, microbial protein, and plasma urea N concentrations in growing beef bulls.[1]

Item	Restriction		Re-alimentation		SEM	*P*-value		
	CON	REC	CON	REC		Treatment	Period	Treatment × Period
pH	6.52^{a}	6.72^{a}	6.67^{a}	6.70^{a}	0.123	0.116	0.183	0.342
NH_3-N, mmol/L	7.35^{ab}	1.52^{c}	7.97^{a}	6.55^{b}	0.378	<0.001	<0.001	<0.001
MCP, g/L	0.30^{a}	0.15^{b}	0.28^{a}	0.32^{a}	0.029	<0.001	<0.001	<0.001
PUN, mmol/L	4.06^{a}	1.17^{c}	3.30^{b}	2.86^{b}	0.286	<0.001	<0.001	<0.001

NH_3-N = ammonia-nitrogen; MCP = microbial crude protein; PUN = plasma urea nitrogen.
Within a row, means with different superscripts differ at $P \leq 0.05$.
[1] CON: a 13% crude protein (CP) diet was fed both in the restriction and re-alimentation period; REC: a 7% CP diet was fed for 4 weeks in the restriction period and a 13% CP diet was fed subsequently for 2 weeks in the re-alimentation period.

Table 4
Effects of dietary protein restriction and re-alimentation on nitrogen balance of growing beef bulls.[1]

Item	Restriction		Re-alimentation		SEM	*P*-value		
	CON	REC	CON	REC		Treatment	Period	Treatment × Period
N intake, g/d	143.0^{ab}	59.5^{c}	146.7^{a}	135.4^{a}	3.21	<0.001	0.039	<0.001
Fecal N, g/d	53.9^{a}	36.7^{c}	52.0^{ab}	43.0^{b}	3.01	<0.001	<0.001	0.004
Urinary N, g/d	54.5^{a}	9.7^{c}	59.1^{a}	41.5^{b}	2.98	<0.001	0.055	<0.001
Total N excretion, g/d	108.4^{a}	46.4^{c}	111.1^{a}	84.5^{b}	3.03	<0.001	<0.001	<0.001
Fecal N/Total N excretion, %	49.7^{b}	79.1^{a}	46.8^{b}	50.9^{b}	2.45	<0.001	<0.001	<0.001
Urinary N/Total N excretion, %	50.3^{a}	20.9^{b}	53.2^{a}	49.1^{a}	2.45	<0.001	<0.001	<0.001
Retained N, g/d	34.6^{b}	13.1^{c}	35.6^{b}	50.9^{a}	3.96	<0.001	<0.001	<0.001
NUE, %	24.2^{b}	22.0^{b}	24.3^{b}	37.6^{a}	2.73	0.069	0.040	0.048

NUE = nitrogen utilization efficiency.
Within a row, means with different superscripts differ at $P \leq 0.05$.
[1] CON: a 13% of crude protein (CP) diet was fed both in the restriction and re-alimentation period; REC: a 7% of CP diet was fed for 4 weeks in the restriction period and a 13% of CP diet was fed subsequently for 2 weeks in the re-alimentation period.

3.4. Effects of dietary protein restriction and subsequent re-alimentation on nitrogen balance

Significant interaction effects were observed on all variables listed in Table 4 ($P < 0.01$). Dietary CP restriction decreased N intake, fecal N, urinary N, total N excretion, the proportion of urinary N to total N excretion, and retained N compared to the CON ($P < 0.05$), and increased the proportion of fecal N to total excreted N ($P < 0.05$). There was no difference in NUE between the REC and the CON in the restriction period. In the re-alimentation period, animals in the REC had lesser urinary N and total N excretion compared to the CON ($P < 0.05$), and greater retained N and NUE ($P < 0.05$). Within the REC, except for the proportion of fecal N to total N excretion, all the variables were greater in the re-alimentation period compared to the restriction period ($P < 0.05$).

3.5. Effects of dietary protein restriction and subsequent re-alimentation on urinary composition

As shown in Table 5, except for uric acid ($P = 0.004$), no significant interaction effects were observed on all the urinary N composition variables. All variables had treatment effects ($P < 0.05$), and all variables had period effects ($P < 0.05$) except creatinine, allantoin, urinary PD and estimated microbial N supply. Compared with the CON, dietary protein restriction decreased urinary excretion of urea, allantoin, uric acid, hippuric

Table 5
Effects of dietary crude protein restriction and re-alimentation on urinary nitrogen compounds of growing beef bulls.[1]

Item	Restriction		Re-alimentation		SEM	*P*-value		
	CON	REC	CON	REC		Treatment	Period	Treatment × Period
Urinary N compound excretion, mmol/d								
Urea	1173.7^{a}	56.1^{c}	1260.9^{a}	665.0^{b}	63.61	<0.001	<0.01	0.086
Uric acid	6.0^{a}	2.52^{b}	4.36^{ab}	4.2^{ab}	0.60	<0.001	0.036	0.004
Hippuric acid	126.3^{ab}	43.1^{c}	158.6^{a}	85.6^{b}	10.69	<0.001	0.024	0.618
Creatinine	105.0^{a}	74.7^{b}	119.9^{a}	95.3^{b}	9.34	0.043	0.065	0.722
Allantoin	99.9^{a}	61.7^{b}	101.4^{a}	95.2^{ab}	8.86	0.002	0.895	0.072
Urinary PD	105.9^{a}	64.2^{b}	105.8^{a}	99.4^{a}	9.07	0.001	0.943	0.051
Nitrogenous compound-N/urinary N, %								
Urea	61.8^{a}	15.2^{b}	76.1^{a}	62.6^{a}	4.78	<0.001	<0.001	<0.001
Uric acid	0.7^{b}	1.5^{a}	0.51^{b}	0.8^{b}	0.13	<0.001	<0.001	0.026
Hippuric acid	3.3^{c}	6.3^{a}	4.7^{b}	4.0^{bc}	0.37	0.002	<0.001	<0.001
Creatinine	8.6^{b}	33.8^{a}	10.8^{b}	13.5^{b}	2.04	<0.001	<0.001	<0.001
Allantoin	10.9^{b}	36.7^{a}	12.1^{b}	17.6^{b}	2.30	<0.001	0.071	<0.001
Estimated microbial N supply,[2] g/d	67.0^{a}	32.8^{b}	65.8^{a}	61.5^{a}	7.58	0.001	0.807	0.048

PD = purine derivatives including uric acid and allantoin.
Within a row, means with different superscripts differ at $P \leq 0.05$.
[1] CON: a 13% crude protein (CP) diet was fed both in the restriction and re-alimentation period; REC: a 7% CP diet was fed for 4 weeks in the restriction period and a 13% CP diet was fed subsequently for 2 weeks in the re-alimentation period.
[2] Microbial N supply was estimated according to Chen and Gomes (1995) based on total urinary excretions of purine derivatives.

acid, creatinine, PD, and estimated microbial N, and the proportion of urea relative to total urinary N ($P < 0.05$), and increased the proportions of allantoin, uric acid, and hippuric acid to total urinary N ($P < 0.05$). Conversely, dietary protein re-alimentation decreased urinary excretion of urea, hippuric acid, and creatinine ($P < 0.05$). Within the REC, urinary excretion of urea, hippuric acid, and the proportion of urea to urinary total N were greater in the re-alimentation period relative to the restriction period ($P < 0.05$), while the proportions of uric acid, hippuric acid, creatinine, and allantoin to total N urinary were lesser ($P < 0.05$).

3.6. Effects of dietary protein restriction and subsequent re-alimentation on targeted metabolomics of amino acids in plasma

Significant interaction effects were observed on lysine, phenylalanine, proline, 3-methyl-L-histidine, and citrulline concentrations in plasma (Table 6). Valine was affected by both treatment and period ($P < 0.05$). Histidine, isoleucine, and alanine concentrations were only affected by period ($P < 0.05$). Dietary protein restriction decreased concentrations of valine and aspartic acid in plasma relative to the CON ($P < 0.05$), and increased the concentrations of proline and 3-methyl-L-histidine ($P < 0.05$). Compared with the CON, dietary protein re-alimentation increased citrulline

Table 6
Effects of dietary protein restriction and subsequent re-alimentation on plasma targeted metabolomics for amino acids in growing beef bulls.[1]

Plasma targets amino acid metabolites	Restriction		Re-alimentation		SEM	*P*-value		
	CON	REC	CON	REC		Treatment	Period	Treatment × Period
Arginine	9.5	8.3	10.5	9.9	0.69	0.410	0.109	0.650
Glycine	4.0	4.2	3.3	3.6	0.31	0.466	0.096	0.948
Isoleucine	7.0^{ab}	5.5^{b}	8.0^{a}	7.5^{a}	0.49	0.067	<0.001	0.273
Histidine	32.6^{a}	30.5^{ab}	21.7^{bc}	18.4^{c}	2.08	0.911	<0.001	0.770
Lysine	7.3	8.5	8.5	6.6	0.65	0.036	0.033	0.016
Methionine	0.8	0.7	0.7	0.6	0.081	0.746	0.342	0.935
Phenylalanine	3.7^{ab}	4.9^{a}	3.9^{ab}	3.1^{b}	0.33	0.083	<0.001	<0.001
Threonine	9.9	7.3	10.1	10.2	1.44	0.930	0.098	0.274
Tryptophan	0.5	0.6	0.9	0.8	0.09	0.415	0.093	0.391
Valine	7.2^{a}	5.5^{b}	7.6^{a}	6.7^{ab}	0.39	0.033	0.017	0.302
Alanine	0.6^{a}	0.5^{ab}	0.4^{b}	0.4^{b}	0.03	0.141	0.018	0.152
Serine	16.5	16.8	16.2	16.7	1.69	0.971	0.944	0.960
Proline	0.6^{b}	0.7^{a}	0.6^{ab}	0.5^{b}	0.03	0.004	0.001	0.014
L-4-Hydroxyproline	5.6	5.0	4.9	4.6	0.59	0.596	0.548	0.758
Asparagine	3.3	3.8	4.3	4.3	0.33	0.232	0.208	0.320
Aspartic acid	0.2^{a}	0.1^{b}	0.2^{ab}	0.2^{ab}	0.02	0.016	0.139	0.060
Glutamine	31.3	26.9	30.5	29.7	2.29	0.786	0.393	0.443
Glutamic acid	5.2	5.2	4.1	4.6	0.53	0.765	0.363	0.629
3-Methyl-L-histidine	3.9^{b}	5.6^{a}	3.7^{b}	3.5^{b}	0.43	0.003	<0.001	0.012
Citrulline	11.7^{b}	9.6^{b}	10.5^{b}	12.6^{a}	0.83	0.014	0.009	0.010
Tyrosine	0.6	0.7	0.7	0.6	0.06	0.397	0.723	0.272
D,L-0-Tyrosine	1.6	1.3	1.6	1.5	0.17	0.354	0.267	0.592

Within a row, means with different superscripts differ at $P \leq 0.05$.
[1] CON: a 13% crude protein (CP) diet was fed both in the restriction and re-alimentation period; REC: a 7% CP diet was fed for 4 weeks in the restriction period and a 13% CP diet was fed subsequently for 2 weeks in the re-alimentation period.

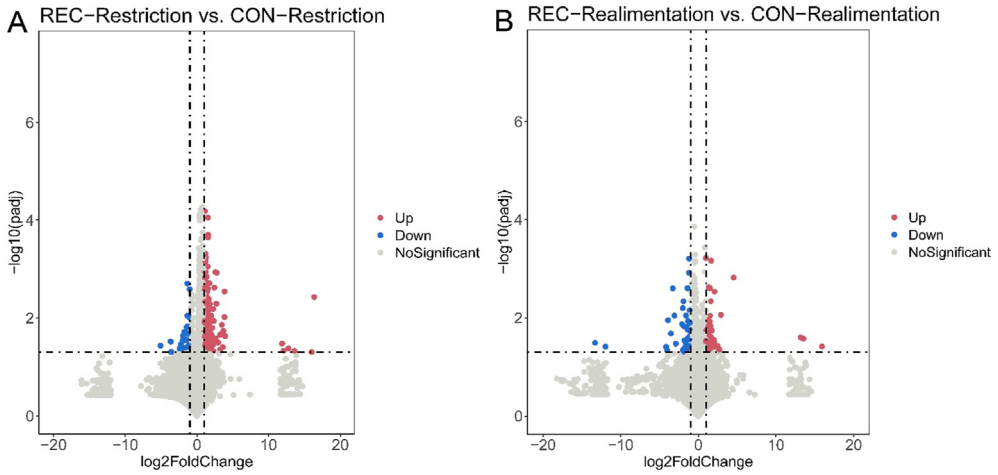

Fig. 3. Volcano plots of differentially expressed genes within the restriction and re-alimentation period. Red points represent upregulated genes, blue points represent downregulated genes, and gray points represent non-differentially expressed genes. CON: a 13% crude protein (CP) diet was fed both in the restriction and re-alimentation period; REC: a 7% CP diet was fed for 4 weeks in the restriction period and a 13% CP diet was fed subsequently for 2 weeks in the re-alimentation period.

concentrations. Within the REC, the concentrations of histidine, phenylalanine, proline, and 3-methyl-L-histidine were lesser ($P < 0.05$) in the re-alimentation period relative to the restriction period, while the concentrations of citrulline and isoleucine were greater ($P < 0.05$).

3.7. Comparison of transcriptome profiles among treatments

In the protein restriction period, a total of 200 DEGs were identified between the REC and the CON (Fig. 3). Compared with the CON, dietary protein restriction upregulated 163 genes and downregulated 37 genes. In the subsequent protein re-alimentation period, a total of 109 DEGs were identified with 59 upregulated genes and 50 downregulated genes relative to the CON. The expression of all identified DEGs is displayed in Fig. S1.

Among all the DEGs, the genes related to protein and AA metabolism pathways were specifically analyzed, which are visualized in Fig. 4. In the protein restriction period, there were 30 DEGs between the REC and the CON. Compared with the CON, dietary protein restriction upregulated 24 genes (such as *ABCA1*, *CASTOR2*, *SLC1A5*) and downregulated 6 genes (such as *TLE6*, *PCK2*, *CAMKK2*). In the protein re-alimentation period, a total of 22 DEGs were identified, which included 13 upregulated genes (such as *ITGA8*, *SLC16A4*, *PTRH1*) and 9 downregulated genes (such as *PFKFB3*, *DDIT4*, *CCL4*).

3.8. Functional enrichment of and pathway enrichment of differentially expressed genes

According to the KEGG enrichment analysis, upregulated genes were significantly enriched in transcription factors (*ARX*, *PLAG1*, and *TP63* etc.), ABC transporters (*ABCA1* and *ABCG4*), transporters (*SLC26A7*, *SLC43A1*, and *SLC22A4* etc.), and protein digestion and absorption (*ATP1A4* and *COL23A1*), while downregulated genes were significantly enriched in the AMPK signaling pathway (*CAMKK2* and *PCK2*) in the restriction period (Fig. 5A). In the subsequent protein re-alimentation period (Fig. 5B), downregulated genes were enriched in the mTOR signaling pathway (*ATP6V1C2* and *DDIT4*), and both upregulated and downregulated genes were significantly enriched in AA transporters (*SLC22A18*, *SLC27A5*, and *SLC7A8* etc.).

The top 30 significantly enriched GO terms are displayed in Fig. S2. In the protein restriction period, the DEGs were significantly enriched in protein localization to the plasma membrane (*CSRP3*),

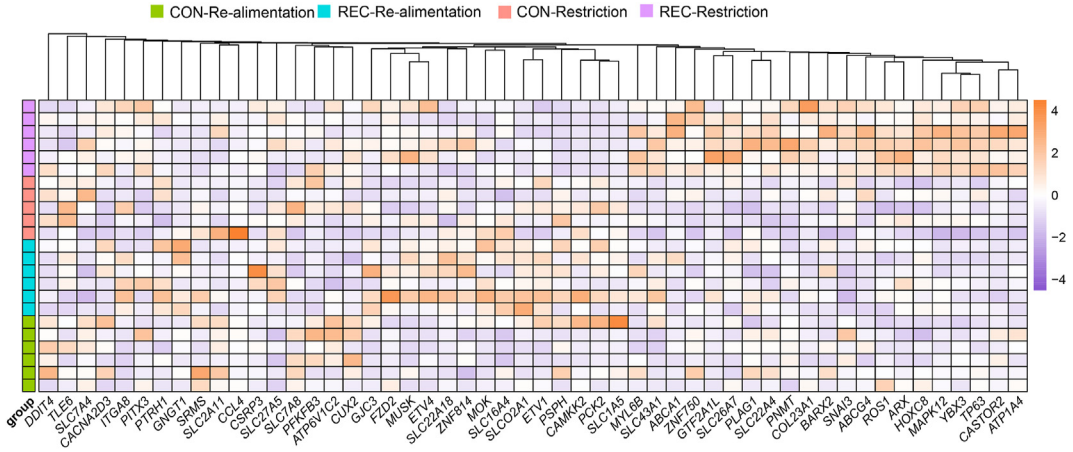

Fig. 4. Heatmap of differentially expressed genes related to protein and amino acid metabolism among treatments. CON: a 13% crude protein (CP) diet was fed both in the restriction and re-alimentation period; REC: a 7% CP diet was fed for 4 weeks in the restriction period and a 13% CP diet was fed subsequently for 2 weeks in the re-alimentation period.

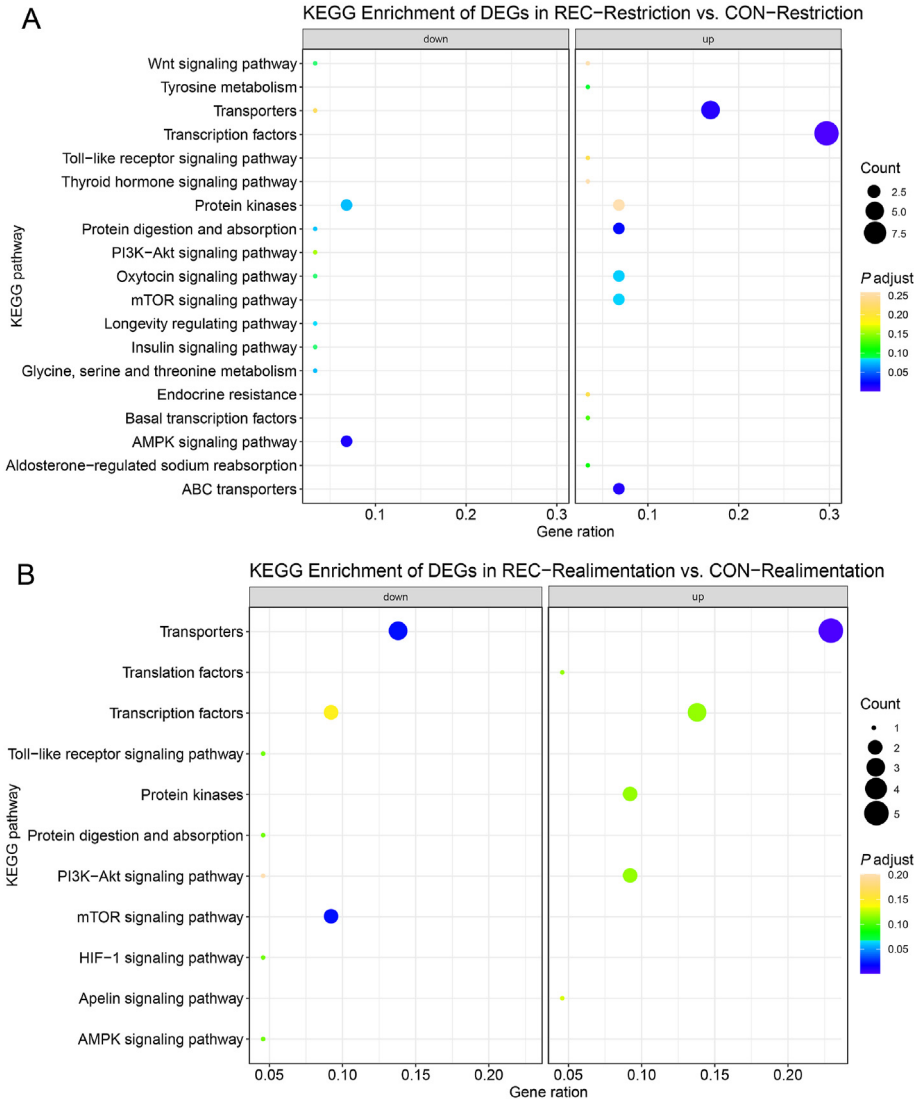

Fig. 5. The enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of differentially expressed genes (DEGs). (A) REC versus CON in the protein restriction period; (B) REC versus CON in the following re-alimentation period. CON: a 13% crude protein (CP) diet was fed both in the restriction and re-alimentation period; REC: a 7% CP diet was fed for 4 weeks in the restriction period and a 13% CP diet was fed subsequently for 2 weeks in the re-alimentation period.

negative regulation of cell migration (*TMEFF2*), Golgi cisterna (*SEC1*), and AA transmembrane transporters (*SLC1A5*). In the protein re-alimentation period, the 30 GO terms included transcription by RNA polymerase II (*EID2* and *ETV1*), signaling receptor activator activity (*INHA*), and response to other organisms (*DDIT4*).

3.9. Correlation analysis between plasma targeted AA metabolites and DEGs

The correlations between plasma targeted AA metabolites and DEGs are visualized in Fig. 6. Proline was negatively correlated with *CAMKK2* and *PSPH*, and positively correlated with *PNMT* ($P < 0.05$). Lysine was negatively correlated with *CAMKK2*, *PCK2*, and *PSPH*, and positively correlated with *PITX3* ($P < 0.05$). Valine was negatively correlated with *FZD2* and *MAPK12* ($P < 0.05$). Histidine was negatively correlated with *PSPH* ($P < 0.05$), and positively correlated with *PFKFB3* ($P < 0.05$). Methionine had a negative correlation with *PSPH* ($P < 0.05$). Arginine was positively correlated with *FZD2* and *SLC43A1* ($P < 0.05$). 3-Methyl-L-histidine was negatively correlated with *CAMKK2* and *PCK2*, and positively correlated with *CASTOR2* and *MAPK12* ($P < 0.05$). Tyrosine had a negative correlation with *PSPH* ($P < 0.05$). Phenylalanine was negatively correlated with *PSPH* and positively correlated with *PNMT*, *PFKFB3*, and *CASTOR2* ($P < 0.05$). Isoleucine was negatively correlated with *FZD2*, *ITGA8*, *MAPK12*, and *SLC43A1* ($P < 0.05$).

4. Discussion

4.1. Dry matter intake, growth performance, and nutrient digestibility

Dietary protein restriction significantly decreased ADG, while subsequent protein re-alimentation increased ADG, suggesting that the compensatory growth model was successfully developed by manipulating dietary protein concentrations in growing beef bulls. Figueiras et al. (2010) reported that feed intake was decreased when the dietary protein concentration was less than 10% of DM. Detmann et al. (2014) demonstrated that increasing dietary CP level from 11.6% CP to 14.5% CP resulted in greater DMI and growth performance of beef cattle. In the current study, the bulls fed with

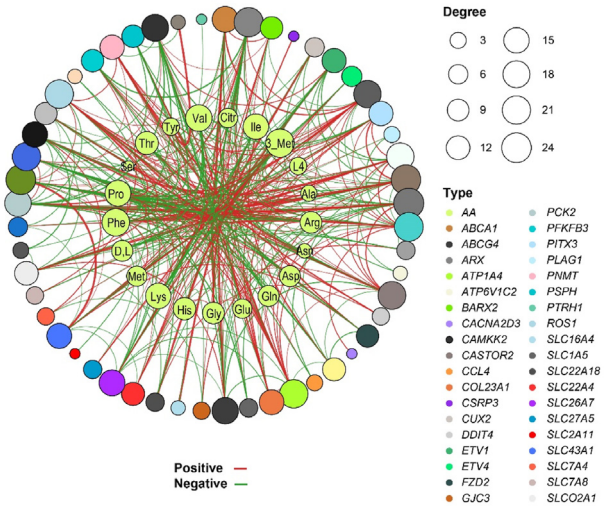

Fig. 6. The correlation network of plasma targeted metabolites of amino acids (AA) and differentially expressed genes (DEGs) in muscle tissue. The thickness of the line represents the size of correlation coefficients. The circle size represents the number of correlated relationships. Arg = arginine; Asn = asparagine; Asp = aspartic acid; Gln = glutamine; Glu = glutamic acid; Gly = glycine; His = histidine; Lys = lysine; Met = methionine; D, L = D, L-0-tyrosine; Phe = phenylalanine; Pro = proline; Ser = serine; Thr = threonine; Tyr = tyrosine; Val = valine; Citr = citrulline; Ile = isoleucine; 3_Met = 3-methyl-L-histidine; L4 = L-4-hydroxyproline; Ala = alanine.

the higher protein diet had a greater DMI and ADG than those fed with the lower protein diet, which was consistent with the previous studies. Hornick et al. (2000) demonstrated that ruminants require approximately one month to achieve the maximum compensatory growth after three months' restriction. In the current study, the animals in the REC did not compensate completely after 2 weeks of protein re-alimentation, which implies the animals may have needed a longer time to recover.

Previous studies reported that total tract CP digestibility was increased alongside dietary CP levels (Singh et al., 2015; Van Dung et al., 2013; Yuangklang et al., 2010). Broderick (2003) demonstrated that increasing dietary protein level could reduce the proportion of endogenous nitrogen in total fecal nitrogen, leading to an increase in apparent total tract protein digestibility. In the current study, dietary protein restriction decreased the apparent total tract CP digestibility, but increased the apparent total tract EE digestibility relative to the CON, likely due to the presence of dried distiller's grains with solubles (DDGS). In the low-protein diet, DDGS were added to replace soybean meal and wheat bran in the REC. Liu et al. (2019) reported that the net protein utilization rate of soybean meal was greater than that of DDGS. Unsaturated fatty acids have a synergistic effect on intestinal fat digestion (Zinn et al., 2000), thus their intestinal digestibility is greater than that of saturated fatty acids. Feeding a diet containing DDGS could increase the levels of polyunsaturated fatty acids and thereby increase post ruminal digestibility of EE (Hart et al., 2019).

4.2. Ruminal ammonia N and plasma urea N

Dietary protein restriction significantly decreased N intake due to the drop in DMI. Consequently, NH_3-N and PUN concentrations were decreased. Previous studies reported that ruminal NH_3-N concentrations were positively correlated with dietary CP or RDP levels (Danes et al., 2013; Javaid et al., 2011). Ruminal NH_3 is generated from dietary protein degradation in the rumen and blood urea entering the rumen (Javaid et al., 2011). Ruminal microbes can use NH_3 as a substrate to synthesize microbial protein, but not all of the NH_3 is captured, and thus a portion is absorbed through the rumen wall (Li et al., 2019b). Therefore, the decreased ruminal NH_3-N, MCP, and PUN concentrations were primarily due to decreased nitrogen intake. These results were consistent with previous studies (Gleghorn et al., 2004; Pilachai et al., 2012).

In the protein re-alimentation period, the ruminal NH_3-N concentration in the REC was lesser than that in the CON. The increased protein supply in the REC could enhance microbial activity and provide sufficient nitrogen sources for microbial protein synthesis, leading to reduced NH_3-N concentrations (Javaid et al., 2011). One could speculate that ammonia was primarily used for microbial protein synthesis, though only numeric differences were observed in MCP and PUN concentrations between the REC and the CON.

4.3. Nitrogen metabolism

Dietary protein restriction decreased N intake, fecal N, and urinary N. As a consequence, protein retention was reduced relative to the control group. However, no significant treatment effect was observed on NUE. Danes et al. (2013) reported that NUE decreased with increased dietary CP supply. Shen et al. (2023) demonstrated a quadratic relationship between N intake and NUE, and the marginal benefit of N intake on NUE was decreased with the increase of N intake.

Subsequent dietary CP re-alimentation following restriction increased protein retention and NUE, primarily due to decreased urinary N excretion. Davidson et al. (2003) reported that urinary N was more sensitive than fecal N when dietary CP level was changed. Cole (1999) demonstrated that oscillating dietary CP could increase retained N by recycling more urea N back to the rumen and decreasing urinary N excretion. Doranalli et al. (2011) also concluded that oscillating dietary CP concentration could increase nitrogen deposition by increasing the circulation of urea back to the rumen. Archibeque et al. (2007) reported that there was no significant difference in urea synthesis in the liver between animals fed oscillating dietary CP and constant dietary CP, but more urea N was utilized by the portal-drained viscera when animals were fed oscillating dietary CP. Increased urea recycling back to the rumen could increase microbial protein synthesis and apparent ruminal N utilization efficiency (Devant et al., 2001; Leupp et al., 2009).

Urea N accounts for 60% to 70% of urinary N (Gardiner et al., 2018). When the supply of rumen NH_3-N exceeds the utilization of rumen microbes, there is an increase in urea excretion (Russell et al., 1992). The changes of urinary urea N are consistent with the changes of PUN and ruminal NH_3-N concentrations. To achieve a continuous supply of N in the rumen to promote microbial growth, ruminants can regulate urea synthesis and recycle urea back to the rumen (Detmann et al., 2014). In the current study, concentrations of urinary urea N and PUN were changed synchronously, which was consistent with the results of Harmeyer and Martens (1980). When CP content in the diet increases, the proportion of recycled urea decreases, resulting in a corresponding increase in urea excreted into the urine. This is consistent with the observed urinary urea N in the protein re-alimentation period.

The levels of creatinine in the urine depend on the glomerular filtration of plasma creatinine which is generated from creatine-phosphate breakdown in the muscle (Braun and Lefebvre, 2008). A reduction in growth performance is likely due to AA deficiency which causes skeletal muscle to break down to meet AA requirements for specific use (Hare et al., 2019). In the protein restriction period, the proportion of creatine in the REC increased, indicating that protein restriction might have increased the protein degradation rate in the muscle, and protein re-alimentation decreased the protein degradation rate in the muscle.

Allantoin and uric acid are the main purine derivatives (PD) in bovine urine. Urinary PD are the end products of microbial protein metabolism in the body. The total PD excretion can be used to estimate the total amount of ruminal microbial N synthesis (Yu et al., 2001). Protein restriction reduced the content of urinary PD, and there was no difference in the content of urinary PD between the two groups during the protein re-alimentation period. This result is consistent with the observed ruminal MCP concentrations, suggesting that the synthesis of MCP is increased with the increase of dietary CP supply (Chanthakhoun et al., 2012; Reynal et al., 2003).

Hippuric acid is an important N-containing compound in urine, which is synthesized by benzoic acid and glycine in the liver (Beyoglu and Idle, 2012). In the current study, dietary protein restriction decreased the concentration of hippuric acid. However, the proportion of hippuric acid in urine was higher in the REC than that in the CON during the restriction period and there was no difference in the proportion between the two groups in the re-alimentation period. Previous studies demonstrated that the excretion of hippuric acid was positively correlated with the content of CP, but its proportion in urine was decreased with increased protein levels (Lantinga et al., 1986; Zhou et al., 2019). The results in the protein restriction period were consistent with the previous studies. The decreased hippuric acid concentration in urine could be explained by the decreased supply of glycine in the liver for hippuric acid synthesis.

4.4. Amino acid metabolism and urea synthesis

Amino acids and protein in blood and skeletal muscle are the main reserve sources of AA (Waterlow, 2006). When an animal is hungry or dietary CP is deficient, the body reduces the degradation and synthesis of muscle protein with the latter reduced more. This results in net losses of muscle protein, and helps maintain plasma AA concentrations when absorbed supplies are low. 3-Methyl-L-histidine is produced by methylation of histidine in actin and myosin in the muscle and can be used to quantitatively indicate whether muscle protein catabolism has changed (Brosnan and Brosnan, 2020). When muscle catabolism occurs, 3-methyl-L-histidine release increases. In this study, protein restriction increased 3-methyl-L-histidine, suggesting that physiological activity is maintained through muscle protein breakdown in the absence of protein. After protein re-alimentation, within two weeks, the 3-methyl-L-histidine decreased to the same level as the CON, implying that dietary protein re-alimentation reduced skeletal muscle catabolism.

Branched-chain AA (valine and isoleucine) are important signal factors, which can activate the mTOR complex 1 pathway and regulate insulin sensitivity, thus regulating tissue protein synthesis and energy metabolism in ruminants (Appuhamy et al., 2012; Toerien et al., 2010). The levels of valine and isoleucine were positively correlated with protein synthesis (Herningtyas et al., 2008). In the restriction period, the levels of valine and isoleucine are low, which could explain the decreased protein retention.

Protein restriction increased the proline content relative to the CON, likely due to high concentrations of proline in the REC diet. Proline and its metabolite hydroxyproline account for 1/3 of AA in collagen, which are important structural and functional AA (Kaul et al., 2008). They play important roles in protein synthesis, structure, metabolism (especially through pyrrolidine-5-carboxylic acid to synthesize arginine, polyamine, and glutamic acid), antioxidant response, and immune response (Wu et al., 2011). van Meijl et al. (2010) found that proline could regulate the mTOR pathway in mammals and cooperate with arginine, glutamine, and leucine to promote protein synthesis in cells and tissues (Wu et al., 2011).

Citrulline is a precursor of L-arginine synthesis in most mammals, which plays an important role in animal intestinal health (Bahri et al., 2013) and urea synthesis (Morris, 2016). The reduction of citrulline can decrease the synthesis of arginine and thus reduce urea production in the liver, which is consistent with the results of PUN concentrations in the restriction period. Supplementing citrulline could increase muscle protein synthesis in rats (Osowska et al., 2006). Previous studies demonstrated that citrulline could activate the phosphorylation of mTOR and directly stimulate protein synthesis (Bahri et al., 2013; Moinard et al., 2007). Therefore, the increased citrulline concentrations could partially explain the protein compensatory growth in the protein re-alimentation period.

4.5. Transcriptional analyses of signaling pathways

Amino acid transporters are membrane-bound transport proteins that regulate transfer of AA into and out of cells or cellular organelles (Kandasamy et al., 2018). Amino acids are not able to diffuse across cell membranes without the assistance of transporters (Bhutia and Ganapathy, 2016). Increased AA delivery to muscle enhances AA sensing and activates protein synthesis (Moro et al., 2016). Members of the SLC6 family (*SLC6A14*/ATB$^{0,+}$ and *SLC6A15*/B^{0}AT2), SLC7 family (*SLC7A5*/LAT1 and *SLC7A8*/LAT2), SLC16 family (*SLCA10*/MCT10), SLC38 family (*SLC38A1*/SNAT1), and SLC43 family (*SLC43A1*/LAT3 and *SLC43A2*/LAT4) mainly mediate the uptake of essential AA (Bodoy et al., 2005; Hyde et al., 2003). *SLC43A1*/LAT3 belongs to the L family, promoting large neutral AA (Ile, Leu, Met, His, and Pro, etc.) diffusion (Bodoy et al., 2013). In response to a His deficiency, the mammary gland's capacity to remove plasma His increased 43-fold, whereas the gland's capacity for other AA declined by 2 to 3-fold (Bequette et al., 2000). Protein restriction upregulated the expression of *SLC43A1*/LAT3 in the present study, suggesting that protein restriction could promote the transportation of relative AA. *SLC7A8*/LAT2 is a Na^{+}-independent AA transporter and is responsible for transporting neutral AA, including Gln, Gly, Ser, Ala, Thr, Asn, Met, Val, Phe, Tyr, Leu, Ile, Trp, and His (Fukasawa et al., 2000; Wang and Holst, 2015). *SLC7A8*/

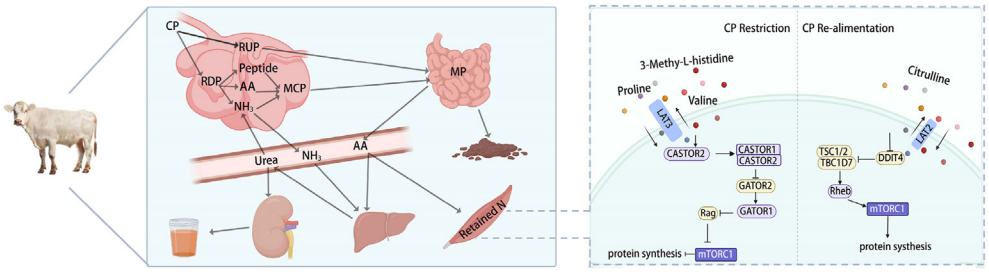

Fig. 7. The underlying mechanisms of nitrogen metabolism and protein deposition during dietary protein restriction and re-alimentation in growing beef cattle. AA = amino acids; CP = crude protein; MP = metabolic protein; RDP = rumen degradable protein; RUP = rumen undegradable protein.

LAT2 was downregulated after protein re-alimentation, implying that less essential AA are exchanged between intracellular and extracellular fluid to maintain protein synthesis in muscle cells.

Amino acids are not only the substrate for protein synthesis, but also the signaling molecule of mTORC1 (Kimball, 2002). mTORC1 could regulate protein synthesis by integrating nutrients and available growth factors in response to various changes in cells and regulating cell activity (Hara et al., 1998; Kimball et al., 1999; Zhang et al., 2021). Protein restriction leads to a deficiency of intracellular AA, thereby inhibiting the activity of mTORC1. As displayed in Fig. 7, *DDIT4* (also known as *REDD1*) can emit signals and activate tuberous sclerosis complex 2 (TSC2; Brugarolas et al., 2004). *TSC2* can inhibit the activity of ras homolog enriched in the brain (Rheb), causing it to lose its function of activating mTORC1 (Inoki et al., 2003). Rheb is one of the regulatory factors of mTORC1, which activates mTORC1 by antagonizing its internal inhibitor (Bai et al., 2007). After protein re-alimentation, *DDIT4* was downregulated, promoting the expression of mTORC1 by regulating the *TSC* and *TBC1D7*, as well as the activity of Rheb. This indicates that dietary protein re-alimentation promotes protein synthesis by activating the mTORC1 signaling pathway, which could explain the increased NUE and nitrogen deposition in the REC.

Archibeque et al. (2007) reported that gradually increasing protein levels through oscillatory feeding could improve retained N in finishing cattle, and the protein digestion was consistent with the digestion of cattle fed with normal protein. One could speculate that changes in protein supply are accompanied by changes in AA balance. Although there was no significant difference in plasma AA concentrations between the two groups after protein re-alimentation, the impact of AA oscillations on signaling pathways might have promoted protein synthesis. Dietary protein re-alimentation increased citrulline concentrations in the plasma. Arginine can activate the mTOR activity in skeletal muscle (Wu, 2009). As a precursor of arginine synthesis, the increased citrulline is conducive to activating the mTOR pathway (Bahri et al., 2013; Yao et al., 2008). The availability of AA, especially branched-chain AA, is essential for the activity of mTORC1. Previous studies indicated that branched-chain AA, such as isoleucine and valine, have a strong ability to activate the mTOR signaling pathway (Appuhamy et al., 2012; Herningtyas et al., 2008). In the current study, the increased plasma isoleucine and valine concentrations might facilitate the phosphorylation of mTORC1 and promote protein synthesis in the muscle. It is worth mentioning that intracellular AA supplies regulate protein synthesis, and the plasma concentrations might not be directly proportional to intracellular concentrations. Yoder et al. (2021) demonstrated that AA uptake is responsive to varying extracellular supplies to maintain homeostasis in mammary epithelial cells. Future kinetic studies are needed to quantify bidirectional AA fluxes and the relationships between protein synthesis and extracellular AA supplies in muscle cells.

The correlation analysis between DEGs and plasma-targeted AA indicated that *FZD2* was negatively correlated with Valine. *FZD2* is a member of the Frizzled gene family (FZDs), and its high expression is positively correlated with the activation of the mTOR signaling pathway (Huang et al., 2022). *FZD2* was significantly upregulated during protein restriction, which might have promoted protein synthesis. However, *CASTOR* could inhibit mTORC1 (Chantranupong et al., 2016). Protein restriction upregulated *CASTOR2* in the REC which might have inhibited protein synthesis. It is worth mentioning that both inhibition and activation of the mTOR pathway were observed in the protein restriction period in the present study. The expression level of *CASTOR2* was greater than *FZD2* (Fig. 4), implying that the inhibition of protein synthesis might take priority over protein synthesis during protein restriction. The upregulated genes related to protein synthesis during restricted feeding might also contribute to the maintenance of CG during re-alimentation (Keogh et al., 2016a).

The enriched GO term is mainly related to functions such as biodegradation, synthesis and metabolism, cell growth and development, and transport carriers. Golgi is a central cell membrane-binding organelle, that has key functions in transporting, processing, and sorting newly synthesized membranes and secreted proteins (Li et al., 2019a). *SEC1*, enriched in Golgi cisterna, is related to the vesicular transport of Golgi, and its down-regulation may decrease protein secretion (Archbold et al., 2014). After protein recovery, DEGs were enriched in terms of transcription by RNA polymerase II, which indicates that additional mRNA is synthesized to synthesize protein (Ren et al., 2023).

5. Conclusion

Protein restriction decreased ADG, while the subsequent protein re-alimentation increased ADG relative to the control group, indicating that a compensatory growth model was successfully developed by manipulating dietary protein supply in growing beef bulls. Dietary protein re-alimentation following restriction promoted retained N and improved NUE, primarily due to reduced urinary urea, hippuric acid, and creatinine excretions, suggesting that the absorbed AA are mainly used for protein synthesis and deposition during the compensatory growth stage. In addition, dietary protein restriction decreased plasma valine and aspartic acid concentrations and increased proline and 3-methyl-L-histidine concentrations, while dietary protein re-alimentation increased citrulline concentrations, implying that protein synthesis and degradation might be dynamically regulated by AA balance in the body. Protein restriction upregulated the *SLC43A1* transporter, while protein re-alimentation downregulated the *SLC7A8* transporter in the muscle cell membrane. Within the muscle cell, the upregulated *CASTOR2* can inhibit protein synthesis by inhibiting mTORC1 phosphorylation in the protein restriction period, and *DDIT4* can promote protein synthesis by activating the mTOR signaling pathway in the protein re-alimentation period. Overall, dietary protein re-alimentation following restriction promotes protein synthesis and reduces muscle breakdown by regulating AA metabolism and functional transcripts related to AA transmembrane transportation and the mTOR signaling pathway.

Data availability

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Credit Author Statement

Jiaqi Wang: Methodology, Investigation, Formal analysis, Visualization, Writing-Original; **Shen Chun:** Methodology, Investigation, Data Curation, Visualization, Writing-Original; **Guangyong Zhao:** Supervision, Writing - Review & Editing; **Mark D. Hanigan:** Writing - Review & Editing; **Mengmeng Li:** Conceptualization, Methodology, Supervision, Writing - Review & Editing, Project administration.

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.

Acknowledgment

This work was supported by the National Natural Science Foundation of China (grant no. 32102573) and the 315 Talent Project at China Agricultural University.

Appendix A. supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2024.04.028.

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